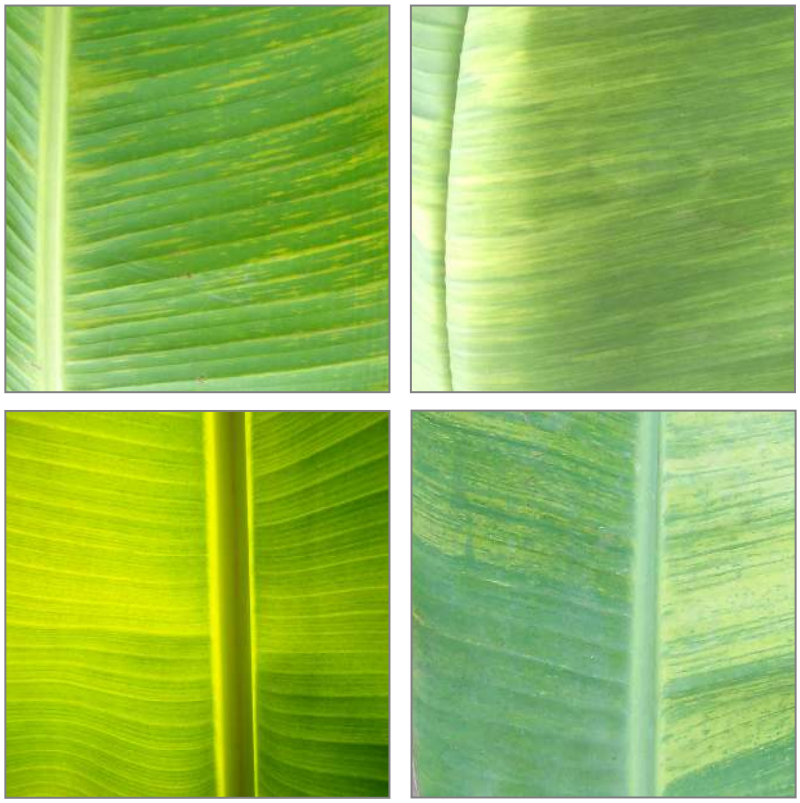
Virus Detection in Banana

A Laboratory Manual



www.iita.org

IITA

Virus Detection in Banana

A Laboratory Manual

Edited and Compiled by

P Lava Kumar
Virology & Molecular Diagnostics Unit
International Institute of Tropical Agriculture
PMB 5320, Ibadan, Nigeria
www.iita.org

About IITA

The International Institute of Tropical Agriculture (IITA) is an international non-profit R4D organization founded in 1967 as a research institute with a mandate to develop sustainable food production systems in tropical Africa. It became the first African link in the worldwide network of agricultural research centers supported by the Consultative Group on International Agricultural Research (CGIAR) formed in 1971. Mandate crops of IITA include banana & plantain, cassava, cowpea, maize, soybean and yam. IITA operates throughout sub-Saharan Africa and has headquarters in Ibadan, Nigeria. IITA's mission is to enhance food security and improve livelihoods in Africa through research for development (R4D), a process where science is employed to identify problems and to create development solutions which result in local production, wealth creation, and the reduction of risk. IITA works with the partners within Africa and beyond. For further details about the organization visit: www.iita.org.

Headquarters:
IITA, PMB 5320, Ibadan, Oyo State, Nigeria
Tel: +234 2 7517472, (0)8039784000
Fax: INMARSAT: 873761798636
E-mail: iita@cgiar.org

International mailing address:
IITA, Carolyn House,
26 Dingwall Road, Croydon
CR9 3EE, England
United Kingdom

Preface

This manual, a modified version of our previous laboratory manual, provides basic principles and offers step-by-step protocols for virus detection in banana (*Musa* spp.) Some molecular methods for the detection of important fungal and bacterial pathogens of banana are also included. The methods described in this manual are based on our experience over the years and are the result of contributions from many of the present and past members of our research units. Some of the descriptions and protocols have been adapted from work done elsewhere and the source of this information has been duly credited. Basic background pertinent to theoretical and practical aspects of plant virology and diagnostics has been provided. Diagnostics is an evolving field and new techniques for detection of banana viruses will continue to emerge. Users are advised to monitor literature for updates on diagnostic techniques.

P Lava Kumar
L.kumar@cgiar.org
IITA-Nigeria

Index

Abbreviations

ACP-ELISA	Direct coated plate-enzyme-linked immunosorbent assay
ALP	Alkaline phosphatase
BTB	Bromothymol blue
cv	Cultivar
DAC-ELISA	Direct antigen coating-enzyme-linked immunosorbent assay
DAS-ELISA	Double antibody sandwich-enzyme-linked immunosorbent assay
DB-PCR	Direct binding-Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
dNTPs	Deoxynucleotide phosphates
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
IC-PCR	Immuno Capture-Polymerase Chain Reaction
IC-RT-PCR	Immuno Capture-Reverse Transcription-Polymerase Chain Reaction
Ig	Immunoglobulin
IgG	Immuno-γ-globulin
mol. wt.	Molecular weight
kb	Kilo base
kbp	Kilo base pair
kDa	Kilo Dalton
PAS-ELISA	Protein-A sandwich-ELISA
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pi	Post-inoculation
PNC	Penicillinase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Scanning electron microscope
TAS-ELISA	Triple antibody sandwich-ELISA
TC	Tissue culture
TEM	Transmission electron microscope
VLP	Virus-like particles

List of pathogens

BBTV	Banana bunchy top virus
BBrMV	Banana bract mosaic virus
BanMMV	Banana mild mosaic virus
BSV	Banana streak virus
BVX	Banana virus-X
BXW	Banana bacterial wilt
CMV	Cucumber mosaic virus

List of Symbols/Units

A	Absorbance
cm	Centimeter
ºC	Degree centigrade
g	grams
h	hours
l	Liter
k	Kilo
lb/sq.in	Pounds per square inch
M	Moles
m	Meter
mM	Millimoles
mm	millimeter
min	minutes
ml	Milliliter
mg	Milligram
µ	Micro
µl	Micro
µg	Microgram
ng	Nanogram
nm	Nanometer
OD	Optical density
pH	Hydrogen ion concentration
%	Percent
rpm	Revolutions per minute
sec	Seconds
v	Volume
w	Weight

I. Background to Virus Disease Diagnosis
1. Diagnosis of Virus Diseases

Plant viruses cause major losses to agricultural crops around the world. Chemical agents similar to fungicides and bactericides are not effective to control virus diseases. Strategies for virus management are mostly aimed at eradicating the source of infection to prevent it from reaching the crop and interfering with the movement of vectors to prevent the spread of the disease. However, the most effective means of controlling virus diseases is through cultivating the virus-resistant varieties. Precise identification of the causal agent is the first step in management of virus diseases. Although accurate description of symptoms is necessary to describe the disease, virus diagnosis should not be based on symptoms alone, because several unrelated viruses cause similar symptoms and same virus or its strains can result in different symptoms on the same host or on different host species. Several diagnostic methods are available for the identification of causal viruses. The choice of test depends on the facilities, availability of reagents, expertise and the amount of known information about the virus or disease.

A) Disease Diagnosis

The terms diagnosis and detection are often used interchangeably. Diagnosis step involve careful examination to determine underlying cause of the disease; whereas detection is to find out the virus. For example, streak disease of maize is *diagnosed* as due to *Maize streak virus* (MSV). Methods such as ELISA are employed to *detect* MSV in diseased plants. Detection of a virus in a diseased plant not necessarily is a proof that it causes the disease. Further careful testing is essential prior to naming a particular virus as cause of the disease. The following steps modified from L Bos (1976) are useful for diagnosing a disease.

1) Observe disease in the field, determine affected plant species and cultivars, disease incidence and distribution within field (random-, clustering-, peripheral-, uniform-distribution of infected plants)
2) Record the symptoms and compare in literature for any similar descriptions on the same host in-country or elsewhere.
3) Study infectivity and transmission tests by grafting; mechanical sap inoculation; transmission through vectors (insects, mites, nematodes or fungi)
4) Inoculate (using plant sap, by grafting or vector) to a range of test plants and back inoculate to a parallel range of test plants to check possible multiple infections and to determine host range and symptoms. Compare symptoms observed on experimental host range in literature for clues to identify the probable virus. Select systemically infected host for virus propagation for purification purpose; local lesion host for virus assays; and diagnostic species, which react uniquely to that particular causal virus.
5) Determine the persistence of infectivity in sap extracts (dilution end point, thermal inactivation point, stability and retention of infectivity upon storage at various temperatures and length of time) and effects of additives on virus infectivity and stability (treatment with organic solvents; stability at various pH, molarity and buffer type; addition of reducing agents).
6) Examine leaf dip preparations under electron microscope to detect any virus particles.
7) Isolate the virus and purify thereafter to determine the physicochemical properties (particle morphology, sedimentation coefficient, buoyant density, number of particle components, number of structural proteins, genome type, number, its polarity and strandedness, sequence information)
8) Study the cytopathology for virus inclusions and cytological changes in affected cells.
9) Produce polyclonal antibodies and develop a serological diagnostic test for virus detection.
10) Assess virus serological relationships using antiserum and inter relationships from nucleotide sequence information to determine virus taxonomic status.
11) Fulfill Kochs' postulates, especially using purified virus or isolated virus cultures if purified virus preparation looses infectivity.

Depending on the virus kind, previous knowledge on virus or knowledge gained from during experimentation, laboratory facilities and expertise, the order of steps described can be changed or few steps can be ignored.

Majority of the plant diseases are caused by specific viruses, often singly (example: banana bunchy top disease is caused by the *Banana bunchy top virus*, genus Babuvirus). Few diseases are caused by mixed infections of unrelated viruses. A good example for this case is groundnut rosette disease, which is caused by three unrelated agents: a luteovirus (*Groundnut rosette assistor virus* – GRAV), an umbravirus (*Groundnut rosette virus* – GRV) and a satellite-RNA, which depends on GRV for its replication. Although 'rosette' symptoms are mainly due to sat-RNA, all the three agents are essential for successful transmission and establishment of the disease under natural conditions. Some diseases are caused by several virus species either alone or in mixed infection. For example, nine virus species belong to the genus *Begomovirus*, are involved in the etiology of cassava mosaic disease (CMD), viz., *Indian cassava mosaic virus* (ICMV), *Sri Lankan cassava mosaic virus* (SLCMV), *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Kenya virus* (EACMKV) and *South African cassava mosaic virus* (SACMV). Each of these viruses is capable of causing CMD on its own or in mixed infection.

Thus it is imperative after purification of virus(es) to show that they can induce characteristic symptoms on natural host and induces the disease, i.e. fulfilling Koch's postulates, they are

A virus isolated:

1) Must be found in all cases of the disease
2) Must be isolated and grown in pure culture
3) Must reproduce the original symptoms when back-inoculated into a susceptible host
4) Must be found in the experimental host, so infected.

When a new disease appear on a host, suspected as due to virus based on symptoms of type never have been described on that particular host in that country, the disease can be considered as new and it can be named. However, conclusion on virus identity should not be drawn without properly diagnosing the disease to identify the actual causal agent. For example, stem necrosis, is a characteristic symptom in groundnut caused by TSV. This disease in groundnut can be named as 'stem necrosis disease', but not as 'stem necrosis virus'.

B) Virus characterization (description)

The properties elucidated during the course of isolation, purification and diagnosis of the virus disease determines the virus relationships with previously characterized viruses and forms a basis to identify it as a new species / an isolate of a virus species / a new strain of a virus species, and to place it into an appropriate taxonomic group in present plant virus classification (see Table 3). The characters commonly used for virus identification are:

(i) Biological characters

Transmission characters

- Mechanical transmission
- Transmission by biotic vectors (insects, fungi, mites, nematodes etc.)
- Transmission by seed or pollen
- Transmission by soil (direct root ingress)
- Transmission by direct contact, plant debris and dodder

Host range

- Symptoms on diagnostic host species (local and systemic infections)
- Reaction on wide range of host plants

In vitro *properties*

- Thermal inactivation point
- Longevity *in vitro* (at various temperatures and time periods in sap extracts and intact plant parts)
- Dilution end point

Symptomatology

- Macroscopic symptoms (on natural hosts and diagnostic hosts)
- Microscopic symptoms (inclusion bodies, cytopathological changes within in the cell)
- Pathogenicity associated with disease
- Tissue tropism

Cross-protection

- Against related strains or non-related viruses

(ii) Physico-chemical properties

- Number of virus components (mono-, di, tri- or multipartite)
- Number and molecular weight of the structural proteins (coat and nucleoproteins)
- Type of nucleic acid (DNA / RNA; single or double; linear or circular; positive or

negative polarity; genome linked structures)
- Number and molecular weight of the virus genome
- Sedimentation coefficient
- Particle buoyant density

Morphological
- Size and shape
- Special features such as lipid membranes

(iv) Inter-relationships

Serology-based
- Serological relationships utilizing polyclonal antibodies or monoclonal antibodies or epitope specific antibodies.
- Relationships by western immuno-blotting
- Mapping epitopes

Nucleic acid-based
- Percent nucleic acid homology by nucleic acid hybridization or direct comparison of nucleotide sequences
- Genome organization and expression
- Amino acid composition

C) Virus detection Methods

Detection of plant viruses included serological laboratory tests since the 1960. The choice of detection method is influenced by facilities and expertise, information on virus suspected to be present, host plant and time for completing the experiment. In general, any detection method should be rapid and highly specific for the target virus, and should detect virus present in low amounts in the plant tissue and detection at an early stage of disease development.

Various methods have been in use for virus detection in plants. They can be broadly categorized as techniques used prior to the development of ELISA (prior to 1976), modern serological assays and nucleic acid-based tools (Table 1).

Some of the techniques have been used for decades without any major changes or improvement, while some are recently introduced. Commonly used diagnostic tools are constantly modified for improvement and optimize the performance. Of various detection methods, ELISA and PCR/RT-PCR are based methods are most widely used, at present. An overview of some of the commonly used detection methods is described here. More details about ELISA and PCR methods are discussed in next chapter 9. Other routinely used assays are briefed below.

(i) Biological assays:

Symptoms on plants are commonly used if they are characteristic of a specific disease. Symptoms are influenced by several biotic and abiotic factors, nutritional deficiencies and some genetic abnormalities can also result in symptoms similar to viruses. Usually symptom based virus diagnosis is done in conjugation with other confirmatory tests

Table 1: The commonly used diagnostic tests

Conventional techniques prior to 1976
Bioassay with indicator hosts
Detection for inclusion bodies
Conventional serological assays
Chloroplast agglutination
Ring precipitation interference test
Agar gel single and double diffusion
Immuno-electrophoresis
Hemagglutination
Bentonite flocculation
Latex agglutination
Serologically specific electron microscopy
Fluorescent antibody-based assay
EM-based
Leaf dips for virus particles
Modern assays
Serological assays
Multiwell plate ELISA (also with fluorescent, gold and radio labelled antibodies)
Dot-blot assay on membranes
Tissue print immuno-blotting
Rapid immuno-filter paper assay
Nucleic-acid based assays
dsRNA analysis
Nucleic acid hybridization
PCR and RT-PCR
Real-time PCR/RT-PCR
Loop-mediate amplification of DNA/RNA
Micro-array hybridization
Nucleotide sequencing

Diagnostic hosts: Mechanical transmission to indicator plants can be done with minimum facilities and characteristic symptoms produced by these plants allow detection and identification of known viruses. Although this may not provide precise virus identification, it is still used as an important assay in virus diagnosis. Viruses that are not transmitted mechanically can be inoculated on to indicator plants by grafting or using vectors. This is relatively complex, as it requires continuous maintenance of vector and virus cultures. It is still being routinely used to assay non-mechanically transmissible viruses.

(ii) Microscopy

Electron microscope (EM) provides useful information on particle morphology in leaf dip preparations. For stable viruses, EM can give rapid results using negative staining technique. When viruses occurring in low concentration are not easily seen. In such case sap from test material needs to be concentrated prior to observation or particles from sap can be trapped using antibody-coated grids (immunosorbent EM) to improve the detection efficiency. However, EM is an expensive to acquire and maintain.

EM is commonly used to study ultracytopathology of virus infected cells also. Although this is not commonly used for diagnostic purpose, unknown viruses can be readily identified based on unique inclusions they produce (e.g potyviruses).

(iii) Serological methods

Polyclonal antibodies raised against structural proteins (coat protein, ribonucleoroteins) in mammalian systems (rabbit, goat, chicken) can be used to develop variety of serological tests. Serological assays are two types, solid phase assays (ELSIA, Western immuno-blotting) and liquid phase assays (agar gel single and double diffusion, ring precipitation or agglutination). (ELISA test is discussed in chapter 9. For more information on some on liquid phase assay refer Hamptom et al., 1990)

Precipitin tests: This assay relies on the formation of a visible precipitate at the point of virus and antibody interaction. In agar gel double diffusion (Ouchterlony) test, antigen (in leaf sap or purified virus preparations) and antibody diffuse through gel matrix and a visible precipitin line appears at the point of interaction. This method is most commonly used to study serological relationships. Although this assay lacks sensitivity, it is most useful to identify viruses that occur in moderate concentration in sap. This assay can be conducted with minimum facilities and expertise, therefore is suitable for diagnosing virus in feebly equipped labs.

Immunoblotting: Dot immunoblotting assay (DIA) can be used to detect virus in plants as well as in vectors. Sap or insect extracts are spotted onto the membrane for detecting virus using homologous antibodies. The principle of DIA is similar to ELISA, except that it is performed on nitrocellulose membranes and precipitable substrates are used for development of positive reaction at the site of reaction. Chemiluminescent or radioactive substrates are also used, but in this case, energy (light or radiation) emitted is captured by exposing it to x-ray film. DIA is as sensitive as ELISA, but it requires optimization and it is not suitable for testing plant tissues, which contain high amount polyphenols that gives of background reaction.

Tissue printing or tissue print immuno-blotting is similar to DIA, but instead of sap extracts, whole tissue is blotted on to the nitrocellulose membrane. Subsequent detection is similar to that of DIA. Tissue print blotting aids in determining virus in the tissues.

Western immuno-blotting (WIB) is another variation of DIA. In this case, proteins separated in polyacrylamide gels are transferred onto nitrocellulose membrane by electrophoresis (Western transfer or Western blotting). Proteins transferred on to the membrane are detected using antibodies (immuno detection). This assay is commonly used to differentiate virus strains, epitope mapping and also for accurate detection of virus from total protein extracts.

There are several variations of immunoblotting techniques. The most commonly used ones are DIA, WIB and tissue printing.

(iv) Nucleic acid (NA) based methods

(Details of NA-based methods are discussed in chapter 9).

Nucleic acid hybridization: The affinity between the complementary strands of DNA/ RNA is very strong and specific. This specificity has been exploited in developing nucleic acid hybridization assays, which are based on the homology between two strands of nucleic acids (DNA:DNA / RNA:RNA / RNA:DNA). A single-stranded complementary NA, either DNA or RNA is labeled with reporter molecule [radioactive (^{32}P) or non-radioactive (digoxygenin)] is used as probe to hybridize with target molecule, and this reaction is detected by various means depending on the reporter molecule.

Dot or slot blot hybridization is most commonly used technique for virus detection. In this target molecule, in total nucleic acid extracts or total RNA or DNA extracts are blotted onto the nitrocellulose or nylon membranes (nylon membranes are durable). Hybridization is allowed to take place at high temperatures (usually 57-65^{o}C) between bound NA and the probe in, hybridization chamber. Target sequences are assayed by detecting the reporter molecule.

NA hybridization take 24-48 h to complete, and requires expertise and well-equipped laboratories.

Detection range of various diagnostic methods is given in Table 2.

Endnote

Virus detection tools are essential to assay infections in seed, testing of stock plants in certification programmes, indexing of commercial crops derived from certification programmes, screening for sources of virus resistance, surveys of virus incidence in crops, weeds, vectors and forecasting of epidemics by direct testing of insect vectors.

Most of the virus detection methods standardized for routine application are ELISA-based. These are simple and convenient for application in developing countries. A low cost enzyme-substrate (penicillinase based reporter system) system has been standardized. This system is cheap and positive and negative reactions can be read by visual observations.

In addition, information bulletins describing typical symptoms of the disease and information on diagnostic host range has been published, for field level disease diagnosis.

Table 2: Detection limits of various virus detection methods (Matthews, 1993)

Method	Detection range
Serological	
Gel double immunodiffusion	2-20 μg/ml
Liquid precipitin tests	1-10 μg/ml
Radial immuno-diffusion	0.5-1.0 μg/ml
Rocket immunoelectrophoresis	0.2 μg/ml -100 ng/ml
Immuno-osmophoresis	50-100 ng/ml
Passive hemaglutination	20-50 ng/ml
Latex test	5-20 ng/ml
ELISA	1-10 ng/ml
Immunoelectron microscope	1-10 ng/ml
Western blotting	1-10 ng/ml
Nucleic acid-based	
Molecular hybridization	<1 pg
PCR/RT-PCR	<1 fg

Table 3. Plant virus classification and their major properties*

Genus	Type species	Transmission	Morphology (nm)
Single stranded (SS) DNA viruses (circular genome, + polarity)			
Family: *Gemniniviridae*			
Begomovirus	*Bean golden mosaic virus*	Wf, Lh (cir)	Geminate, 18x30
Mastrevirus	*Maize streak virus*	Lh (cir, n.prop)	Geminate, 18x30
Curtovirus	*Beat curly top virus*	Lh (cir, n.prop)	Geminate, 18x22
Topocuvirus	*Tomato pseudo curly top virus*	Th	Geminate, 18x22
Family: *Nanoviridae*			
Nanovirus	*Subterranean clover stunt virus*	Ap (cir.)	Icos 17-20
Babuvirus	*Banana bunchy top virus*	Ap (cir.)	Icos 17-20
Double stranded (ds) DNA viruses (with reverse transcription activity)			
Family: *Caulimoviridae*			
Caulimovirus	*Cauliflower mosaic virus*	Ap (np, sp)	Icos, 40-50
Soymovirus	*Soybean chlorotic mottle virus*	No vector	Icos, 45-50
Cavemovirus	*Cassava vein mosaic virus*	No vector	Icos, 45-50
Petuvirus	*Petunia vein clearing virus*		Icos, 45-50
Badnavirus	*Commelina yellow mottle virus*	Mb (sp)	Bacilliform,130x13 Few 900nm long
Tungrovirus	*Rice tungro bacilliform virus*	Lh (sp)	Bacilliform, 60x18nm
Family: *Pseudoviridae*			
Pseudovirus	*Saccharomyces cerevisiae Ty1 virus*	No vector	Icos, 30-40nm
Sirevirus	*Glycine max SIRE1 virus*	No vector	Icos, 30-40 nm
Family: *Metaviridae*			
Metavirus	*Saccharomyces cerevisiae Ty3 virus*	No vector	Ribonucleo protein particles (poorly understood)
Double stranded (ds) RNA viruses			
Family: *Reoviridae*			
Phytoreovirus	*Wound tumor virus*	Lh (cp)	Icos, 2 protein shells c. 70-75
Fijivirus	*Fiji disease virus*	Ph (cp)	Icos, 65-70
Oryzavirus	*Rice ragged stunt virus*	Ph (cp)	Icos, 75-80
Family: *Partitiviridae*			
Alphacryptovirus	*White clover cryptic virus 1*	Seed	Icso,
Betacryptovirus	*White clover cryptic virus 2*	Seed	Icso,
Unassigned genus			
Endornavirus	*Vicia faba endornavirus*		No true virus particles
Single stranded (ss) RNA viruses (Negative sense genome)			
Family: *Rhabdoviridae*			
Cytorhabdovirus	Lettuce necrotic yellows virus	Ap (per)	Bullet shaped, Env. 160-380X60
Nucleorhabdovirus	*Potato yellow dwarf virus*	Ap (per), Lh	Bullet shaped, Env. 50-90X90

Family: *Bunyaviridae*			
Tospovirus	*Tomato spotted wilt virus*	Sap, Th (prop)	Env, Icos. 80-100
Unassigned genera			
Ophiovirus	*Citrus psorosis virus*	Unknown	Thin filaments
Tenuivirus	*Rice stripe virus*	Ph (prop)	Thin filaments, 3-10
Varicosavirus	*Lettuce big-vein virus*	Fungus	Rod shaped, 350-360X 18nm.
Single stranded RNA viruses (Positive polarity)			
Family: *Bromoviridae*			
Bromovirus	*Brome mosaic virus*	Sap, beetles	Icos, 28-30
Cucumovirus	*Cucumber mosaic virus*	Sap, Ap (np)	Icos, 28-30
Alfamovirus	*Alfalfa mosaic virus*	Ap (np), seed,	Bacilliform, 4 particles, 30-57X18
Ilarvirus	*Tobacco streak virus*	pollen	Icos, 3 particles,
Oleavirus	*Olive latent virus 2*	Unknown	Bacilliform, multipartite
*Anulavirus***	*Pelargonium zonate spot virus*	Seed, pollen	Bacilliform, 35 nm
Family: *Comoviridae*			
Comovirus	*Cowpea mosaic virus*	Sap, Bt,	Icos, 28-30
Nepovirus	*Tobacco ringspot virus*	Nematodes, pollen, seed	Icos
Fabavirus	*Broad bean wilt virus 1*	Ap (np)	Icos
Family: *Closteroviridae*			
Closterovirus	*Beet yellows virus*	Ap (sp), Mb, Wf	flexuous filaments, 1250-2000,monopartite
Crinivirus	*Lettuce infectious yellows virus*	Ap (sp), Mb, Wf (sp)	flexuous filaments, bipartite, 700-900 & 650-850,
Ampelovirus	*Grapevine leafroll-associated virus 3*	*Pseudococcus longispinus, Planococcus ficus* (Hemiptera)	Flexuous filaments, 1800-2200nm
Family: *Luteoviridae*			
Luteovirus	*Barley yellow dwarf virus-PAV*	Ap (cir, np)	Icos, 25-28
Polerovirus	*Potato leafroll virus*	Ap (cir, np),	Icos, 24,
Enamovirus	*Pea enation mosaic virus-1*	Ap	Icos, 25-28
Family: *Tymoviridae*			
Tymovirus	*Turnip yellow mosaic virus*	Bt,	Icosahed, 30
Marafivirus	*Maize rayadofino virus*	Lh	Isom, 28-32
Maculavirus	*Grapevine fleck virus*		
Family: *Sequiviridae*			
Sequivirus	*Parsnip yellow fleck virus*	Aphids (sp, np, cir.) depending on helper virus)	Icos, 30
Waikavirus	*Rice tungro spherical virus*	Lh (sp)	Icos, 30
Family: *Tombusviridae*			
Tombusvirus	*Tomato bushy stunt virus*	few by seed, pollen, few by fungi.	Icos, 32-35
Carmovirus	*Carnation mottle virus*	Fungi	Icos, 32-35
Necrovirus	*Tobacco necrosis virus A*	Fung.	Icos, 28
Machlomovirus	*Maize chlorotic mottle virus*	Seed	Icsos, 30

Dianthovirus	*Carnation ringspot virus*	Soil, no vectors, some times nematode	Icos, 30
Avenavirus	*Oat chlorotic stunt virus*	Soilborne, zoosporic, fungi,	Isom, Icos, 35
Aureusvirus	*Pothos latent virus*	Soilborne, no vector	Isom, Icos, 30
Panicovirus	*Panicum mosaic virus*		Isom, Icos, 30
Family: *Potyviridae*			
Potyvirus	*Potato virus Y*	Ap (np), some by seed also	flexuous filaments 680-900X11-13
Rymovirus	*Ryegrass mosaic virus*	Mt (per)	Filamentous 690-720X11-15
Bymovirus	*Barley yellow mosaic virus*	Fungi	flexuous filaments 250-300 & 500-600 both are 15 width.
Macluravirus	*Maclura mosaic virus*	Ap (np)	flexuous filaments 650-675X13-16
Ipomovirus	*Sweet potato mild mottle virus*	Wf (np),	flexuous filamentous, 800-950
Tritimovirus	*Wheat streak mosaic virus*	Mt (per)	flexuous filamentous, 690-700
Family: *Flexiviridae*			
Potexvirus	*Potato virus X*	Contact	Slight flexuous rods, 470-500X13
Carlavirus	*Carnation latent virus*	Ap (np)	slightly flexuous filaments, 600-700nmX12-15
Allexivirus	*Shallot virus X*	Mt	flexuous filaments, 800X12
Capillovirus	*Apple stem grooving virus*	No vector	flexuous filaments, 640-700X12
Trichovirus	*Apple chlorotic leaf spot virus*	Nematodes	Flexuous filaments, 720-740 nm
Vitivirus	*Grapevine virus A*	Pseudococcidae	flexuous filaments, 800X12
Foveavirus	*Apple stem pitting virus*	No vector	flexuous filaments, 800X12
Mandarivirus	*Indian citrus ringspot virus*		flexuous filaments, 650nm
Unassigned genera (single stranded (ss) RNA genomes with positive polarity)			
Tobravirus	*Tobacco rattle virus*	Nematodes	Rigid rods, bipartite, L-180-215 & 48-115
Tobamovirus	*Tobacco mosaic virus*	contact, no vectors	rigid rods, mono partite, 300-350X18
Hordeivirus	*Barley strip mosaic virus*	Sap, contact	NE, rigid rods, tri partite, 110-150X20
Furovirus	*Soil-borne wheat mosaic virus*	Fungus (*Polymyxa graminis*)	Rod shaped, bipartite, 260-300X20 140-160X20
Pomovirus	*Potato mo-top virus*	Fungus	Rod shaped, tripartite 1) 290-310 2) 150-160 3) 65-80
Pecluvirus	*Peanut clump virus*	Fungus	Rod shaped, 2 predominant length, 245 & 190 with dia-21

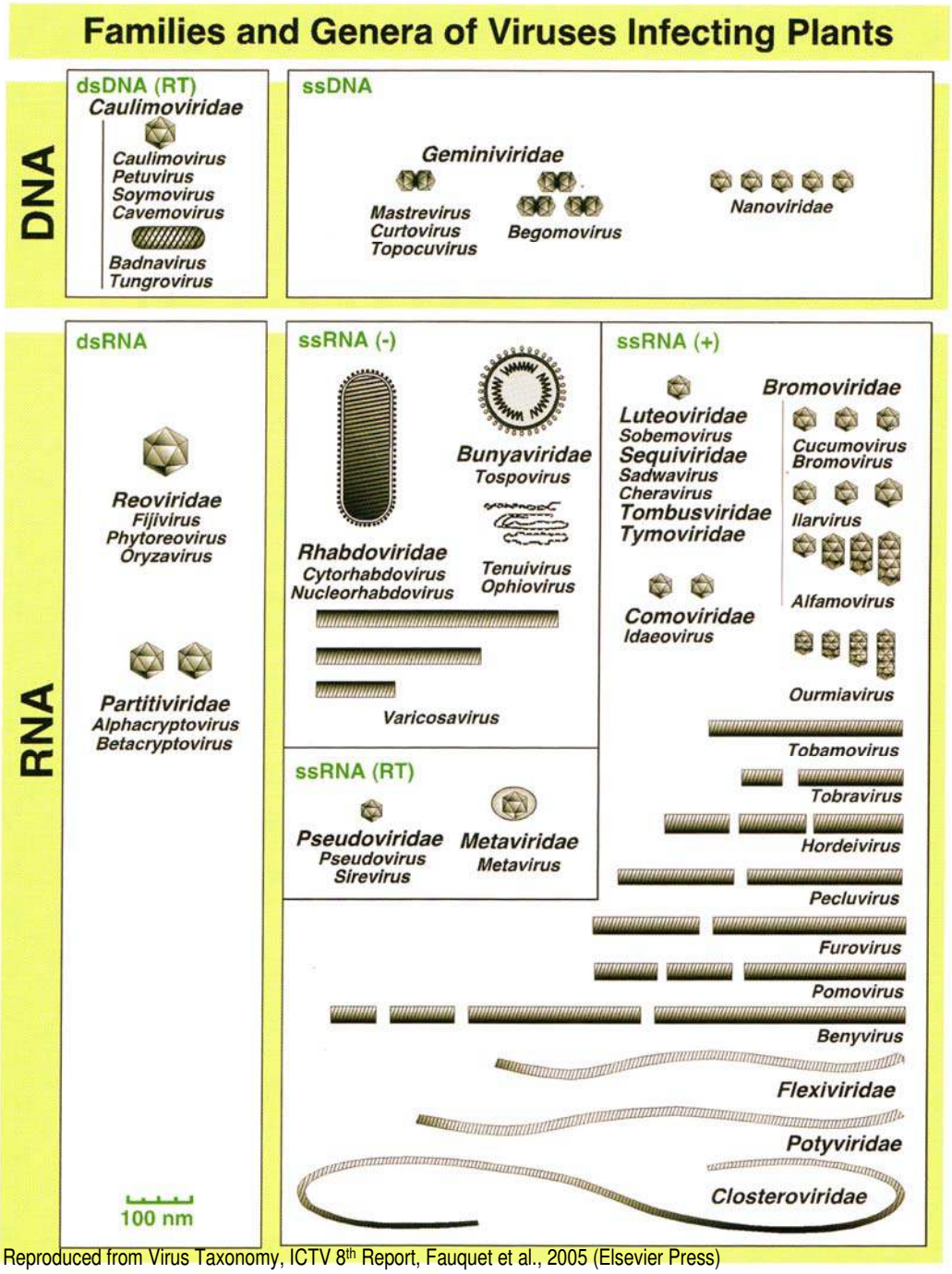
Benyvirus	*Beet necrotic yellow vein virus*	Sap, fungus	Filamentous, 4-5 particles-390,265,100,85X20
Sobemovirus	*Southern bean mosaic virus*	Bt	Icos, 30
Idaeovirus	*Raspberry bushy dwarf virus*	Pollen , seed	Isom, 33
Ourmiavirus	*Ourmia melon virus*	Unknown vector, seed transmission	bacilliform, multipartite, 28 in dia, length of 55, 43, 43, 37
Umbravirus	*Carrot mottle virus*	No vectors, Helper virus dependent	No specific particles
Cheravirus	*Cherry rasp leaf virus*	Nematode	Isometric, 30 nm
Sadwavirus	*Satsuma dwarf virus*	Seeds (in French bean)	Icosahedral, 26nm

Viroids

Family: *Pospiviroidae*			
Pospiviroid	*Potato spindle tuber viroid*	Contact, seed, pollen, aphids, vegetative propagation	No specific particles
Hostuviroid	*Hop stunt viroid*	-do-	No specific particles
Cocadviroid	*Coconut cadang cadang viroid*	-do-	No specific particles
Apscaviroid	*Apple scar skin viroid*	-do-	No specific particles
Coleviroid	*Coleus blumei viroid 1*	-do-	No specific particles
Family: *Avsunviroidae*			
Avsunviroid	*Avocado sunblotch viroid*	-do-	No specific particles
Pelamoviroid	*Peach latent mosaic viroid*	-do-	No specific particles

Ap = Aphid; **Th** = Thrip; **Lh** = Leaf hopper; **Mb** = Mealy bug; **Wf** = Whitefly; **Bt** = Beetle; **Mt** = eriophyid mites; **s.per**: Semi persistent; **np** = Non persistent; **n.prop** = non-propagative; **per** = persistent; **cir** = circulative; prop = propagative; **Isom** = Icosahedron; **Icos =** Isometric particles; **Env** = Enveloped (unless stated, rest are non-enveloped); Genus unassigned to any family is in parenthesis **Important note:** Classification as per the ICTV – 8th Report (2005).

*Fauquet, CM, Mayo, MA, Maniloff, J, Desselberger, U, and Ball, LA. 2005. Virus Taxonomy: 8th Report of the International Committee on Taxonomy of Viruses. Elsevier Press (USA), 1162pp.
**Not in the 8th ICTV report.



Reproduced from Virus Taxonomy, ICTV 8th Report, Fauquet et al., 2005 (Elsevier Press)

2. Plant Virus Isolation and Purification

Isolation of a virus in its purest form from a diseased plant newly recognized in the field is called *isolation*. Obtaining virus in most pure form from the host plant is called *purification*. These two steps are prerequisite for characterization and identification of disease causative agent.

A) Isolation

In order to isolate a virus, certain aspects, such as means of its transmission, knowledge on vector, its spread in the field is advantageous. The virus from the diseased plant is isolated by sap inoculation to the healthy homologous and selected diagnostic/indicator host plants, using infected tissue sap extracted in water or buffer. If virus is not sap transmissible, virus culture is established by grafting or using vector, onto the homologous and other test plants. Inoculated plants are maintained in isolation to prevent contamination with other pathogens. The development of the disease in the laboratory inoculated plants indicates successful isolation of virus(es) from the field infected plants.

The second step is to check for the homogeneity of the isolated virus(es). Diseased plants in the field may contain more than one virus or strains of the same virus, and they need to be separated by inoculating the sample to a range of differential host plants and back inoculation to the original host to check for conformation of isolation of disease causing virus. Appearance of the disease with original symptoms indicates isolation of the virus involved in the disease etiology. The 'isolated' virus is purified by established cultures with sap prepared from single lesion (or individual vector) by transferring serially for 4 to 5 times on a suitable local lesion host or by exploiting different virus-vector transmission mechanisms. Such pure isolate can then be propagated on a suitable host for bulking the material for further investigations and purification.

Certain properties of the virus can be studied without purifying the virus. These include biological characters of the virus, such as longevity *in vitro* [in detached leaf, sap extract, lyophilized tissues]; virus stability and infectivity [sensitivity to organic solvents, thermal inactivation point]; host range [local lesion hosts, diagnostic hosts, propagative hosts, non-hosts]; modes of transmission [vector (arthropod, nematode, fungi) and non-vector (mechanical sap inoculation, grafting, contact, soil]; symptomatology [macroscopic symptoms (visual changes on the plants) and cytological (virus inclusions and cytological changes)] and observation of sap extracts for virus particles under electron microscope. These properties would aid in developing a method for virus purification and also provide clues to the virus identity.

B) Purification

Purified virus preparations are essential to study virus properties at biochemical level. Virus purification aims at the separation of virus from host constituents without affecting its structure and infectivity. Choice of purification method depends on the virus as well as host plant. The number of purification methods in use exceeds total number of virus species. Because different procedures are required to purify same virus from different host plants or for the strains of the same virus. Some knowledge on the virus being purified would aid in devising a suitable purification protocol and also provide indicators to monitor the quality and quantity of virus at various stages. Lack of any information, would sometimes result in unusually longtime to devise a suitable purification method.

The most common steps in the purification of the plant viruses are:

1) Establishment of biologically pure virus culture in a suitable propagation host.
2) Extraction of the cultured virus into a selected buffer medium that can protect virus from the deleterious effects of host components and retain virus infectivity.
3) Clarification of the extracted sap to remove as much of the host material with minimum loss of virus.
4) Concentration of the virus from the clarified extract by chemical precipitation or by differential centrifugation or by gel permeation/ affinity chromatography (for labile viruses) or combination of one or more of these methods.
5) Further purification of the virus by rate zonal or equilibrium density gradient centrifugation.
6) Final pellets of the virus obtained by high speed centrifugation are used to determine physico-chemical properties of the virus and its infectivity.

Virus purification is performed at low temperatures (usually 4^0C) to minimize the deleterious effects on virus particles.

(i) Extraction

The composition of the virus extraction medium (buffer molarity and pH, additives) should be

compatible to the host and also to virus and yield infective virus in high quantities. Buffers at high concentration (0.2-0.5 M) and pH of 7.0-9.0 are usually used for the initial extraction of the virus from the plant tissues. Additives that are generally incorporated into the extraction buffer are: β-mercaptoethanol, monothioglycerol, sodium sulphite, ascorbic acid, glutathione, EDTA and DIECA at different concentrations. Some times detergents like Triton X-100 and Tween-80 are used. On occasions protein denaturing agents such as urea or polyvinyl pyrilodine are included into the extraction or resuspension buffers to minimize the aggregation of virus particles. To release some viruses from host components it may be necessary to treat extracts with enzymes such as drysilase. Plant material is extracted in electric blenders in presence of the selected buffer.

(ii) Clarification

Following extraction, coarse host components are removed by different clarification methods. This include low speed centrifugation, filtration through a filter paper supporting a pad of celite, emulsification with organic solvents like chloroform, n-butanol or carbon tetrachloride, followed by centrifugation. Organic solvents are not used for the purification of enveloped viruses (if the aim is to isolate particles with intact membranes; otherwise only nucleoprotein particles of virus would result).

The virus present in the clarified aqueous extract can be concentrated either by precipitation of the virus with chemicals like ammonium sulphate or polyethylene glycol (PEG) or by differential high speed pelleting of the virus. In some cases, especially if virus is highly unstable clarification can be achieved by gel permeation/affinity chromatography. The concentrated virus is resuspended in a suitable buffer and subject to further purification.

The impurities present in the clarified extracts can be minimized by pelleting the virus through sucrose cushion. The virus obtained in this step may still contain pigments and plant molecules. Therefore, further purification of the virus is generally achieved by rate-zonal sucrose density gradient (usually 10-40% w/v) centrifugation (@26,000 rpm, 2hr.) or by equilibrium density gradient centrifugation in heavy salt gradients of cesium chloride or cesium sulphate at 25,000-30,000 rpm, over night. Depending upon the nature of the virus (mono-, bi-, multi-partite components) and associated impurities, clarified virus resolves as different light scattering zones. This separation is based on the sedimentation coefficient or particle buoyant densities. Virus from the light scattering zones are collected separately, and concentrated by centrifugation. Various tests are used to determine the infectious nature of the virus and its purity

(iii) Virus purity

The purity and virus yield vary with virus-host combinations. The virus purity usually examined by UV spectrophotometry, serology, electron microscopy, analytical ultracentrifugation and gel electrophoresis. If the purified virus contains impurities, preparations are subjected to second cycle of either rate-zonal or equilibrium density gradient centrifugation, followed by final high speed pelleting of the virus.

Infectivity of the purified virus can be assessed by inoculation on the host plants and also on diagnostic host. It is vital to inoculate the purified virus onto host plant and reproduce the disease to fulfill the Koch's postulates. Certain viruses, though intact loose infectivity during purification.

Purified virus can be stored for long term as aliquots at -20^0C or in lyophilized form. Some viruses are highly sensitive to freezing and thawing process. Such viruses are processed, immediately after purification, as per the need (denatured proteins or as nucleic acids) and virus components can be preserved for downstream applications.

Endnote

Virus isolation and purification is a complex process. Depending on the virus and host, it can be achieved in short period or sometimes it would take extremely long periods. Several factors can influence the ease with which virus isolation and purification can be achieved. Stable viruses that reach high concentration in host plants are easy to purify. Whereas some viruses are very difficult to purify, owning to their labile nature and occurrence in low concentration. Virus purification from herbaceous hosts (such as tobacco plants) is relatively simple due to low percent of host interfering material, whereas purification from woody plants are difficult due to hardy nature of the tissue, and to the deleterious host interfering material, such as polyphenols and tannins. There is no universal purification procedure that suits all viruses. Each and every virus and host system needs unique procedure to achieve optimum results.

3. ELISA and RT-PCR Methods for the Detection of Plant Viruses

Diagnosis is as much an *art* as it is *science*. The 'scientific' part is the technology used to detect pathogens. The art lies in the synthesis of information obtained from the case history, symptoms and results of laboratory tests to determine the virus(es) involved in inducing disease. Detection of a virus in a plant does not necessarily prove that the virus causes the disease. To establish that the virus detected causes the disease, Koch's postulates should be proved. Nevertheless constant association of a virus with a set of symptoms is often used as the '*proof*' that the virus detected causes the disease. Disease diagnosis based on symptoms is unreliable for the reason that different viruses may cause similar symptoms and that different symptoms may be induced by one virus. Many abiotic stresses and other pathogens such as phytoplasma may cause symptoms characteristic of virus infection. Even after one become familiar with the symptoms typically caused by a virus in a particular plant, it is essential to confirm the diagnosis with reliable methods.

Several factors influence the method to be used for virus detection. These include;

- Facilities and expertise available
- Type of virus suspected to be present
- Host plant
- Time available

Any detection method should be rapid and highly specific for the target virus, and should detect virus present in low amounts in the plant tissue and detection at an early stage of disease development.

Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most widely used virus detection methods because of their rapidness and
sensitivity. However, PCR-based methods require expensive laboratory equipment, whereas ELISA requires little or no special equipment and is particularly suitable for use in developing countries.

A) ELISA: A serology-based method
Principles of antibody production

An antigen is a molecule that can elicit production of antibodies when introduced into warm-blooded animals. Proteins, peptides, carbohydrates, nucleic acids, lipids, and many other naturally occurring or synthetic compounds can act as antigens, especially those having a molecular weight of 10,000 Daltons or higher with a definite molecular structure and which are not normal constituents of the animal being immunized. Antibodies are glycoproteins, which are produced as a result of immune response following introduction of antigens. Blood serum containing antibodies is referred to as antiserum.

When antigens are introduced, into an animal, a series of interactions between macrophages, T lymphocytes, and B-lymphocytes lead to antibody production. The first exposure of animals to antigens leads to a relatively weak reaction, referred to as the primary response. A series of specialized events occur during the primary response. These events prepare the animal to respond with quick and intense production of antibodies (secondary response) when the antigen is reintroduced. Both the primary and secondary responses occur in plasma cells. When antigens are first introduced, antigen presenting cells (APCs), (Langerhans cells in the skin, dendritic cells in the spleen and lymph nodes and monocytes in the blood), T cells and B cells act in concert to stimulate the production of antibodies. Many techniques for the preparation and introduction of antigens, such as selection of appropriate injection site (intramuscular, subcutaneous, intravenous, intraperitoneal etc.), mixing of antigen with adjuvants etc. influence the uptake of antigen by the APCs. Adjuvants act by protecting the antigen from being rapidly degraded in the blood stream, and they also contain substances that stimulate the secretion of host factors that facilitate the macrophage movement to the site of antigen deposition and increase the local rate of phagocytosis.

After an antigen is engulfed by APCs, it is partially degraded, appears on the cell surface of APC and binds to it with a cell-surface class II glycoprotein. In the next step, antigen-glycoprotein complex on the APC binds to T-cell receptors. This leads to T-cell proliferation and differentiation. While T-cells are proliferating, antigens are also processed by virgin B-cell lymphocytes in a similar manner as by APC's. However, the uptake of antigen by B-cells is specific, unlike that by APC'$_S$. As in the case of APC'$_S$, the antigen forms a complex with a surface antibody (Class II protein) on the B-cell surface. This complex also stimulates the same helper T-cells, which now bind to B- cells. This leads to division of B-cells and the production of the antibodies. Therefore the contact between B

cells and helper T-cells is a major event in the regulation of production of antibodies.

In order for a compound to be good antigen, it should possess one or more epitopes (an antigenic determinant of defined structure), which can bind to the surface antibody on virgin B cells. After the antigen is dissociated, each epitope should be able to bind simultaneously to both the Class II protein and T- cell receptor. Any epitope that is exposed is expected to stimulate strong response to antibody production.

Structure of immuno-gammaglobulins and function

Antibodies are glycoproteins present in the serum and tissue fluids of mammals. They are referred to as immunoglobulins (Igs) because of their role in adaptive immunity. Although all antibodies are immunoglobulins, it is important to realize that not all the immunoglobulins produced by a mammal have antibody activity. There are five classes of antibodies, IgG, IgM, IgA, IgE, and IgD, separated on the basis of the number of Y-like units and the type of heavy-chain polypeptide they contain. There are also significant differences within each class of gammaglobulins.

The basic polypeptide structure of the immunoglobulin molecule is shown in the Fig 1. It contains a unit of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulfide linkage. The class and subclass of an immunoglobulin molecule are determined by the type of heavy chain. The most common immunoglobulin is IgG and therefore the description given is for IgG.

IgG molecule contains one structural "Y" unit (Fig. 1). The two arms of Y are made of two identical light chains of molecular weight 23,000 daltons and two identical heavy chains of molecular weight 53,000 daltons. Each light chain is linked to the heavy chain by non-covalent bonds and by one covalent disulfide bridge. Each light-heavy chain pair is linked to another IgG by disulfide bridges between the heavy chains. Carboxytermini of the two heavy chains fold together and form the "FC" domain. The region between the Fab and Fc fragments is called the "hinge". Digestion of IgG with pepsin yields two Fab fragments attached to each other by disulfide bonds and an Fc fragment.

In both heavy and light chains, at the N-terminal portion, the amino acid sequences vary greatly from IgG to IgG. In contrast, in the Fc portion (C-terminal portion of both heavy and light chains) the sequences are identical. Hence the Fab domain contains "Complementary Determining Regions (CDRs)" or hypervariable regions. The six CDR's (three on either side of Fab) comprise the antigen combining site or "paratope" region of IgG. The antigen binds to IgG at this paratope region. The paratope is about 110 amino acid residues in length (both for light and heavy chain). The constant region of the light chain is also about 110 amino acids but the constant region of the heavy chain is about 330 amino acid residues in length.

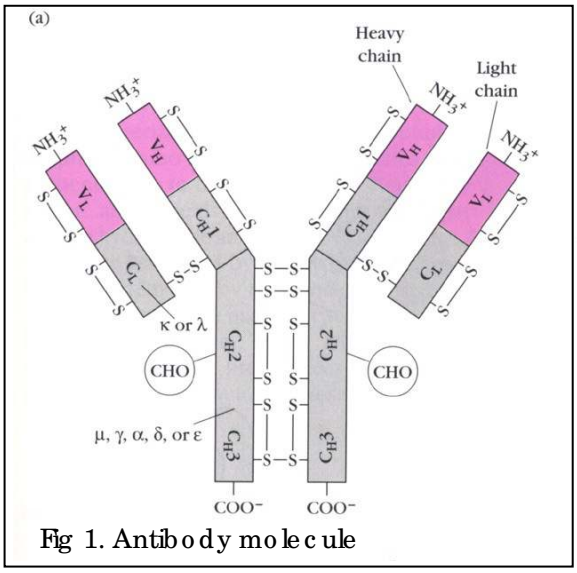


Fig 1. Antibody molecule

The antigen-combining site (paratope region in IgG) is a crevice between the variable regions of the light and heavy-chain pair. The size and shape of crevice can vary because of differences in the variable light and variable heavy regions, as well as differences in the amino acid sequence variation. Therefore specificity between antigen and antibody results from the molecular complementarity between determinant groups on the antigen (called "Epitope") and the paratope region of the IgG. A single antibody molecule has the ability to combine with a range of different antigens. Stable antigen-antibody complexes can result when there are a sufficient number of short-range interactions between both, regardless of the total fit. This interaction can be as a result of non-covalent bonds (hydrogen bonds, salt bridges, electrostatic charges), hydrophobic bonds, van der Waals' forces and so on. Therefore it is important to realize that the interaction between antigen and antibody is not covalent and therefore is reversible. Various factors such as pH, temperature,

detergents, and solvent conditions can influence these interactions.

Polyclonal antibodies
These are obtained from serum of an animal following injection with an antigen, which contains many antigenic sites. Therefore the antibodies produced react with more than one epitope.

Monoclonal antibodies
They are produced by a single antibody-producing B lymphocyte, immortalized either by mutation or fusion with a myeloma cell line. They react with a single epitope.

Production of polyclonal antibodies to viruses
If it possible to use both polyclonal and monoclonal antibodies (MAbs) for virus detection. Polyclonal antibodies are cheaper to produce than MAbs and also can be highly specific when made to highly purified antigen. Since polyclonal antibodies consist of heterologous populations of antibodies with variable sensitivities, they tend to be broadly specific and widely applicable to different serological tests. Therefore for routine virus detection polyclonal antibodies are highly suitable.

Preparation of virus antigens for antibody production
The viral genome can code for a number of proteins. Of all the proteins, the structural protein(s) [coat protein or capsid protein or nucleoprotein] or non-structural proteins, such as inclusion body proteins accumulate to a high concentration in the plants compared to other proteins encoded by the virus genome. The majority of antisera produced for plant viruses are to the coat protein(s). Inclusion body proteins can also be used for antibody production (eg. potyviruses).

The best source from which to obtain large quantity of coat protein is the purified virus, largely devoid of host plant components. Purification of viruses is accomplished by various physcio-chemical techniques. There are several important points to consider prior to purifying viruses from plants. They include selection of suitable host plant for virus maintenance, procedures for purification and methods for monitoring purity. The quality of the antiserum produced will depend largely on the purity of the virus preparation used for immunization.

Recombinant antigens
Recombinant DNA technology allows cloning of plant viral nucleic acids and express their genes in prokaryotic and eukaryotic systems. This facilitates large-scale expression of proteins *in vitro*. For this it is essential to know the sequence of protein encoding gene (for example, coat protein sequence, if the antibodies are to be produced to the coat protein). The gene of interest is inserted at a suitable site in an expression vector (eg. pET, pRSET) to express in *Escherichia coli*. This leads to production of virtually unlimited quantities of gene product of interest. Expressed protein can be purified and utilized in the production of antiserum.

Choice of animals
Any warm blooded animal can be used for antibody production e.g., Rabbits, chickens, guinea pigs, rats, sheep, goats and horses. When small animals such as rats and mice are used, only small quantity of serum can be obtained. Although large animals such as goats and horses can provide large volumes of serum, large amounts of antigen are required for immunizing these animals. The rabbit is the most commonly used animal for antibody production.

Immunization
Injection of an antigen into an animal is accomplished either by intramuscular or subcutaneous injections or intravenous.

For injection the antigen preparation should be emulsified with an adjuvant (1:1 proportion). The most commonly used adjuvant is Freund's adjuvant, which consists of paraffin oil and an emulsifier, mannide monooleate (incomplete). Complete adjuvants, in addition to these two components, contain heat-killed *Mycobacterium tuberculosis*, or *M. butyricurn* or a similar acid-fast bacterium. Emulsification with adjuvants results in very slow release of antigen, thereby stimulating excellent immune response. Antigen concentration required may vary from 100 μg/ml to 500 μg/ml. A normal immunization schedule followed for rabbits is given below.

- Four subcutaneous injections (multiple sites) at weekly intervals (for first injection use Freund's complete adjuvant and for the 2nd, 3rd and 4th use incomplete adjuvant). Five injections are usually adequate to obtain good immune response.
- If the titer of the antibody is low, either an intravenous (for intravenous injection adjuvants should not be used) or an intramuscular injection should be given as a booster.

Blood collection and serum preparation
Blood is collected from rabbits by making an incision in the marginal vein of the ear. It is preferable to collect the blood in sterile containers. The blood is allowed to clot at room temperature for 2 - 3 h (this can also be done by exposure at 37°C for 30 min). After overnight refrigeration, the serum is collected with a Pasteur pipette and then centrifuged at 5,000 rpm for 10 min.
Note: It is important to starve rabbits for at least 24 h before blood collection to minimize concentration of lipids

Storage of antisera
- For long-term storage of antisera at 4°C it is essential to add either glycerol (1:1) or. sodium azide to a concentration of 0.02%.
- In lyophilized form antisera can be stored at –20°C indefinitely for many years without losing potency.
- Antisera can be stored at –70°C.
- It is advisable to store serum in small aliquots of 1.0 ml or less.
- Antisera should not be frozen and thawed repeatedly. This leads to aggregation of antibodies thereby affecting antibody activity by steric interference of the antigen-combining site or by generating insoluble material, which may sediment during centrifugation.

Enzyme-linked immunosorbent assay
Enzyme-linked immunosorbent assays are solid-phase assays in which each successive reactant is immobilized on a plastic surface and the reaction is detected by means of enzyme-labelled antibodies. The principle of amplification of the reaction between viral antigens and their antibodies by utilizing an enzyme and its substrate, was described by Avrameas (1969). The microplate method currently being used widely for virus detection and the term ELISA was introduced by Voller *et al*. (1976).

ELISA is one of the most widely used serological tests for the detection of plant viruses because of its simplicity, adaptability and sensitivity. In this immunospecificity is recognized through the action of the associated enzyme label on a suitable substrate. ELISA detects only viral antigens and it does not give a measure of infective virus concentration.

The basic principle of the ELISA lies in immobilizing the antigen onto a solid surface, or capturing antigen by specific antibodies, and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction is detected by adding the suitable substrate. The enzyme converts substrate to product, which can be easily recognized by its colour. There are two types of ELISA procedures; 'direct' and 'indirect' ELISA. In the 'direct' procedure, IgG's extracted from virus-specific antiserum or in some cases polyclonal antiserum, are used for coating the solid surface to trap the antigen, and the same IgG's labelled with an enzyme are employed for detection. In this case the antigen gets sandwiched between IgG's and thus is referred to as the double-antibody sandwich (DAS) form of ELISA. The DAS-ELISA has limitations in that test is not suitable for (a) virus detection in disease surveys unless it is targeted to a specific virus, (b) when adequate antisera are not available for IgG extraction and conjugation and (c) for probing a single antigen with several different antisera.

In the simplest 'indirect' ELISA procedure, antigen is bound to the solid surface of ELISA plate. In the second step unconjugated antigen-specific detecting antibodies (primary antibody) is added. Primary antibody is detected by the enzyme-labelled second antibody (anti Fc or anti IgG). The second antibody is produced in a different animal than that used for producing primary antibody. The main advantage of the indirect ELISA procedure is that one enzyme conjugate (of antiglobulin antibody or protein A) can be utilized with all the systems. This assay is particularly suitable for (a) virus detection in disease surveys, (b) testing the presence of virus in seed and (c) for determining serological relationships, particularly when specific conjugates cannot be prepared. It is also more economical to perform than the DAS form.

Choice of antibodies
Antibodies produced in any experimental animal are suitable for ELISA. In some test procedures crude antisera can be used. For DAS-ELISA only purified IgGs can be used for conjugation with an enzyme. IgG's produced in a heterologous animal or second antibody (eg., anti-rabbit IgGs produced in goat) used in the 'indirect ELISA' procedure are commercially obtained.

Choice of antigens
One of the major advantages of ELISA is that it can be used on crude plant/insect extracts, and on partially purified and purified virus preparations.

Choice of enzyme labels

The two-enzyme labels that are widely used are alkaline phosphatase (ALP) and horseradish peroxidase (HRP). Urease and penicillinase (β-lactamase) have subsequently been introduced. Reaction kinetics of HRP is not linear and some of its substrates are hazardous to the operator. Urease and isozymes of peroxidase are known to be present in seeds and plant extracts, thus limiting their application in plant virus detection. ALP and its substrate, p-nitrophenyl phosphate, are very expensive and are not readily available in developing countries. ALP has certain limitations for use in the detection of viruses in insects.

Penicillinase has several advantages over the ALP system;

- It is less expensive than ALP and HRP
- Enzyme and substrate are available in some developing countries
- Penicilloic acid produced as a result of penicillinase activity on penicillin substrate is less toxic
- The substrate has longer shelf-life than the other enzyme substrates
- Visual reading of results is easier than for the ALP system
- Penicillinase is not known to occur in higher plants.

Penicillinase breaks down penicillin into penicilloic acid, and this is detected either by the rapid decolorization of a starch-iodine reagent or by utilizing acid-sensitive pH indicators.

B) PCR: A nucleic acid-based virus detection method

Nucleic acid-based methods

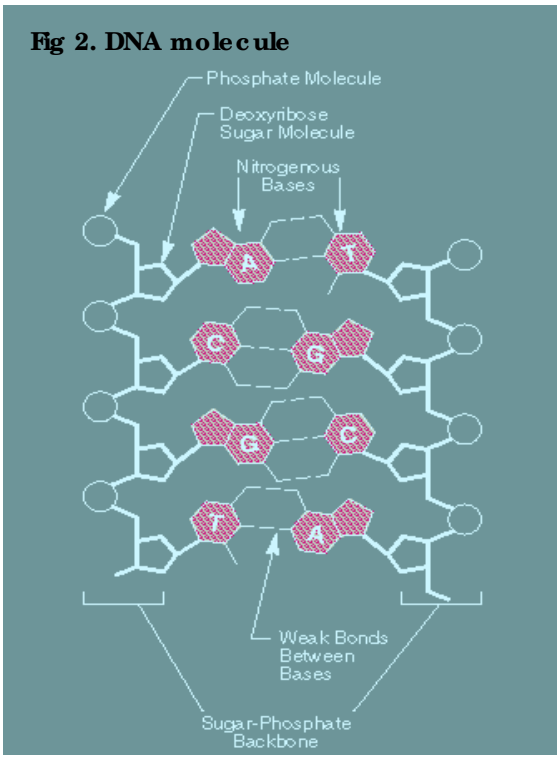
Serological methods have major disadvantage that they are based on the antigenic properties of the virus structural proteins**.** Thus immunological approaches ignore the rest of the virus genome. It is possible that viruses that are distantly related or not related, as determined by serological methods, may have highly conserved sequences in the genes other than the coat protein gene or that serologically related viruses may have very little sequence homology. In addition, there are instances where immunological procedures have limited application such as the detection of viroids, satellite RNAs, viruses that lack particles (eg. *Groundnut rosette virus*), viruses which occur as extremely diverse serotypes (eg. *Indian peanut clump virus*) and viruses that are poor immunogens or are difficult to purify. For these agents, detection is often possible only by using nucleic acid-based methods such as nucleic acid hybridization assays and PCR.

In instances where nucleic acid-based methods and serological methods provide similar information, detection sensitivity, and specificity, and are equally convenient, serological methods like ELISA be the preferred method. This is particularly so in developing countries because serological methods are easier to perform, cost effective and the required reagents are readily available.

The composition of nucleic acids

Nucleic acids are polynucleotides, i.e. they consist of nucleotides joined together in a long chain. Each nucleotide is made up of a base, a sugar and a phosphate group. The differences between DNA and RNA (i) the sugar is ribose in RNA but deoxyribose in DNA, (ii) the bases in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T) but in RNA the bases are A, C, G and Uracil (U) in place of T. In polynucleotide the bases are side branches on a 'backbone' chain made of alternating sugar and phosphate groups. The

Fig 2. DNA molecule



carbon atoms in the sugar molecule are numbered by convention. Thus the backbone is constructed by joining the 3' and 5' carbon atoms through a phosphate. As a result every linear nucleic acid molecule that has 5'-end usually terminating in a phosphate group and a 3' end, which usually terminates in a hydroxyl (OH) group.

Because of their structure, bases are able to join in particular pairs by hydrogen bonding. This is called base pairing. Adenine (A) will bond to T (in DNA) or U (in RNA) by making two bonds, G will bond to C by making three bonds. The bonds form between polynucleotide chains running in opposite direction (Fig. 2). The bonding can be with in a molecule, which will make a loop, or between separate molecules. When two sequences of nucleotides are able to base pair they are said to be complementary, the structure formed is double- stranded molecule. The process of two polynucleotides joining to form a double-stranded structure is called 'annealing' (renaturation), the reverse process, when chains separates to from a single stranded molecules, is called 'melting' (denaturation).

Polymerase chain reaction

The PCR provides a simple ingenious method to exponentially amplify specific DNA sequence by *in vitro* DNA synthesis. The three essential steps to PCR include (a) melting of target DNA, (b) annealing of two oligonucleotide primers to the denatured DNA strands and (c) primer extension by a thermostable DNA polymerase. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis as the three steps are repeated up to 35 times. The specificity of the method derives from the synthetic oligonucleotide primers, which base pair to and defines each end of the target sequence to be amplified. PCR has the power to amplify a specific nucleic acid present at an extremely low level, from a complex mixture of heterologous sequences. PCR has become an attractive technique to exploit for the diagnosis of viruses through the detection of the viral genome.

Basic PCR

PCR process amplifies a short segment of a longer DNA molecule. A typical PCR reaction includes thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphophstes (dATP, dGTP, dCTP and dTTP collectively termed dNTPs), reaction buffer, magnesium and optional additives and the template. The components of the reaction are mixed and the reaction is placed in a thermal cycler, which is automated instrument that takes the reaction through a series of different temperatures for varying periods of time. This series of temperatures and time adjustments is referred to as one cycle of amplification. Each PCR cycle doubles the amount of template sequence (amplicon) in the reaction.

Each cycle of PCR consists of initial denaturation of the target DNA by heating to >90^{o}C for 15 seconds to 2 min. In this step, the two intertwined strands of DNA separate from one another. In the second step, the temperature is reduced to approximately 45-60^{o}C. At this step oligonucleotide primers can form stable associations (anneal) with the separated target strands and serve as primers for DNA synthesis. This step lasts approximately 30-60 seconds. Finally, the synthesis of new (primer extension) DNA begins when the reaction temperature is raised to the optimum for the thermostable DNA polymerase, which is around 70-74^{o}C. This step lasts for 30-120 seconds depending on the amplicon size. This step completes one cycle. After 20-35 cycles, the amplified nucleic acid can be analyzed for size, quantity, sequence or can be used for further experimental procedures such as cloning.

PCR optimization

The following factors influence the amplification of products during PCR;

- Magnesium ion concentration
- Reaction buffer
- Enzyme choice and concentration
- Primer design
- Template
- Cycle parameters
- Nucleic acid cross-contamination

Magnesium ion concentration: It is the critical factor affecting the performance of *Taq* DNA polymerase. Reaction components, including template, chelating agents present in the sample (eg., EDTA), dNTPs and proteins, can affect the amount of free magnesium. In the absence of adequate free magnesium, *Taq* DNA polymerase is inactive. Excess free magnesium reduces enzyme fidelity and may increase the non-specific amplification. For this reason it is important to determine empirically, the optimal concentration of $MgCl_2$ for each reaction. This can be done by preparing a reaction series in 0.5 mM increments by adding 2, 3, 4, 5 or 6 μl of a 25 mM $MgCl_2$ stock to a 50 l reaction.

Reaction buffer: The basic ingredients of a PCR reaction buffer are; NaCl, KCl, EDTA, DTT, Triton X-100, Nonidet-P 40, Tween-20, glycerol and tris-HCl, pH 8. The composition of these components varies depending on the type of thermostable polymerase in consideration. The manufacturer supplies reaction buffer in 10x concentration along with the thermostable DNA polymerase. For most of the PCRs, use of this buffer at

recommended concentration yields good amplification.

Enzyme: The choice of the enzyme to use depends on the several factors. *Taq* DNA polymerase is the most popular thermostable DNA polymerase. This enzyme possesses relatively high processivity and is the least expensive enzyme. However, this enzyme lacks 3'-5' exonuclease (proof reading) activity and it has high error incorporation rate compared to other enzymes. For accurate amplification of the PCR product thermostable enzymes with proof reading activity are recommended (eg: *Pfu. Tli*).

Generally, 1 U of Taq DNA polymerase in a 50 μl reaction is sufficient for good yield of product. Inclusion of more enzyme does not significantly increase product yield. Further, this lead to likelihood of generating artifacts associated with 5'-3' exonuclease activity associated with *Taq* DNA polymerase resulting in smearing in agarose gels. Pipetting errors are the most frequent cause of excessive enzyme levels. Accurate dispensing of submicroliter volumes of enzyme solutions is difficult. We strongly recommend the use of reaction master mixes, sufficient for the number of reactions being performed to overcome this problem. The master mixes will increase the initial pipetting volumes of reactants and reduce pipetting errors.

Primer design: PCR primers (oligomers or oligonucleotides) generally range in length from 15-30 bases and are designed to flank the region of interest. Primers should contain 40-60% G+C and care should be taken to avoid sequences that would produce internal secondary structure. The 3'-end of the primers should not be complementary to avoid the production of primer-dimers in the PCR reaction. Ideally both primers should anneal at the same temperature. The annealing temperature is dependent upon the primer with the lowest melting temperature. Regardless of primer choice, the final concentration of the primer in the reaction must be optimized. We recommend adding 50 pmol of primer (1 μM final concentration in a 50 μl reaction) as a starting point for the optimization.

Template: successful PCR amplification depends on the amount and quality of the template. Reagents commonly used to purify nucleic acids (salts, guanidine, proteases, organic solvents and SDS) are potent inhibitors of DNA polymerases. The amount of template required for successful amplification is dependent upon the complexity of the DNA sample and depends on percent target DNA of interest. Too much of target DNA or too little, results in poor or no amplification.

Cycle parameters: The sequence of the primers is major consideration in determining the temperature of the PCR amplification cycles. For primers with a high melting temperature it may be advantageous to use high annealing temperatures. The higher temperature minimizes nonspecific primer annealing, increasing the amour of specific product and reduce primer-dimer formation. Allow a minimum extension time of 1 min for a cycle and increase it by a min for every 1 kb of amplicon (2 min extension for 2 kb target).

Certain unwanted reactions can occur in PCR, and these usually begin at room temperature once all components are mixed. These unwanted reactions can be avoided by incorporating 'hot start' method. In this thermostable enzyme is added into the reaction mixtures after heating the reaction minus enzyme to 90^{o}C. However, this method is tedious and can increase the chances of contamination.

Nucleic acid cross-contamination: It is important to take great care to minimize the potential for cross-contamination between samples and to prevent carryover of RNA and DNA from one experiment to another. Use positive displacement pipettes or aerosol resistant tips to reduce contamination during pippetting. Wear gloves and change them often. Wherever possible prepare master mixes by mixing all reagents and at the end, add template into the reaction tube.

RT-PCR

Most of the viral and sub-viral pathogens have RNA genome. In this case RNA is first reverse transcribed in order to produce a complementary (c)DNA copy using the enzyme reverse transcriptase and a primer. In the first cycle of PCR thermostable DNA polymerase synthesis complementary strand to the first strand cDNA. The resultant double stranded cDNA is amplified exponentially by PCR process.

RT-PCR uses *Moloney murine leukemia virus* (MoMLV) or *Avian myeloblastosis virus* (AMV) reverse transcriptase (RT). *Taq* DNA polymerase performs second strand cDNA and subsequent amplification during PCR. The viral RT enzymes are inactivated at elevated temperatures. Therefore first strand reaction must be performed at 37-48^{o}C. The maximum recommended temperature for optimum RT enzyme activity is 42^{o}C. Efficient first cDNA can be completed in 20-60 min.

RNA exhibiting significant secondary structure must be denatured for efficient reverse transcription. Generally, incubation at 42^{o}C for 45 min yields good yield of first strand cDNA. For RNA templates with high secondary structures, a denaturation step can be incorporated by incubating primers and RNA in a separate tube at 70^{o}C for 10 min, then quench on ice and proceed to RT step.

The purity and integrity of the total RNA extracted from the leaf tissue of interest is critical for successful and consistent results in RT-PCR. The extraction procedure for RNA isolation consists of (a) effective disruption of tissue, (b) inactivation of ribonuclease (RNase) activity and (c) separation of RNA from protein, carbohydrates, polysaccharides etc. It is very difficult to inactivate RNase and hence several precautions have to be followed to prevent RNA degradation due to RNase activity, during or after extraction. Use autoclaved solutions and baked glassware (bake in an overnight 200^{o}C overnight). Always use disposable gloves as a precaution against RNase in the fingertips. Include potent RNase inhibitors (SDS, guanidine thiocyanate, β-mercapthoethanol) in the extraction buffer to inactivate the enzyme and carry all steps at 4^{o}C to minimize RNase activity.

Controlling banana virus diseases through identification and elimination of infected planting material

A wide range of disease including fungal, viral, bacterial and nematode diseases affects banana production worldwide (Table 1). The four major diseases of concern to banana production in Africa are banana bunchy top disease, black sigatoka, bacterial wilt and Panama disease. Banana streak although is endemic in sub-Saharan Africa (SSA) but the virus (*Banana streak virus*) is not known to cause serious epidemics and pathogens responsible for bract mosaic (*Banana bract mosaic virus*), moko, blood, freckle and certain races of Panama disease are not present in SSA (Table 1). Specific details of banana diseases and pathogens can be found in literature elsewhere.

Tissue culture process eliminates pests like weevils and nematodes, bacterial and fungal pathogens through shoot tip culture, but viral pathogens are difficult to eliminate. Therefore, principal approach to control viruses is through indexing planting material to identify infected material and eliminate them from the subsequent use.

An effective indexing depends on reliable diagnostic methods that can lead to correct identification of the pathogen (Table 2). Various methods have been established based on the biological, serological and nucleic acid properties of the viruses and these methods are continuously improved and optimized. Specificity (accuracy to detect the target species/strain) and sensitivity (ability to detect lowest amount of target) are the two important attributes that determines the selection of test for virus indexing. Choice of test depends on the objective of the indexing: for instances tests aimed at detecting BSV should be sensitive and broad-specific to capture all the virus variants. Application of diagnostic test specific to one strain, for example to BSGFV, would not ensure freedom from other BSV strains. Similarly, strains specific diagnostics are essential for specific identification of Fusarium oxysporum Tropical Race-4, which is a quarantine pathogen of high importance. Since virus indexing is routinely performed for selecting material free of viruses, ideally broad-specific diagnostic tools that can capture all known strains/variants of the virus species are highly desired

Indexing for banana viruses is performed using bioassays (mechanical inoculation/grafting to indictor plants), enzyme-linked immunosrobent assay (ELISA), electron microscopy (EM), polymerase chain reaction (PCR) and nucleic acid spot hybridization (NASH). Choice of test for pathogen detection depends on host, virus (RNA or DNA genome), stage of testing and tissue material. Maximum sensitivity is the primary goal of the virus indexing programs (see Table 2).

Bioassays, although are useful for the detection of CMV and BBrMV, is seldom used because of poor sensitivity and risk of failure to detect virus in the potentially infected plants. ELISA and variants (DAS-ELISA, TAS-ELISA and PAS-ELISA) are the most frequently used assays for virus detection and such methods are available for the detection of CMV, BBrMV, BBTV and BSV. However, because of its relatively low sensitivity its use in reliable indexing of foundation/mother stocks is limited. PCR and variants (RT-PCR, IC-PCR and IC-RT-PCR) are increasingly adopted for banana virus indexing. PCR-based assays are available for the detection of all the banana pathogens (Table 2). Because of endogenous BSV sequences PCR do not provide reliable detection of episomal (infectious particles) form of BSV. Immuno-capture-PCR that first captures virus particles using BSV antibodies, followed by the PCR detection of the viral genome present inside the virus particles, has been the method of choice for indexing.

In this manual, methods commonly used for the detection of viruses in our research unit are provided.

Table 1. Continental distribution of quarantine pathogens of banana*

Disease	Pathogen	Africa	Asia	Australia & South Pacific	Latin America
Fusarium wilt	*Fusarium oxysporum* f. sp. *cubensem* Race 1 and Race 2	+	+	+	+
Sigatoka (yellow)	*Mycosphaerella musicola*	+	+	+	+
Sigatoka (black)	*Mycosphaerella fijiensis*	+	+	+	+
Banana streak	*Banana streak virus*	+	+	+	+
Banana mosaic	*Cucumber mosaic virus*	+	+	+	+
Mild mosaic / latent infection	*Banana mild mosaic virus*	+	+	+	+
Nk (latent?)	*Banana virus – X*	+	+	+	+
Bunchy top[1,2]	*Banana bunchy top virus*	+	+	+	-
Bacterial wilt[1]	*Xanthomonas capestris* pv. *Musacearum*	+	-	-	-
Eumusae leaf spot[1,2]	*Mycosphaerella eumusae*	+	+	-	-
Fusarium wilt[1,2]	*Fusarium oxysporum* f. sp. *Cubense* Sub-Tropical Race 4	+[3]	+[3]	+[3]	-
Fusarium wilt[2]	*Fusarium oxysporum* f. sp. *Cubense* Tropical Race 4	-	+	+	-
Freckle[2]	*Guignardia musae*	-	+[4]	+	?
Moko[2]	*Ralstonia solanacearum* Race 2	-	-	+	+
Blood[2]	*Ralstonia* sp.	-	-	+	-
Bract mosaic[2]	*Banana bract mosaic virus*	-	+	+	-
Mosaic[2]	*Abaca mosaic virus*	-	+	-	-

*These pathogens have very restricted spread even within the continents and countries of occurrence. + = present; - = absent; ? = unconfirmed report.
[1]Concern for exports from Africa; [2]Concern for imports from Africa;
[3]Reported in South Africa & Canary islands in Africa; Taiwan in Asia; and Australia.
[4]Reported from some islands in Pacific.
nk = not known; Banana virus- X was detected in germplasm collections. Information on natural distribution is not known

Table 2. Diagnostic methods for indexing pathogens in banana

Pathogen / disease	Culture-based	EM/ISEM	ELISA	PCR	RT-PCR	IC-PCR/ IC-RT-PCR	NASH
BSV (integrated)	na	na	-	+[3]	na	na	+
BSV (episomal)	-[1]	+[2]	+	+	na	+[3]	+
CMV	-[1]	+[2]	+	-	+[3]	+	+
BBrMV	-[1]	+[2]	+	-	+[3]	+	+
BBTV	na	+[2]	-	+[3]	na	-	+
AbMV	na	-	-	+[3]	na	-	+
FoC Race 1 & Race 2	+	na	-	+[3]	na	na	-
FoC TR 4	+	na	-	+[3]	na	na	-
BXW	+	na	-	+[3]	na	na	-
M. fijiensis *M. musicola* *M. eumusae*	+	na	-	+[3]	na	na	-

na= not applicable; + = suitable method; - = not suitable / available
EM = electron microscopy; ISEM = immuno-electron microscopy; NASH = Nucleic acid spot hybridization; PCR = polymerase chain reaction; RT-PCR = reverse transcription PCR; IC-RT-PCR = immunocapture RT-PCR; IC-PCR = immunocapture PCR

II. Laboratory Protocols

5. Bioassay by Mechanical Sap Inoculation for Virus Detection

Mechanical inoculation to a range of experimental hosts is the most widely used method for the detection and identification of viruses. It can be used to know about the presence or absence of virus, to assess the infectivity of the virus and nucleic acids in a variety of sources (leaf sap / plant tissue extract / purified virus preparations / nucleic acids purified from the virus preparations / total nucleic acids extracted from the infected leaves / extracts of vectors, etc.). Bioassays are performed on host plants that produce local lesions, however, use of systemic hosts are not uncommon. In local lesion host the number of lesions produced is governed by the infective virus particle concentration in and thus this test can also be used as quantitative assay to determine the number of infectious units present in the inoculum.

Bioassays can only be used with viruses that can multiply in epidermal and mesophyll cells, those viruses transmitted in non-persistent manner by insect vectors and those that reach to moderate concentration can be transmitted by mechanically sap inoculation (eg. *Cucumber mosaic virus*). Viruses that are restricted to conductive tissues, that exists in low concentration and those transmitted in persistent manner cannot be transmitted by mechanical inoculations (eg. *Banana bunchy top virus*). Generally, sap transmissible viruses produce, mosaic, mottling, ringspots, necrotic spots and general necrosis and chlorosis on the infected leaves. Non-mechanically transmissible viruses are restricted to xylem and they cause yellowing symptoms, vein clearing and stunting. Most of the mechanically transmissible viruses contain positive polarity genome (eg. *Cucumber mosaic virus*). Viruses with negative polarity genomes are difficult to transmit by mechanically sap inoculation and requires special care (eg *Tomato spotted wilt virus*). Virus stability *in vitro*, and host components also influence the rate of mechanical transmission. Some mechanically sap transmissible viruses have wide host range (eg. *Cucumber mosaic virus*) and some viruses have narrow host range (eg. *African cassava mosaic virus*). All these factors influences application of bioassays by mechanical inoculation for virus detection, yet this assay is very useful tool in identifying viruses in the planting material whose phytosanitary status is unknown.

This assay is performed on test plants that are dusted with abrasives [carborundum (silicon carbide), corundum (aluminium oxide), celite (diatomaceous earth)] to induce micro-wounds to facilitate virus particle entry. Extracts are applied to test plants with fingers or pads made with muslin cloth or cotton swab. Inoculated plants produce symptoms if infectious virus particles are present in the source plant.

Leaf tissue or pseudostem sap of *Musa* plants can be used for bioassays by mechanical sap inoculation. CMV, BBrMV and other sap transmissible viruses infecting banana can be detected by this assay. However, it is not useful for the detection of BSV and BBTV, which are phloem limited and occur in low-concentration. Due to the availability of other easy-to-use sensitive virus detection tools, bioassays are seldom used for detecting viruses in banana.

Materials

- Pestles and mortars
- Muslin cloth
- Carborundum or corundum
- Chemicals for appropriate buffers

Inoculation buffer

Choice of inoculation buffer depends on the virus and host system. Most commonly used inoculation buffer composition is given here.

Phosphate buffer (0.05 M)

To 1L dH_2O add 2.4 g of $KH_2PO4$2.4g; 5.4g of K_2HPO4; and 0.75 ml of thioglycerol (or 1.56 ml of β-mercaptoethanol). Not necessary to adjust pH (if compounds are accurately measured) and store the buffer at 4°C.

Selection of experimental hosts
Choice of plants depends on the virus in question. Cowpea (*Vigna unguiculata* cv Ife Brown), *Chenopodium* spp, *Nicotiana* species are commonly used for this purpose. For list of host plants to select against each group of viruses refer Matthews (1993). Always select healthy looking plants raised in well-fertilized soils.

Choice of infected tissue and inoculum preparation
Select young infected tissues showing clear symptoms. Young leaves contain high virus concentration and less inhibitory substances. Ensure that source is not contaminated with other pathogens (eg. fungi). Using a mortar and pestle macerate leaf tissue to fine homogenate under chilled conditions, using cold inoculation buffer. Usually for every 100 mg leaf tissue 1 ml buffer is used (1:10 w/v). Apply this inoculum immediately onto the leaves of abrasive dusted test plants.

Procedure
1. Dust abrasive (corundum or carborundum or celite) sparingly on leaves of the test plants to be inoculated.
2. Use disposable gloves to inoculate plants. If not, wash hands thoroughly with soap.
3. Make sure that mortar and pestle used for preparing inoculum is clean and free of any residual virus contaminants from previous experiments.
4. Support the leaf to be inoculated with one hand and apply inoculum on the leaf with fingers of other hand or muslin cloth or thick end of a pestle or with cotton swab.
5. Inoculate at least 5 plants per each treatment. Label the pots containing the test plants or plants individually with date and inoculum details.
6. Rinse the inoculated leaves with tap water and cover the plants with sheets of paper (old news papers) overnight.
7. Wash hands thoroughly with soap (or with trisodium phosphate when highly infectious are handled) and then with water.
8. Soak mortars and pestles used for inoclum preparation in trisodium phosphate or 1% bleach solution overnight to eliminate any residual virus contaminants.

Observations
- Monitor test plants regularly and record time and appearance of first symptoms and the symptom type (mosaic, ringspots, necrosis, chlorosis etc).
- Observe for symptoms on inoculated leaves and newly produced leaves on the plants
- When local lesions are produced record their diameter and colour, concentric rings or haloes
- Observe for systemic symptoms such as vein clearing, mosaic, line patterns, chlorosis, leaf deformation, puckering, enations, etc.
- Check both inoculated leaves and newly produced leaves on the plants by ELISA or by back inoculating on to the local lesion assay plant as described above.

Classification of hosts:
- Symptoms only on the inoculated leaves: *Local lesion hosts*
- Symptoms on inoculated leaves as well as newly developed leaves: *Systemic host*
- No symptoms on inoculated leaves, but symptoms on newly developed leaves: *Systemic host*
- No symptoms on inoculated leaves or on newly developed leaves. Assay both leaves by ELISA using antibodies of virus in question.
  - Inoculated and newly developed leaves are ELISA-negative: *Non-host*
  - Only inoculated leaves are positive: *Asymptomatic local host*
  - Both inoculated and newly developed leaves test positive: *Asymptomatic systemic host*
    Note: Asymptomatic infections should be confirmed by back inoculation with the sap extracted from such hosts, onto symptom producing hosts.

Note 1: Symptom development depends on the virus incubation period in each host. Usually it would be 4-20 days depending on virus and host. Some viruses would take long time (>40 days) to produce symptoms. At that stage of the crop growth, symptoms may not appear clearly, and/or are difficult to notice due to growth related changes.

Note 2: Some viruses are erratically distributed in plants; leaves on one or few branches may produce symptoms. Careful monitoring is essential in such cases.

Note 3: Symptoms can be enhanced by pruning the plants. New growth of pruned plants generally shows good symptoms.

Note 4: Record symptoms on each host by taking colour and black white photographs with contrasting and neat background.

Precautions

- Good greenhouse facilities are required to maintain test plants rose for the biological assays. At the least, test plants should be kept in cages protected with wire-mesh to control insets.
- Only seeds obtained from genetically pure-lines should be used for this assay to avoid confusion due to symptom variation as result of genetic impurity.
- When highly infectious viruses such as *Tobacco mosaic virus*, tested, all the equipment should be soaked in sodium hypochlorite overnight, they should be washed and autoclaved prior to next use. Inoculated plants should be kept well away from other plants to avoid contact transmission.
- Care should be taken to avoid contact transmission by handling such plants with gloves.

5.2. Virus Detection by Enzyme-linked Immunosorbent Assay (ELISA)

The basic principle of ELISA technique involves immobilizing the antigen onto a solid surface or captured by specific antibodies bound to the solid surface and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction is detected by adding a suitable substrate. The enzyme converts the substrate to a product, which can be recognized by its colour. ELISA is performed in several different formats depending on the target virus and tissue material. The Direct Antigen Coating (DAC) [also known as Plate Trapped Antigen (PTA)] ELISA is the most commonly used format of ELISA.

Leaf tissue or pseudostem sap of *Musa* plants can be used for virus detection by ELISA. Although, ELISA-based methods are useful for the detection of CMV, BSV, BBrMV, BanMMV and BBTV in plants but these are less sensitive than PCR-based detection methods. Several ELSIA procedures have been described for the detection of viruses infecting bananas. Methods we routinely used in our lab are described below.

6.1. Direct antigen coating (DAC)-ELISA [*Cucumber mosaic virus* (CMV) as example]: This is the simplest of ELISA, and also referred as Antigen Coated Plate (ACP)-ELISA. Antigen is bound to the plate surface. In the second step, polyclonal antiserum (primary antibody usually produced in a rabbit or mice) or IgGs are used to detect the trapped homologous antigen (Fig. 1). Primary antibody is detected by the enzyme-labeled secondary antibody produced in a different animal (goat). Then enzyme substrate is added to detect the positive reactions. The main advantage with DAC-ELISA is one secondary antibody (anti-rabbit or anti-mice) can be utilized with several systems. This is the most widely used assay. However, certain virus cannot be detected by DAC-ELISA (such as luteoviruses).

Polyclonal antibodies to CMV have been produced in a rabbit. In the first step, virus antigen in leaf sap extract bind to the plate. In the second step, primary antibody (anti-CMV (rabbit) antibodies) is used to detect the plate-bound CMV antigen. In the third step, secondary antibody, the alkaline phosphatase (ALP)-labelled anti-rabbit (goat) antibodies, are used to detect the positive reactions. Enzyme substrate, p-nitro phenyl phosphate (PNPP) is added into the ELISA wells to develop positive reactions. Substrate turns to deep yellow in case of strong positive reactions, and it remains colorless to light yellow in negative and weak reactions, respectively. In ALP system colour difference between positive reaction and negative reaction is difficult to read visually. Plates must be read in an ELISA plate reader fitted with a 405 nm filter for accurate assessment of the results.

Experiment procedure

Materials

- **ELISA plates:** Several brands are available. 'Nunc-Maxisorp' plates are recommended.
- **Micropipettes:** 1-40 µl, 40-200 µl and 200-1000 µl single channel pipettes. 40-200 µl multichannel pipette. Several brands are available (eg: Eppendorf, Finpipette, Gilson). Those with adjustable volumes are preferable.
- **ELISA plate reader:** Manual or automatic fitted with 405 nm filter.
- CMV polyclonal (rabbit) antibodies; Alkalinephosphotase (ALP)-labelled anti-rabbit (goat) antibodies. Whole molecule (available from several commercial companies, eg. SIGMA)
- Mortars and pestles; Muslin cloth; pH meter; p-nitrophenyl phosphate (PNPP); Light box; Incubator

Solutions

Carbonate buffer or coating buffer, pH 9.6

Na_2CO_3 1.59 g
$NaHCO_3$ 2.93 g
Distilled water to 1 l [No need to adjust pH]

Note: Add diethyldithiocarbamate (DIECA) at 1.71 M concentration (1.71 gm for 1 L) when this buffer is used for extracting antigen. DIECA is not necessary for coating antibodies. Store buffer at room temperature.

Phosphate buffer saline (PBS), pH 7.4

Na_2HPO_4	2.38 g
$KH_2\ PO_4$	0.4 g
KCl	0.4 g
NaCl	16.0 g
Distilled water	to 2 l

No need to adjust the pH

Phosphate buffered saline Tween (PBS-T)

PBS	1 l
Tween-20	0.5 ml

Antibody buffer (PBS-TPO)

PBS-T	100 ml
Polyvinyl Pyrrolidone (PVP) 40,000 MW	2.0 gm
Ovalbumin (Sigma Cat. No. A5253)	0.2 gm

Distilled water - Tween (dH_2O-T)

Distilled water	2 l
Tween 20 (0.05% v/v)	1 ml

Substrate buffer (diethanolamine buffer) for ALP system

Prepare 10% diethanolamine in distilled water and adjust pH to 9.8 with con.HCl. Store at 4 °C.

Substrate solution

Prepare 0.5 mg/ml p-nitro phenyl phosphate (PNPP) in 10% diethanolamine, pH 9.8 (for each 15 mg table 30 ml substrate buffer is required). This solution should be prepared fresh prior to use.

> **Note:** Diethanolamine is toxic and harmful to eyes. Take necessary care to avoid contact with skin. PNPP convert to p-nitrophenol after reacting with ALP. p-nitrophenol is corrosive and thus plates after adding substrate must be handled extremely carefully.

Precautions

- Perform all incubation steps in a humid chamber to provide uniform temperature [a small plastic box suitable to fit ELISA plate, with moist paper towels covering the bottom of the box].
- Use new ELISA plates as supplied by the manufacturer. Do not wash or rinse them prior to use.
- In each plate always include positive (eg. CMV infected sample), negative (eg. healthy banana) and buffer controls.
- Unless antiserum used is very high quality, cross adsorption of crude antisera with healthy plant extract is recommended for primary antibodies used for DAC-ELISA. This is done by grinding healthy leaves in 1:50 dilution (W/V) buffer, then filter it by passing through musclin cloth. Use this for diluting the antiserum appropriately and incubate by shaking at room temperature or at 37 °C for 1h and use it for coating into wells of ELISA plates.
- Working dilution of antibodies depends on various factors and is usually established by the antibody developer. Users must use antibodies at dilutions specified by the supplier.

Cross-adsorption of virus-specific antiserum

To minimize the non-specific reaction as a result of presence of antibodies to host plant antigen, cross-adsorption of virus specific antibodies with homologous healthy leaf extract is strongly recommended. For this, collect fresh healthy leaves and grind in antibody buffer to give 1:10 w/v dilution (10 mg/ml), then filter through a double layer of muslin cloth. Use this extract for preparing the required dilution of anti-virus antibody or enzyme conjugate. This step reduces the non-specific reaction due to neutralization of antibodies to host antigen. **Note:** Cross-adsorbed enzyme conjugates can be stored for a maximum period of 1 weeks at 4 °C. However, fresh extracts are recommended.

DAC-ELISA Procedure

1. **Coating ELISA plates with antigen:** Grind test leaves (banana) in carbonate coating buffer at a rate of 100 mg/ml buffer (1:10 w/v) and dispense 100 µl into each well of the new ELISA plate. Incubate the plate in a humid chamber for 1 h at 37 °C or in a refrigerator (4 °C) overnight.
2. Wash the plate with three changes of PBS-T, allowing 3 min for each wash.
3. Prepare 1:10 (w/v) healthy cowpea leaf extract in PBS-TPO for cross-adsorption of CMoV polyclonal antibodies. Dilute CMV polyclonal antiserum to 1:10,000 (this dilution depends on the CMV serum batch) in health cowpea leaf extract and incubate at room temperature or 37ºC for 30 to 60 min with gentle shaking. Dispense 100 µl of this into each well of the ELISA plate. Cover the plate, place it in a humid chamber and incubate at 37 °C for 1 h or in a refrigerator (4 °C) overnight.
4. Wash the plate with three changes of PBS-T, allowing 3 min for each wash.
5. Depending on the detection system (ALP), select the anti-rabbit enzyme conjugate and substrate system.

Detection using ALP-system

1. Dilute anti-rabbit ALP-conjugate to 1:15,000 (v/v) (this dilution depends on the conjugate batch) in PBS-TPO and dispense 100 µl of this into each well of the ELISA plate. Keep the plates in a humid chamber and incubate at 37 ^{0}C for 1 h or in a refrigerator (4 ^{0}C) overnight (note: conjugate after incubation can be collected for reuse at least 3-4 times, if stored properly at 4 ^{0}C, within a period of 2 weeks from the date of first use)
2. Wash the plate with three changes of PBS-T, allowing 3 min for each wash.
3. Add 100 µl of pNPP substrate into each well and cover the plates and incubate in dark at room temperature. (**Note:** Substrate solution turns yellow when exposed to light for long time)
4. Observe plate on X-ray film light box for recording color changes. Results recorded after long intervals (>4 hrs) may not be accurate. Overnight incubation at 4 ^{0}C can also be allowed for ALP system. Measure absorbance at 405 nm in an ELISA plate reader. **Note:** Absorbance value of the positive reaction will be higher than negative reactions.
5. In the case of positive reaction the colourless-substrate will turns to light yellow and then to deep-yellow colour. Light yellow colour indicates week positive and deep- yellow indicates a strong positive. The reaction can be stopped by adding 50 µl of 3M NaOH per well.
6. Samples with A405nm values twice or more than healthy sample reading are considered as virus positive.

6.2. Triple Antibody Sandwich (TAS)-ELISA

1. TAS-ELISA is commonly used for the detection of BSV and BBTV. This method is also applicable for the detection of other viruses, including CMV.
2. Dilute polyclonal antibodies for BSV/ BBTV in coating buffer at 1:1000 (or antibody dilution recommended by the supplier) and dispense 100 µl into each of ELISA plate wells.
3. Cover plate and incubate at 37°C for 2 hours (or overnight at 4°C).
4. Wash plate three times at three minutes interval with PBS-T and tap dry.
5. Prepare 5% skimmed milk in PBS-T and add 200 µl/ well of the solution to block any empty sites. Cover and incubate plate for 30 minutes at 37°C
6. Then wash the plate PBS-T as detailed before.
7. Grind 01. to 0.2 g fresh *Musa* leaf in 1 ml PBS-T containing 2% polyvinyl pyrrolidone and 1% sodium sulphite and add 100 µl of ground banana sap into microtitre plate well
8. Incubate plate at 37°C for 2 h or overnight at 4°C
9. Dilute mouse monoclonal IgG for BSV/ BBTV in PBS-T at 1µg/ml (or dilution specified by the supplier) and dispense 100 µl per microtitre plate well and incubate at 37°C for 2 h.
10. Wash plate three times with PBS-T as described before.
11. Prepare goat anti-mousse alkaline phosphatase conjugate at 1:6000 (v/v) (or dilution specified by the supplier) in conjugate buffer, and dispense 100 µl into wells of the ELISA plate.
12. Incubate the plate at 37 °C for 2 hours, and wash plates with PBS-T as described before.
13. Incubate the plate at room temperature and take readings after 1, 2, 3 hours and overnight

14. For overnight reading, keep plate at 4°C and take reading 30 minutes after exposure at room temperature the following day.
15. Perform subsequent steps as described previously (section: Detection using ALP-system) and record the results.
16. An A_{405} reading twice the value of the corresponding healthy control is considered as positive.

6.3. Protein A-Sandwich ELISA (PAS-ELISA)

Protein A derived from the *Staphylococcus aureus* has high affinity to Fc region of the antibody molecule. ELISA plates coated with Protein A bind with Fc portion of the antibody introduced subsequently into the wells leaving the Fab portion to bind the antigen introduced in further step. This method is used to increase the specificity and sensitivity of the ELISA system for the detection of viruses occurring in low-titer in plants, such as *Banana streak virus* (BSV) which is used as an example for this type of ELISA.

1. Dilute Protein A (purchased from SIGMA chemicals) at 1 µg/ml in coating buffer.
2. Dispense 100 µl of diluted protein A into wells of a 96 well micro-titre plate and incubate the plate at 37°C for 2 h (or 4°C overnight).
3. Wash plate three times with PBS-T as described before.
4. Dilute BSV (CMV or BBTV) rabbit polyclonal antibody at 1:1000 (or as specified by the supplier) in PBS-Tween and dispense 100 µl into the wells of the ELISA plate.
5. Incubate the plate at 37°C for 2 h.
6. Wash plate three times with PBS-T as described before.
7. Grind banana leaf sample in extraction buffer at a ratio of 1:10 (w/v) and dispense 100 µl of test sap to each well and incubate plate at 37 for 2 °h or overnight at 4°C.
8. Wash plate three times with PBS-T as described before.
9. Dispense 100 µl of BSV (or CMV or BBTV, depending on the virus in question) polyclonal antiserum diluted at 1:1000 (or as specified by the supplier) in PBS-T.
10. Incubate the plate at 37°C for 2 h.
11. Wash plates three times with PBS-T as described before.
12. Prepare anti-goat alkaline phosphatase conjugate at 1:6000 (v/v) (or dilution specified by the supplier) in conjugate buffer, and dispense 100 µl into wells of the ELISA plate.
13. Incubate the plate at 37 °C for 2 h, and wash plates with PBS-T as described before.
14. Keep plate at room temperature and take readings after 1, 2, 3 hours and overnight
15. For overnight reading, keep plate at 4°C and take reading 30 minutes after exposure at room temperature the following day.
16. Perform subsequent steps as described previously (section: Detection using ALP-system) and record the results.
17. An A_{405} reading twice the value of the corresponding healthy control is considered as positive.

6.4. Virus Testing in Banana Seed Samples

Banana streak virus and *Cucumber mosaic virus* are known to be transmitted through true seed of *Musa*. In order to assess seed for virus-infection it is recommended to germinate the seed in screenhouse and test the germinated plants for CMV, BSV and any other virus by ELISA / PCR-based methods. Direct detection of viruses in *Musa* seed is not practicable. This testing is possible for relatively large seeds with soft tissue, such as groundnut, and thus it has limitations for application in banana.

> **Note 1:** ELISA/PCR procedures described in previous/subsequent sections are suitable for testing *Musa* seedlings for CMV and BSV.
> **Note 2:** It is advised to test all the seed received. If the lot consists of too many seeds (>100), test 10% of randomly selected seeds and release virus tested plants.
> **Note 3:** The level of seed transmission of each virus varies (it ranges from 0.1 – 50% depending on the virus and host). This factor influences the final outcome.

Grow-out test.

1. Sow the seed in a contained greenhouse or field.
2. Monitor the germinated plants for any symptoms and growth abnormalities at regular intervals. Common symptoms of virus infection in *Musa* are, mosaic, streaks (continuous/non-continuous brown or white), chlorotic streaks on pseudostem and bunching.
3. After 4 – 5 weeks of germination, collection small amount of leaf or pseudostem tissue and test for virus(es) by ELISA or PCR described in other sections.
   Note: If the plants are free from virus – seed lot will be approved for release or they will be permitted for seed production in quarantine zone. Seed thus produced will be approved for release.

Note: In every ELISA test plate make sure to incorporate healthy (*Musa* plant known to be free of viruses), positive (*Musa* plant known to be infected with the virus being assayed) and buffer controls. At the end of the experiment, healthy and buffer control reading should be lower and positive control should react with the antibodies. Positive reaction in healthy and/or buffer control and negative reaction in virus infected control (positive control) suggests error in the test.

Table 1. Summary of various steps involved in different ELISA formats*

ELISA type	Step – 1	Step – 2	Step – 3	Step – 4	Step – 5	Step -6
Direct DAC-ELISA	Antigen (virus) coating	**Enzyme-labelled virus specific rabbit antibody**	Substrate			
DAC-ELISA	Antigen (virus) coating	Virus specific rabbit antibody	**Enzyme-labelled anti-rabbit, goat antibody**	Substrate		
DAS-ELISA	Virus specific rabbit antibody	Antigen (virus) coating	**Enzyme-labelled virus specific rabbit antibody**	Substrate		
TAS-ELISA	Virus specific rabbit antibody	Antigen (virus) coating	Virus specific mouse antibody	**Enzyme-labelled anti-mouse, goat antibody**	Substrate	
TAS-ELISA (*FAB*)	Virus specific rabbit ***FAB*** antibody	Antigen (virus) coating	Virus specific rabbit antibody	**Enzyme-labelled anti-rabbit, goat antibody**	Substrate	
Direct PAS-ELISA	Protein A coating	Virus specific rabbit antibody	Antigen (virus) coating	**Enzyme-labelled virus specific rabbit antibody**	Substrate	
PAS-ELISA	Protein A coating	Virus specific rabbit antibody	Antigen (virus) coating	Virus specific rabbit antibody	**Enzyme-labelled virus specific rabbit antibody**	Substrate

Notes: Step 1 must be performed using carbonate coating buffer and all other steps must be performed using antibody buffer (PBS-TPO) unless recommended otherwise. For substrate step, use recommended buffer based on the type of substrate. Wash plates between each step with PBS-T. Recommended incubations period are 37°C for 1 h minimum.
FAB = fraction antibody binding region of the IgG.
*Only few of these formats are described in this manual.

Fig. 1
CMV detection by DAC-ELISA using ALP-System

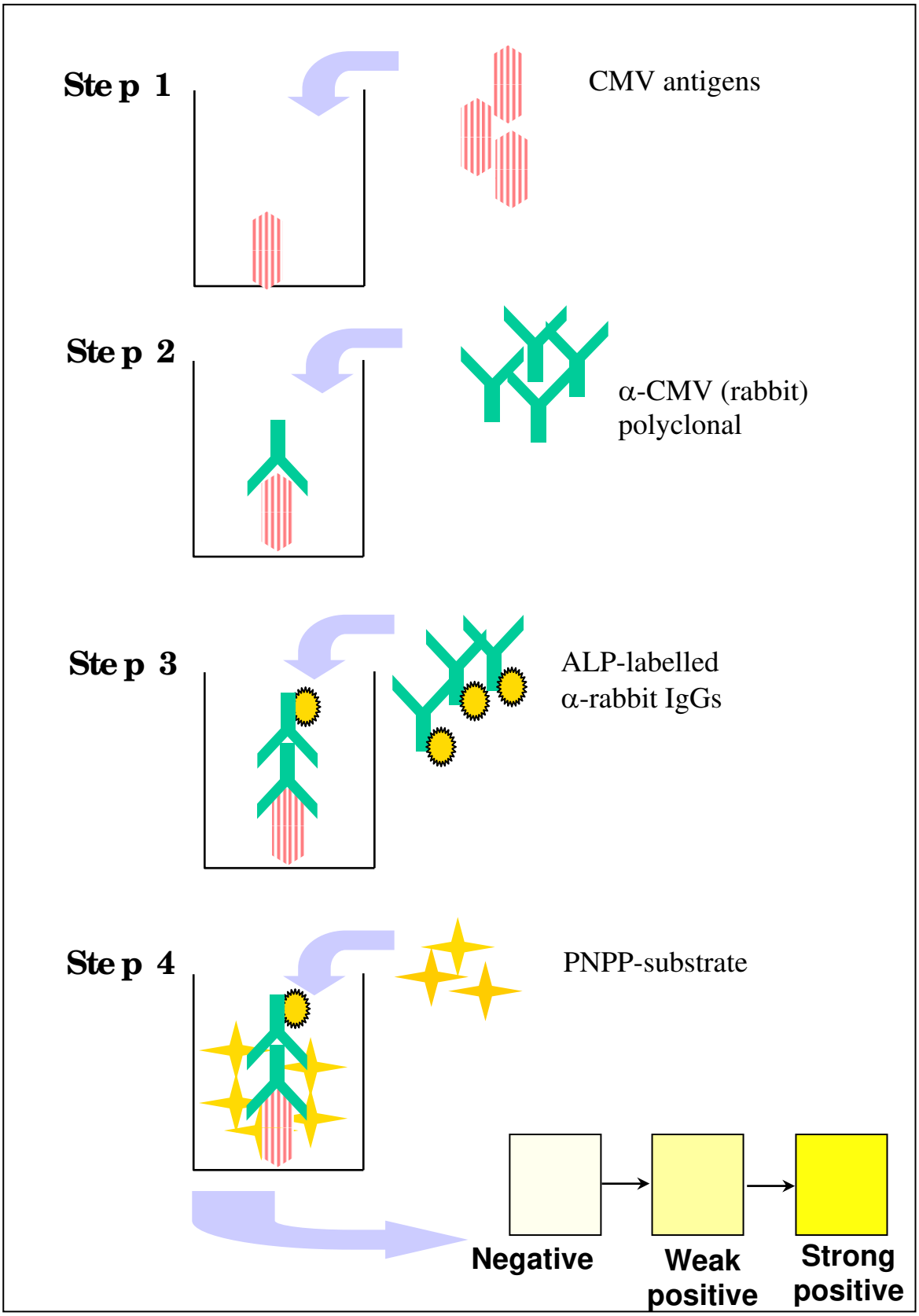
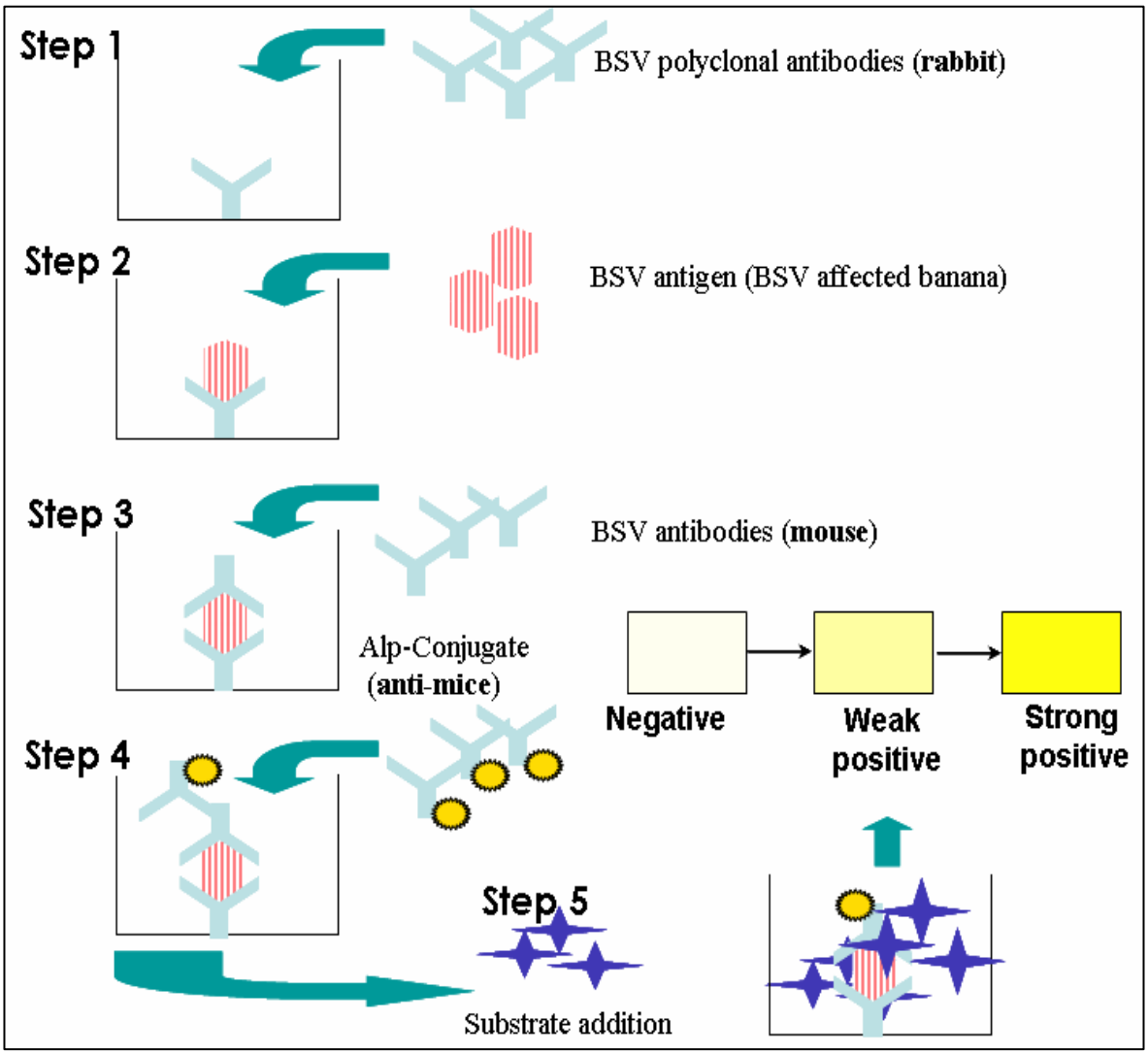


Fig. 2. BSV Detection by TAS-ELISA



7. Nucleic Acid-based Methods

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. This involves repeated cycles of heat denaturation of the DNA, annealing of primers to the complementary sequences and extension of the annealed primers with thermostable DNA polymerase (*Taq* polymerase) in the presence of four deoxyribonucleotides (dNTPs). Since the extension products are complementary to and capable of binding primers, subsequent cycles of amplification double the amount of target DNA synthesized in the previous cycle (Fig. 1). The result is exponential accumulation of the specific target DNA. Reverse Transcription (RT)-PCR is used for the amplification of RNA. In this case, first RNA is converted into single stranded DNA known as complementary DNA or cDNA in a process termed as reverse transcription by the enzyme reverse transcriptase. During the first cycle of PCR, a second strand of the DNA is synthesized from the first-strand cDNA. The resultant dsDNA copy is then amplified *in vitro* by PCR by the simultaneous primer extension of complementary strands of DNA, as in PCR. Since the extension products are complementary to and capable of binding primers, subsequent cycles of amplification double the amount of target DNA synthesized in the previous cycle (Fig. 1). The result is exponential accumulation of the specific target DNA of interest, which in essence has originated from RNA.

PCR is used for amplification of genome of DNA containing viruses (eg. *Banana bunchy top virus* and *Banana streak virus*). RT-PCR is used for the amplification of viruses containing RNA as their genome (eg. *Cucumber mosaic virus, Banana bract mosaic virus, Banana mild mosaic virus* and *Banana virus X*). During RT-PCR, the target RNA is first reverse-transcribed to a complementary DNA (cDNA) copy using the enzyme, reverse transcriptase (RT). PCR is also used for the detection of fungi, bacteria, nematodes and other pests and pathogens of banana.

PCR-based assays require nucleic acids (DNA or RNA) which will serve as templates for primers to amplify the target molecule. Therefore, the first step always is isolation of DNA or RNA. We also perfected methods that can bypass nucleic acid extraction step. Various procedures used commonly in our Unit are given below. Numerous protocols 100s of protocols have been described in the literature for the same purpose. Users are advised to make appropriate selection. Depending on the equipment, reagents and enzymes numbers variants of PCR-based methods have been described for the amplification and detection of targeted molecules. Users are advised to refer to pertinent literature for further details.

7.1. Isolation of total RNA from leaf tissue

Obtaining high quality intact RNA is the first and the critical step in performing RT-PCR. Many procedures are currently available for the isolation of total RNA from prokaryotes and eukaryotes. The essential feature of any protocol is to obtain large amount of intact RNA by effectively lysing the cells, avoiding the action of contaminating nucleases, in particular RNase. RNA isolation is difficult when processing certain tissues like banana, which is rich in polyphenols, tannins, polysaccharides and nucleases making it difficult to get clean RNA preparations. The protocols described here for RNA isolation from banana are being used successfully for RT-PCR.

Precautions

- Use autoclaved solutions, glass- and plastic ware.
- Always wear disposable gloves as a precaution to avoid RNase contamination.
- Where possible use DEPC-treated water.

1. Isolation of total RNA using Qiagen plant RNeasy RNA isolation kit

This kit is designed to isolate high quality total RNA from small amounts of starting material. The procedure is simple and fast (<30 min). In this procedure, leaf material is first lysed and homogenized in the presence of a denaturing buffer, which rapidly inactivates the RNase to ensure isolation of intact RNA. Ethanol is added to the lysate to provide appropriate binding conditions and the sample is then applied to an RNeasy minicolum built with a silica-gel-based membrane. Total RNA binds to the membrane and contaminants are efficiently removed. High-quality RNA is then eluted in distilled water.

Materials

- QIAGEN Plant RNeasy mini kit (Genetix, New Delhi, India)
- Variable speed microcentrifuge (table top model)
- Sterile 1.5 ml and 2 ml eppendorf tubes
- Sterile mortars and pestles
- Liquid nitrogen
- Absolute ethanol (molecular biology grade)

Procedure

1. Grind 100 mg of leaf material under liquid nitrogen to a fine powder using a mortar and pestle.
2. Transfer the tissue powder to a 2 ml eppendorf tube.
3. Add 450 µl of RLT buffer (supplied with the kit) and 5 µl of µ-monothioglycerol (or µ-mercaptoethanol) and mix vigorously (in a vortex shaker).
4. Transfer the lysate into the QIAshreder spin column (supplied with the kit) and centrifuge for 2 min at maximum speed (14,000 rpm) in a microcentrifuge.
5. Transfer flow-throw fraction (lysate) from QIAshreder to a new 2 ml tube without disturbing the cell-debris pellet.
6. Add 0.5 volumes (usually 250 µl) of absolute ethanol to the lysate and mix well by pipetting.
7. Apply the sample into an RNeasy mini spin column (supplied with the kit) and centrifuge for 15 sec at 10,000 rpm.
8. Discard the flow-throw.
9. Add 700 µl of RW1 buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
10. Discard the flow-throw
11. Add 500 µl of RPE buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
12. Discard the flow-throw.
13. Repeat the steps 11 and 12.
14. Transfer the RNeasy column into a new 1.5 ml collection tube and centrifuge for 1 min at 10,000 rpm to dry the RNeasy membrane.
15. Transfer RNeasy column into a new 1.5 ml tube and add 30-50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge at 10,000 rpm for 1 min to elute RNA.
16. Store RNA at –20 ^{0}C.

2. Isolation of total RNA by phenol-chloroform method

This is a relatively inexpensive procedure to separate RNA from proteins and other contaminants. In this RNA from leaf extract is selectively partitioned into the aqueous phase after extracting in the presence of phenol-chloroform. RNA from aqueous phase is precipitated in the presence of salt by adding 2.5 volumes of ethanol.

Materials

- Sterile mortars and pestles
- Sterile eppendorf tubes 0.5 ml, 1.5 ml and 2 ml
- **1 M Tris-HCl, pH 8.0 :** Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to 8.0 with conc. HCl. Adjust volume to 1 l with distilled water. Sterilize by autoclaving. Store at room temperature
- **0.1 M Trish-HCl, pH 7.6:** Dissolve 12.11 g of Tris base in 800 ml of distilled water. Adjust the pH to 7.6 with conc. HCl. Adjust volume to 1 l with distilled water. Sterilize by autoclaving. Store at room temperature
- **10% SDS:** Dissolve 10 g of sodium dodecyl sulfate (SDS) in 1 l of autoclaved distilled water. Warm to assist dissolution of SDS. No need to sterilize by autoclaving. Store at room temperature
- **0.5 M EDTA:** Add 186.1 g of EDTA to 800 ml water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8 with 1 M NaOH (EDTA dissolves in solutions above pH 8). Make up to 1 l with distilled water. Sterilize by autoclaving. Store at room temperature
- **3 M sodium acetate:** Dissolve 24.612 g of sodium acetate in 80 ml distilled water. Adjust the pH to 5.2 with glacial acetic acid. Adjust volume to 100 ml. Sterilize by autoclaving. Store at room temperature

- **Phenol:chloroform:** Mix equal amounts of redistilled phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris-HCl, pH 7.6. Store the mixture under 0.01 M Tris-HCl pH 7.6 at 4 ^{0}C in a dark bottle.
  Caution: Phenol is highly corrosive, can cause severe burns and is carcinogenic. Wear gloves and protective clothing when handling phenol. Any areas of skin that comes in contact with phenol should be rinsed with a large volume of water. DO NOT USE ETHANOL. Carry all steps involving phenol-chloroform in a fume hood. Care must be taken in disposing phenol-chloroform solutions.
- DEPC-treated water.
- Chloroform: Isoamyl alcohol (IAA) (24:1 v/v) mixture: To 96 ml of chloroform add 4 ml of IAA. Store the bottle at 4 ^{o}C.

Procedure

1. Grind 150 mg leaf material in liquid nitrogen to a fine powder.
2. Add 1 ml of extraction buffer (0.1 M Tris-HCl, pH 8.0 containing 2% SDS and 2 mM EDTA) and 1 ml of phenol-chloroform mixture (1:1 v/v).
3. Transfer the contents into a 2 ml eppendorf tube, vortex vigorously and then heat the samples at 70 ^{0}C for 5 min.
4. Centrifuge at 12,000 rpm for 10 min in a microcentrifuge.
5. Collect the upper aqueous phase carefully and add equal volumes of phenol-chloroform mixture and vortex vigorously.
6. Centrifuge at 12,000 rpm for 5 min.
7. Take the upper aqueous phase carefully and add equal volumes of chloroform and vortex vigorously.
8. Centrifuge at 12,000 rpm for 5 min.
9. Carefully collect the upper aqueous phase and to this add 1/10 (v/v) 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold absolute ethanol. Store at –20 ^{0}C overnight.
10. Centrifuge at 12,000 rpm for 10 min. Carefully discard the supernatant. Rinse the pellet with 70% ethanol. Carefully discard the supernatant.
11. Dry the pellet at room temperature and resuspend the pellet in 100 µl of RNase-free water and store at –20 ^{0}C.

3. Isolation of total RNA using TriZol® Reagent:

Materials

- Sterile mortars and pestles
- Sterile eppendorf tubes 0.5 ml, 1.5 ml and 2 ml
- Table top microcentrifuge
- TriZol® Reagent (GIBCO, Invitrogen Croporation Inc.)
- Chloroform (molecular biology grade)
- Isopropanol
- 75% (v/v) ethanol

Procedure

- ◊ In a sterile mortar, ground 100 mg leaf tissue to fine powder using liquid nitrogen. Immediately transfer the powder into 2 ml microcentrifuge tube and add 1 ml of TriZol® reagent.
- ◊ Vortex the tube thoroughly and incubate for 5 min at room temperature.
- ◊ Add 200 µl of chloroform into the mixture and shake the tube vigorously for 15 sec and incubate for 3 min at room temperature.
- ◊ Centrifuge the tube at 12,000x g for 15 min. Collect the upper aqueous phase into a fresh 2 ml sterile tube. To this add 500 µl of isopropanol and incubate for 10 min at room temperature.
- ◊ Centrifuge tubes at 12,000x g for 10 min. Discard the supernatant and add cold 75% (v/v) ethanol and centrifuge at 7,500x g for 5 min.
- ◊ Discarded the supernatant. Air dry the RNA pellet by keeping at 37^{o}C for 10 min and dissolve the pellet in 20 µl of RNase-free water by passing the solution a few times through a pipette tip and if required heat at 50^{o}C for 10 min. Then store sample at -20^{0}C

4. CTAB method for RNA Extraction (Maruthi et al., 2010, J. Vir. Met 163:353-359)

Reagents

CTAB extraction buffer
100mM Tris-HCl, pH8
1.4M NaCl
20mM EDTA
2% CTAB
0.2% 2-mercaptoethanol (add prior to use)
Store the buffer at room temperature

For other items, see previous sections.

Procedure
1. Grind 100 mg (w/v) leaf tissue thoroughly in a 1 ml of CTAB buffer
2. Transfer extract into 1.5 ml eppendorf tube, mixed and incubated at 60 ◦C for 10 min.
3. To this extract add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vortex it, and centrifuge at 12,000×g for 10 min.
4. Collect the supernatant into fresh 1.5 ml tuber and add 0.6 (v/v) volumes of ice cold isopropanol.
5. Incubate the samples at −20 ◦C for at least 1 h.
6. Centrifuge tubes at 12,000×g for 10 min and carefully discard the ethanol and add 0.5 ml ice-cold 70% ethanol into the tubes and centrifuge it for 5min.
7. Carefully discard the ethanol and air dry the tubes (or incubate at 37°C for 10 min) to remove the last traces of ethanol.
8. Dissolve the pellet in 1x TE buffer and stored at −20 ◦C for further analysis.

Note: Depending on the user choice, availability of reagents and virus in question, any of the above methods can be chosen by the user

7.2. Procedure for the isolation of total DNA for virus detection by PCR

Suitable for DNA extraction from young leaves of cassava, coco, cowpea, maize, musa, rice, soybean and yam.

Reagents & Buffers:
- Extraction buffer
  100 mM Tris (pH 8.0)
  500mM NaCl
  8.5 mM EDTA
  (Sterlize by autoclaving)
  10 mM β-mercaptoethanol (add just before use)

- TE Buffer
  10 mM Tris, pH 8.0
  1 mM EDTA
  (Sterilize by autoclaving)

- 2 ml or 1.5 ml microfuge tubes
- 95% (v/v) Ethanol in sterile distilled water
- 70% (v/v) Ethanol in sterile distilled water
- 5M potassium acetate
- Iso-propanol
- Micropipettes (10 – 1000 µl)
- Mortars and pestles (sterilize in autoclave prior to use)
- Table top centrifuge (12,000 or more rpm)
- Refrigerator
- Waterbath & Vortex mixer

Procedure:

1. Grind about 50 – 100 mg of young material in 1 ml of extraction buffer
2. Transfer contents into a microfuge tube
3. Add 66 µl of 10% SDS and vortex and incubate in 65°C water bath for 10 min
4. Allow tubes to cool to room temperature and then add 160 µl of 5M potassium acetate
5. Vortex and centrifuge at 10,000 *g* (or 12,000 rpm) for 10 min.
6. Collect supernatant into a separate microfuge tube
7. Add 200 µl of cold iso-propanol and mix gently and incubate on ice or at 4°C for 20 min.
8. Centrifuge at 10,000 *g* for 10 min to precipitate DNA
9. Carefully decant the supernatant without disturbing the pellet (whitish to cream-colour pellet can be seen at the bottom; sometime pellet may not be visible; pellet can be slimy and slide)
10. Add 500 µl of 70% ethanol into the tubes and centrifuge at 10,000 *g* for 5 min.
11. Carefully decant the ethanol to the last drop, without disturbing the pellet (whitish to cream-colour DNA pellet can be seen at the bottom; sometime pellet may not be visible; pellet can be slimy and slide)
12. Allow the tubes to dry at room temperature or at 37°C to remove final traces of ethanol (it takes about 10-15 min).
13. Resuspend DNA pellets in 50 µl of TE buffer and store tubes at -20°C until further use.
14. For very long term storage: To DNA containing microfuge tubes, add 5M Potassium acetate to a final concentration of 0.5 M and 2.5 volumes of 95% ethanol, mix well and store at -20°C. To recovery DNA, centrifuge tubes at 12,000 *g* (or 14,000 rpm) for 15 min, decant ethanol as stated in step 10; and continue steps 11-14.

7.3. Extraction of Total Nucleic Acid (TNA)

This method provides both DNA and RNA templates for PCR reaction

Procedure:

1. Grind 1 g frozen banana sample in a chilled mortar and pestle using 2 ml extraction buffer and centrifuge the extract for 2 min at 12,000 *g.*
2. Collect 300 µl of supernatant and add 30 µl of 10% SDS and 600µl phenol/chloroform/iso-amyl alcohol (25:24:1).
3. Vortex mixture for 1 min and centrifuge at 12,000*g* for 5 min
4. Collect upper aqueous phase and add equal volumes of phenol/chloroform/iso-amyl alcohol and spin at 12,000*g* for 5min.
5. Collect aqueous phase and add 3M sodium acetate to a final concentration of 0.3 M and 2.5 volume of ice-cold 95 % ethanol and store tubes at -20°C for 1 h. Then centrifuge at 12,000*g* for 10min.
6. Carefully decant the ethanol and add 0.5 ml of 70% ethanol and centrifuge at 12,000*g* for 5 min. Carefully decant the ethanol and air dry tubes at room temperature or in 37°C to remove final traces of ethanol.
7. Dry pellet at room temperature and resuspend in 20 – 30 µl sterile TE buffer and store the sample at -20C or -70°C till used.
8. Take out 1 µl of this stock and dilute to 1:10 (v/v) or 1:50 (v/v) ssolve in 50 µl TE buffer and use for PCR or RT-PCR applications.
9. Primer sequences include:

7.4. Direct Sample Preparation for PCR/RT-PCR Assays

GEB buffer, pH 9.6

Na_2CO_3	1.59 g
$NaHCO_3$,	2.93 g
PVP-40 (2%)	20 g
Bovine serum albumin (BSA) (0.2%)	2 g
Tween-20 (0.05%)	0.5 ml

Sterile distilled water to 1 Liter
Sterilize by filteration and store this buffer at 4ºC

GES buffer, pH 9.0

0.1M glycine	7.507 g
50mM NaCl	2.922 g
1mM EDTA	0.372 g

Adjust pH to 9.0, and make the volume to 955 ml with distilled water. Sterilize by autoclaving. Then add 5 ml of Triton X-100 to (0.5% v/v) and store this buffer at 4ºC. Prior to use, add 2-mercaptoehtanol to a final concentration of 1% (v/v).

Procedure for preparing tissue samples for PCR/RT-PCR:

Step 1: Grind leaf tissue at a ratio of 1:20 (w/v) in GEB buffer. Use this extract immediately or distribute into aliquots and store at −80/-20ºC for subsequent use.

Step 2: Take 5 μl of the extract from step 1 (stored extracts should be thawed and mixed well) and mix with 25 μl of GES buffer. Vortex and heat denature at 95ºC for 10 min in a water bath or heating block. Then place tubes on ice for 5 min. Use 2-4 μl of this preparation as template in PCR or RT-PCR reactions.

Procedure for preparing FTA samples for PCR/RT-PCR:

Step 1: Punch 0.5 cm FTA card sample (make sure to take punch from area where samples are spotted) and soak it in 500 μl of GEB buffer in a microfuge tube for about 15-30 min at room temperature, with occasional shaking/vortexing. Use this extract immediately or store the tubes at -20ºC for subsequent use.

Step 2: Take 5 μl of the extract from step 1 (stored extracts should be thawed and mix well) and mix with 25 μl of GES buffer. Vortex it and heat denature at 95ºC for 10 min (in a water bath). Then place tubes on ice for 5 min. Use 2-4 μl of this preparation as template in PCR or RT-PCR reactions.

8. Polymerase Chain Reaction (PCR)

PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. This involves repeated cycles of heat denaturation of the DNA, annealing of primers to the complementary sequences and extension of the annealed primers with thermostable DNA polymerase in the presence of four deoxyribonucleotides (dNTPs). Since the extension products are complementary to and capable of binding primers, subsequent cycles of amplification double the amount of target DNA synthesised in the previous cycle. PCR is used for the detection of BSV and BBTV and RT-PCR is used for the detection of CMV, BBrMV, BanMMV and BVX.

Materials

Thermal cycler
Sterile 0.2 ml Eppendorf tubes
Oligonucleotide Primes (see table 2)
Taq Polymerase (supplied by several commercial companies)
100 mM stock of four deoxynucleotide triphosphates (dATP, dGTP, dCTP, dGTP)

Solutions

dNTP mixture

Mix 10 µl of each dATP, dCTP, dGTP and dTTP from a 100 mM stock into 60 µl of sterile water. The final concentration of each dNTP in this mixture is 10 mM.

25 mM $MgCl_2$

Usually provided along with Taq enzyme.

8.1. PCR for the detection of *Banana bunchy top virus* (BBTV)

PCR offers the most sensitive detection for BBTV in banana plants. The following procedure is routinely used in our lab for BBTV detection. This protocol uses internal control primers and increases the robustness of the results.

Template: Extract total DNA using any of the procedures described in the previous sections

PCR primers

BBTV primers; from Rep-coding:
Virion sense: BBTV-1: 5'GCGTGAAACGCACAAAAGGCC 3'
Complementary Sense: BBTV-2: 5' GCATACGTTGTCAAACCTTCTCCTC 3'
Results in 239 bp product

Internal controls (primers for host DNA; for Brep repetitive genes in Musa spp.)
BrepF: 5' GATTTTGTAGATTTTGGACACCG 3'
BrepR: 5' GAATAACAAATATGCTCCAATACCC 3'
Results in 401 bp product

PCR reaction mixture

PCR reaction mix (All the reagents are from Promega; other commercially available enzymes can also be used). Add the following in a sterile 0.2 ml Eppendorf tube:

5X PCR Buffer*	2.5 µl
25mM $MgCl_2$*	2.4 µl
10mM dNTPs	0.25 µl
10 µM BBTV-1 Primer	0.25 µl
10 µM BBTV-2 Primer	0.25 µl
BrepF**	0.25 µl
BrepR**	0.25 µl
Taq Polymerase	0.06 µl
Templated DNA	1 or 2 µl

Sterile double distilled water to final volume of 12.5 µl

*Some buffers are pre-mixed with $MgCl_2$. In such case, do not add $MgCl_2$ into the PCR mix.

**Internal control primers are optional, but we recommend you to include in the reaction mix. Internal control primers are designed to host genome, and they amplify host DNA. Amplification on internal control primers suggests successful PCR and increases the confidence of the results.

Tightly cap the tubes and keep them in a thermal cycler installed with the following PCR program.

PCR cycling conditions:
94°C for 5 min: One cycle
94°C for 45 sec: 50°C for 30 sec: 72°C for 30 Sec: for 35 cycles
72°C for 5 min: one cycle

Analysis of PCR products
Analyze 8-12 μl of PCR products in a 1.5% agarose gel as described in the next section.

Note: Poor template DNA, especially when isolated from decomposed material results in non-specific bands and sometimes no amplification.

Precautions: PCR is a highly sensitive technique. Care must be taken to avoid cross contamination to prevent false amplifications. The following tips aids for better PCR

- Autoclave all solutions used in PCR. This degrades any extraneous DNA to very low molecular weight oligomers.
- Divide reagents into aliquots to minimize the number of repeated samplings necessary.
- Avoid splashes by using tubes which do not require much effort to open and collecting the contents to the bottom by brief spinning.
- Use positive displacement pipettes with disposable tips, preferably plugged at the top.
- Prepare master reaction mixture by premixing all reagents except DNA. Add DNA at the end.
- Always use a positive control (infected/disease control; sample that must result in DNA amplification), a negative control (healthy control; sample that should not result in amplification) and buffer control (water or buffer as template; there should not be any amplification in this sample).

8.2. Reverse Transcription (RT)-PCR for the detection of *Cucumber mosaic virus*

Precautions
PCR and RT-PCR is a highly sensitive technique. Care must be taken to avoid cross-contamination and carryover of template, to prevent false amplifications. The following tips may help in getting good results with RT-PCR.

- Autoclave all solutions used in PCR. This degrades any extraneous DNA/RNA and nucleases.
- Divide reagents into aliquots to minimize the number of repeated samplings necessary.
- Avoid splashes by using tubes, which do not require much effort to open and collect the contents to the bottom by brief spinning before opening the tubes.
- Ensure that all the reaction components are added as per the required concentration. Failure would result in blank PCR gel.
- Use positive displacement pipettes with disposable tips.
- **Wherever possible, prepare master reaction mixture by premixing all reagents except template. Distribute into individual reactions then add the template directly into each tube.**
- Always use a positive control (known positive) and a negative control (no 'template' control) to ensure the specificity of the RT-PCR reaction. A successful RT-PCR should give amplification in positive control and there should not be any bands in negative control and buffer control.
- Note: The procedure for two steps RT-PCR given below is generally applicable for the detection of most viruses. However, users are strongly advised to use specific protocol recommended for the detection of specific virus.

Materials

- Thermal cycler
- Sterile 0.2 ml, 0.5 ml and 1.5 ml Eppendorf tubes

- Oligonucleotide primers (select based on the virus)
- Template RNA
- Moloney murine leukemia virus-RT (MoMLV-RT. Cat.# M1701, Promega)
- RNase inhibitor (Rnasin Cat.# N251A, Promega)
- Dithiothreitol (DTT) (Sigma grade)
- *Taq* Polymerase (Cat.# M668, Promega)
- Four deoxynucleotide triphosphates, 100 mM stock (Promega, Cat.# U1330)
  (dATP, dGTP, dCTP, dGTP)
- RNase free water
- Mineral oil (optional)
- Crushed ice
- Micropipettes (1-10 μl, 1-40 μl, 40-200 μl and 200-1000 μl single channel pipettes).

Solutions

RNase free water

Treat distilled water with 0.1% diethylpyrocarbonate (DEPC; Sigma) for 12 h at 37 ^{0}C. Then autoclave for 15 min at 15 lb/sq.in to destroy DEPC.

Caution: DEPC is a suspected carcinogen and should be handled with care.

Note: DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris. Autoclaving degrades DEPC and therefore is safe to use DEPC-treated autoclaved water for preparation of Tris buffers.

10 mM dNTP mixture

Mix 10 μl of each dATP, dCTP, dGTP and dTTP from a 100 mM stock and makeup to 100 μl with RNase free water. The final concentration of each dNTP in this mixture is 10 mM.

25 mM $MgCl_2$

Usually supplied with *Taq* enzyme by the manufacturer.

If necessary, prepare by dissolving 0.508 g of $MgCl_2.6H_20$ in 100 ml RNase-free water. Sterilize by autoclaving, aliquot and store at -20 ^{0}C.

Note: Magnesium chloride solution can form a gradient of different concentrations when frozen. Therefore vortex well prior to using it.

0.1 M DTT

Dissolve 154 mg of DTT in 10 ml of RNase-free water, aliquot and store at -20 ^{0}C

RT-PCR procedure

Template: Isolate total RNA using any of the methods described for RNA extraction in the previous section.

Primers

CMV 1 - 5' GCC GTC AGC TGG ATG GAC AA – 3'
CMV 2 - 5' TAG GAT AAG AAG CTT GTT TCG CG- 3'
Results in about 485 bp product

RT-PCR reaction mix: Add the following in the 0.2 ml tubes.

5X reaction buffer (supplied with the enzyme)	2.5μl
10mM dNTPs	0.25μl
10mM CMV-1 Forward Primer	0.25μl
10mM CMV-2 Reverse Primer	0.25μl
Reverse transcriptase (100 U)	0.06μl
Taq Polymerase (1 U)	0.06μl
Sterile distilled water	8.38μl
Total volume	12.5 μl

Tightly cap the reaction tubes and keep them in a termal cycler installed with the following PCR program.

RT-PCR programme cycle 1
42 ^{0}C for 30 min: One cycle
95 ^{0}C for 1 min: One cycle
95 ^{0}C for 1 min; 55^{0}C for 1 min; 72^{0}C for 1 min: 35 cycles
72C ^{0}C for 5 min: One cycle

Analysis of RT-PCR products
Analyze 8-12 μl of PCR products in a 1% agarose gel as described in the next section. CMV primers results about 485 base pair product.

8.3. RT-PCR for the detection of *Banana bract mosaic virus*

(Rodoni et al: Plant Dis. 81:669-672, 1997)

Template: Isolate total RNA using any of the methods described for RNA extraction in the previous section.

Primers
Bract1: 5' GAC ATC ACC AAA TTT GAA TGG CAC ATG G 3'
Bract2: 5' CCA TTA TCA CTC GAT CAA TAC CTC ACA G 3'
Results in 604 bp product

RT-PCR reaction mix: Add the following in the 0.2 ml tubes.

5X reaction buffer (supplied with the enzyme)	2.5μl
10mM dNTPs	0.25μl
10mM BanCP-1 reverse primer	0.25μl
10mM BanCP 2 forward primer	0.25μl
Reverse transcriptase (100 U)	0.06μl
Taq Polymerase (1 U)	0.06μl
Sterile distilled water	8.38μl
Total volume	12.5 μl

Tightly cap the reaction tubes and keep them in a termal cycler installed with the following PCR program.

RT-PCR programme cycle 1
42 °C for 45 min: One cycle
94 °C for 3 min: One cycle
94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s: 35 cycles
72 °C for 10 min: One cycle

Analysis of RT-PCR products
Analyze 8-12 μl of PCR products in a 1% agarose gel as described in the next section. CMV primers results about 604 base pair product.

8.4. RT-PCR for the detection of *Banana mild mosaic virus*

(Teycheney et al: J. Gen. Virol. 86: 3179–3187, 2005)

Template: Isolate total RNA using any of the methods described for RNA extraction in the previous section.

Primers
BanCP1: 5' GGA TCC CGG GTT TTT TTT TTT TTT TTT 3'
BanCP2: 5' TAT GCN TTY GAY TTC TTR GAY G 3'

Results in 311 bp product

RT-PCR reaction mix: Add the following in the 0.2 ml tubes.

5X reaction buffer (supplied with the enzyme)	2.5µl
10mM dNTPs	0.25µl
10mM BanCP-1 reverse primer	0.25µl
10mM BanCP 2 forward primer	0.25µl
Reverse transcriptase (100 U)	0.06µl
Taq Polymerase (1 U)	0.06µl
Sterile distilled water	8.38µl
Total volume	12.5 µl

Tightly cap the reaction tubes and keep them in a thermal cycler installed with the following PCR program.

RT-PCR programme cycle 1
42 °C for 45 min: One cycle
95 °C for 3 min: One cycle
95 °C for 30 s, 56 °C for 1 min, 72 °C for 30 s: 35 cycles
72 °C for 10 min: One cycle

Analysis of RT-PCR products
Analyze 8-12 µl of PCR products in a 1% agarose gel as described in the next section. CMV primers results about 311 base pair product.

8.5. Immunocapture (IC-PCR) for episomal *Banana streak virus* (BSV) detection

(Harpet et al.,1999; J. Vir. Met. 79:1-8)

IC-PCR combines the principle of ELISA and PCR (Fig. 1). The advantage of IC-PCR is that it is simple to perform and eliminates DNA extraction step and also improves the detection sensitivity. This assay is particularly useful for the differential detection of episomal BSV infection in *Musa* genotypes with integrated BSV forms. PCR can either be performed with tubes with IC product or with tubes coated with extracted DNA. The following were added into each 12.5 µl reaction tube using a Go Taq DNA polymerase (Cat No: M3008).

Requirements

- List of PCR of Reagents as described under section 5.4.1
- BSV polyclonal antibodies
- PBS-T (section 5.2)
- Sterile distilled water

Primers: The primers described here can detect all the BSV strains.
Primer 1: Badna 1A 5'-ATG CCI TTY GGI ITI AAR AAY GCI CC-3'
Primer 2: Badna 4A 5'-CCA YTT RCA IAC ISC ICC CCA ICC-3'
Primers results in 600 bp product

Procedure

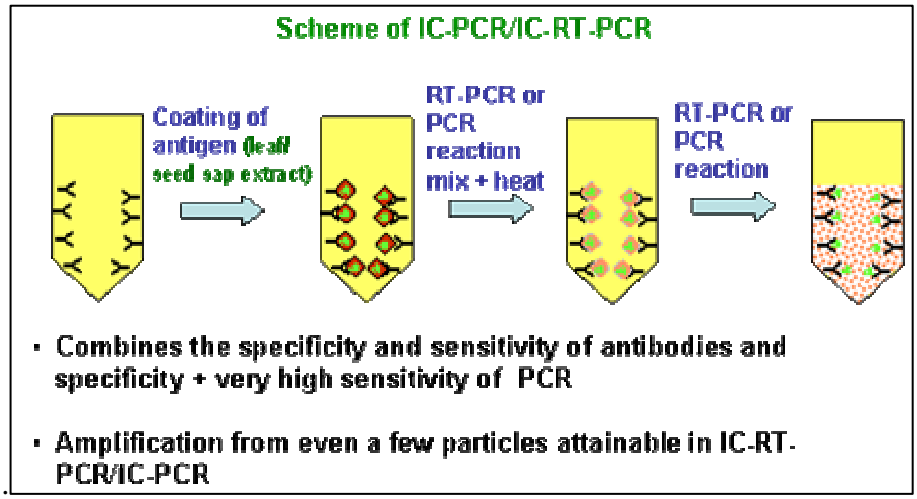
1. Dilute BSV rabbit polyclonal antibody at 1:1000 (v/v) (or dilution specified by the supplier) in PBS-T and dispense 20 µl of diluted antibody to each PCR tube.
2. Incubate the tubes at 37°C for 3 h or 4°C overnight.
3. Wash the tubes three times with PBS-T as described in ELISA section. Make sure to prepare PBS-T in sterile distilled water [prepare 1 L of 1x PBS and sterilize by autoclaving. Allow the solution to cool to room temperature and then add 5 ml of Tween-20 (v/v) and store the solution at room temperature].
4. Grind test sample in extraction buffer at a ratio of 1:10 (w/v) [Extraction buffer: PBS-T containing 2% Polyvinyl pyrrolidone (PVP) and 1% sodium sulphite]
5. Add 50 µl of extracted sap to antibody coated tubes and incubate the tubes overnight at 4°C.
6. Wash the tubes three times with PBS-T and a final wash with sterile distilled water.
7. Into these tubes, add PCR reaction mix as indicated below:

Sterile distilled water	7.94 µl
10x Taq buffer (supplied with the enzyme)	2.6 µl
10 mM dNTPs	0.25 µl
Taq polymerase	0.06 µl
25 mM MgCl2	0.75 µl
Primer 1 (Badna 1A)	0.5 µl (50 pmol)
Primer 2 (Badna 4A)	0.5 µl (50 pmol)
Total volume	12.5 µl

Tightly cap the tubes and place them in a termal cycler installed with the following PCR program.

PCR program
94°C for 10 min: one cycle
94°C for 30s; 37°C for 30s; 72°C for 90s for 5 cycles
94°C for 30s; 50°C for 30s; 72°C for 60s for 5 cycles
72°C for 5 min: one cycle

Analyze the PCR product in 1% agarose gel as described in the next section.



8.6. PCR for the Identification of Fungal & Bacterial Pathogens in Banana

8.6.1. Identification of *Fusarium oxysporum* f. sp. *Cubense* race 4

(Lin et al., 2009; European J Plant Pathol. 123:353–365)

The procedure described here is suitable for the detection of *Fusarium oxysporum* race 4 (FoC TR4) from pure culture established from the host plant and also in the infected plant tissue.

PCR primers
Foc-1: CAGGGGATGTATGAGGAGGCT
Foc-2: GTGACAGCGTCGTCTAGTTCC
Results in PCR product of 242 bp

PCR reaction mixture
Mix the following reagents in a 0.2 ml PCR tube

	Volume (µl) for one reaction
5x PCR Reaction buffer ($MgCl_2$ free)	2.5
25 mM $MgCl_2$ (supplied by the manufacturer along with the enzyme)	0.75

10 mM dNTP mix (Promega)	0.25
10 pM Foc-1	0.25
10 pM Foc-2	0.25
Taq polymerase (Cat. # M3008)	0.06
DNA template (usually 1:100 dilution of stock DNA works best; if necessary adjust dilution accordingly)	2.0
sterile distilled water (or adjust according to the final volume)	6.44
Total reaction volume	12.5

Thermal cycle conditions
94 °C for 1:00 min: one cycle
94 °C for 30 s; 68 °C for 30 s; 72 °C for 1:30 min: 35 cycles
72 °C for 10:00 min: one cycle

Analyze the PCR products in 1.2% agarose gel. A 242 bp product results in samples positive to FoC-TR4.

8.6.2. Identification of *Mycosphaerella fijiensis, M. musicola and M. eumusae* (Sigatoka leaf spot disease)

(Arzanlou et al. 2007; *Phytopathol.* **97**: 1112–1118)

The procedure described here is suitable for the detection of *Mycosphaerella spp.* from pure culture and infected banana plant tissue.

PCR primers
The following pair of primers is useful for the detection of *M. fijiensis*.

	Primer name	Sequence (5'→3')	Product size (bp)
	Mycosphaerella fijiensis		
1F	ActR	GCAATGATCTTGACCTTCAT	500
1R	MFactF	CTCATGAAGATCTTGGCTGAG	
2F	MFBF	CGACACAGCAAGAGCAGCTTC	134
2R	MFBRtaq	TTCGAAAGCCTTGGCACTTCAA	
	Mycosphaerella musicola		
3F	MFactF2	ACGGCCAGGTCATCACT	200
3R	MMactRb	GCGCATGGAAACATGA	
4F	MMBF	CACACATCAAGAGCAGCACAG	142
4R	MMBRtaq	TGGCACTTGGCGGAAGTTTG	
	Mycosphaerella eumusae		
5F	ActF	TCCAACCGTGAGAAGATGAC	600
5R	MEactR	GAGTGCGCATGCGAG	
6F	MEBFtaq	CACCTCAAGAGCAGGAGTGGAA	134
6R	MEBRtaq	TTGGCAATTGGAGGTAGTTGTCC	

PCR reaction mixture
Mix the following reagents in a 0.2 ml PCR tube.

Components	Volume (µl) for one reaction
5x PCR Reaction buffer ($MgCl_2$ free; supplied by the manufacturer along with the enzyme)	2.5
25 mM $MgCl_2$ (supplied by the manufacturer along with the enzyme)	0.75
10 mM dNTP mix	0.25

10 pM Primer 1 (select from the list above)	0.25
10 pM Primer 2 (select from the list above)	0.25
Taq polymerase	0.06
DNA template (usually 1:100 dilution of stock DNA works best; if necessary adjust dilution accordingly)	2.0
sterile distilled water (or adjust according to the final volume)	6.44
Total reaction volume	12.5

Thermal cycle conditions
Set the following thermal cycling conditions in the PCR machine.

1 cycle of: 95 °C for 5:00 min
36 cycles of: 94 °C for 30 s; 60 °C for 30 s; 72 °C for 1:00 min
1 cycle of 72 °C for 7:00 min

Analyze samples in 1.5% (w/v) agarose gel in TAE buffer as described previously.

8.6.3. Identification of *Xanthomonas campestris* pv. *Musacearum*

(Lewis et al., 2010; Plant Dis. 94:109-114)

The procedure described here is suitable for the detection of BXW from pure culture and infected banana plant tissue.

PCR primers
BXW-1: GTCGTTGGCACCATGCTCA
BXW-3: TCCGACCGATACGGCT
Results in 214 bp product

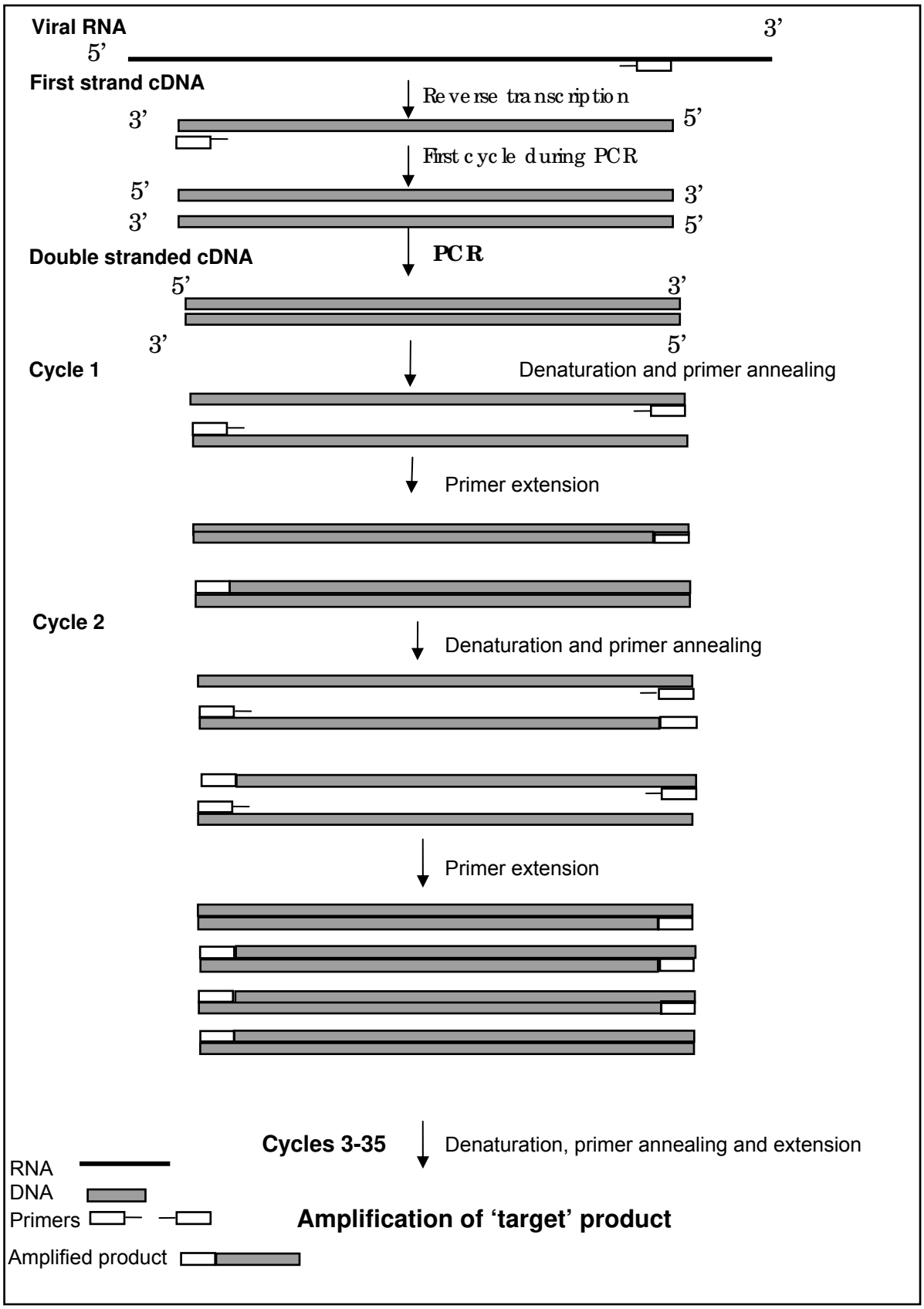
PCR reaction mixture
Mix the following reagents in a 0.2 ml PCR tube (when analyzing multiple samples, we recommend making master mixture).

Components	Volume (µl) for one reaction
5x PCR Reaction buffer ($MgCl_2$ free; supplied by the manufacturer along with the enzyme)	2.5
25 mM $MgCl_2$ (supplied by the manufacturer along with the enzyme)	0.75
10 mM dNTP mix (Promega)	0.25
10 pM BXW-1	0.25
10 pM BXW-3	0.25
Taq polymerase (Cat. # M3008)	0.06
DNA template (usually 1:100 dilution of stock DNA works best; if necessary adjust dilution accordingly)	2.0
sterile distilled water (or adjust according to the final volume)	6.44
Total reaction volume	12.5

Thermal cycle conditions
Set the following thermal cycling conditions in a PCR machine.
1 cycle of: 95 °C for 5:00 min
30 cycles of: 95 °C for 30 s; 55 °C for 30 s; 72 °C for 30 s
1 cycle of 72 °C for 5:00 min

Analyze samples in 1.5% (w/v) agarose gel in TAE buffer as described previously.

Schematic representation of various steps involved during the first few rounds of RT-PCR



9. Gel Electrophoresis of PCR and RT-PCR Products

Electrophoresis through agarose or polyacrylamide gels is the standard method used to analyse PCR amplified products. The phosphate groups in the DNA backbone carry uniform net negative charge at neutral or alkaline pH. During electrophoresis regardless of base composition, the DNA molecules move towards anode under a constant driving force provided by the net negative charge. Consequently, the rate of migration of DNA molecules depends on its size (length) than on the molecular weight, the smallest moving fastest. However, the migration rate is affected by such factors as, DNA conformation, buffer composition and presence of intercalating dyes. These techniques are simple, rapid to perform and DNA in the gel can be identified by staining with low concentrations of intercalating fluorescent dyes, such as ethidium bromide. As little as 1 ng of DNA can be detected in the gels by direct observation under ultraviolet light. The choice of gels to be used depends on the size of the fragments being separated. Polyacrylamide gels have high resolving power and are most effective for separating DNA fragments differed by 1-500 bp. These are run in a vertical configuration in a constant electric field. Agarose gels have low resolving capacity than polyacrylamide gels but are easy to prepare and has greater separation range. These are run in a horizontal configuration. For routine separation of RT-PCR and PCR products agarose gels are preferred. Procedure for separation of DNA in agarose gels is given below.

Agarose Gel Electrophoresis

Agarose gels are prepared by melting agarose in the desired buffer until a clear transparent solution is obtained. The molten agarose solution is poured into a mould (boat) and allowed to harden. Upon hardening the agarose forms a matrix, the density of which depends on the concentration of the agarose.

Materials

- Horizontal electrophoresis unit
- Power supply
- Agarose (electrophoresis grade)
- UV Transilluminator (302 nm wave length)

Solutions

Electrophoresis buffer

Two types of buffers are used for gel electrophoresis. Tris-borate buffer and Tris-acetate buffer. Users can choice either of these buffers.

10x Tris-borate electrophoresis buffer (TBE buffer, pH 8.3)

Tris base (0.45 M) 54 g
Boric acid (0.45 M) 27.5 g
0.5 M EDTA, pH 8 (0.01 M) 20 ml
Distilled water to 1 liter
It is not necessary to adjust pH. Sterilize by autoclaving and store at room temperature.

TBE working solution (0.5x)

To 5 ml of 10x TBE buffer add 95 ml of sterile distilled water. The final concentration of Tris-base, boric acid and EDTA in working solution is 0.0225 M, 0.0225 M and 0.0005 M, respectively.

50x Tris-acetate buffer (TAE buffer, pH 8.3)

Tris base 242 g
Glacial acetic acid 57.1 ml
0.5 M EDTA, pH 8 (0.01 M) 100 ml
Distilled water to 1 liter
It is not necessary to adjust pH. Sterilize by autoclaving and store at room temperature.

Working solution (0.5x)

To 1 ml of 50x TAE buffer add 95 ml of sterile distilled water.

5x Sample buffer (Gel loading buffer)

Bromophenol blue (0.25%) 5 mg
Xylene cyanol FF (0.25%) 5 mg

Glycerol (30%) 3 ml
Sterile distilled water to 10 ml

1% Ethidium bromide solution
Ethidium bromide 100 mg
Distilled water 10 ml
Store in a dark coloured bottle at 4 ^{0}C.

Working solution (0.5 μg/ml)**:** To 100 ml water or molten agarose, add 5 μl of 1% ethidium bromide.

Caution: Ethidium bromide is a carcinogen. Gloves should be worn when handling and care must be taken to dispose materials containing this substance.

Procedure
1. Prepare agarose at the desired concentration (w/v) in 1x TBE buffer (for 1% gel, dissolve1 g agarose in 100 ml buffer) and boil in a microwave oven or on a hot water bath, with intermittent shaking until all the agarose is completely dissolved. Replace evaporation loss with distilled water. **Note:** Ethidium bromide can be directly added into molten agarose [8 μl (0.05μg/ml) /100ml]. This is kind of staining is used for routine analysis. However, if the gel is for estimating molecular size of DNA fragments, do not add ethidium bromide into the gel. It affects DNA migration in gel.
2. Seal the edges of the gel tray with a tape and place the comb at one end of the tray surface.
3. Cool the agarose solution to about 50 ^{0}C and pour into the gel tray to a thickness of 4-5 mm and allow the gel to set. **Note:** It will take about 20 min for agarose to harden.
4. Remove the tape and place the gel tray in the electrophoresis unit and fill the unit with 0.5x TBE buffer so that there is 2-3 mm of buffer over the gel surface. Then remove the comb carefully. **Note:** Wells should be towards cathode end (black colour leads). The migration of DNA will be towards anode (red colour leads)
5. Mix 6 μl of loading buffer to 30 μl of PCR product and load slowly into the wells (**Note:** according to the sample volume adjust loading buffer concentration). Avoid overloading of the wells.
6. Load DNA molecular weight marker.
   6.2.2. **Note:** Make sure to record the order of sample loading in the gel.
7. Connect electrophoresis unit to the power pack and turn on power supply until the bromophenol blue dye reaches the bottom of the gel. (Approximately 60 min at 100 V, for DNA to migrate 7 cm from the wells in a 1% gel)
8. Remove the gel from the tray and stain in ethidium bromide solution (0.5 μg/ml) in water for 15 min with gentle agitation. Then destain by soaking the gel in water.
9. Observe the gel under UV Transilluminator using UV protective goggles or a full safety mask that efficiently blocks UV light. Photograph the gel using an orange filter fitted camera.

Caution: UV radiation is very dangerous to the skin and particularly to the eyes. **It is absolutely essential to use UV-protective goggles. Direct exposure to UV light can result in blindness.**

5.6. Quarantine Monitoring for Plant Viruses

Note: Only authorized government, international and officially recognized non-governmental/private agencies can perform virus indexing for quarantine and certification purpose. Information provided here is for academic purpose. Consult appropriate authorities in your country/region for further details on quarantine monitoring and certification schemes.

Virus Spread & Distribution
Plant viruses are not capable of moving on their own, but they are moved from place-to-place by vectors (insects, fungi, nematodes etc.), host plant material (pollen, seeds, stem cuttings, bulbs, rhizomes, tillers, buds etc.) and rarely by inert material (soil particles and water).

Natural pathways: Certain viruses are carried over large distances by natural vectors such as aphids, leafhoppers, plant hoppers, thrips and other arthropod vectors, fungal spores etc. Pollen- and seed-transmitted viruses are carried in infected seeds and pollen (through wind, water and even inadvertently by animals), and some viruses through soil and water. Generally, it is difficult to monitor plant virus dissemination through natural pathways.

Human-made pathways: The carriers which pose the greatest risk are plant parts imported (seed or vegetative material) for propagation. All viruses are transmitted through vegetative material. Only a few viruses (about 110) are known to be transmitted through seed and even fewer (about 20) viruses through pollen. Of various virus sources, vegetative propagules and seed pose major risk of virus introductions in new regions. Various systems are in place for the safe movement of plant material. Of these, Quarantine Regulation is paramount.

Quarantine Monitoring for Exclusion of Plant Viruses
Quarantine monitoring plays a major role in preventing virus infections through principle of Exclusion. This involves isolation of imported plant material from domestic plants and vectors until standard virus detection methods establish virus-free status.

Viruses are most difficult pathogens for quarantine control. Unlike fungi, nematodes, arthropods, weeds, viruses are invisible to naked eye or even with assistance of a light microscope. The often present inside the cells and difficult to destroy by standard physical or chemical treatments. Rarely diagnostic symptoms can be found on plant parts. Consequently, virus presence cannot be confirmed by inspection or they can be eliminated by application of certain treatments.

Therefore, it is necessary to apply testing procedures to demonstrate the absence of virus and virus-like pathogens in germplasm. The standard testing procedures used are

- Enzyme-linked immunosorbent assay (ELISA)
- Polymerase chain reaction (PCR)
- Grow-out tests
- Grafting to indicator plants/bioassays

Application of testing procedures requires good knowledge on viruses infecting crop species, their distribution and means of spread and availability of sensitive virus detection procedures. Procedures given in this manual is generally applicable for this purpose.

Appendices

A1. List of Commonly Used Methods for the Detection of Plant Viruses

- **Biological methods**
  - Visual detection based on symptoms
  - Transmission to indicator hosts

- **Microscopic methods**
  - Light microscopy of inclusions
  - Transmission Electron microscopy

- **Serological methods (protein-based)**
  - Polyclonal antibodies, monoclonal antibodies and recombinant antibodies
  - Agar gel single/double diffusion test
  - Immuno-fluorescent microscopy
  - Immuno filter paper assay
  - Enzyme immuno assays
    - Direct and indirect ELISAs
    - Dot immunobinding assay
    - Electroblot immunoassay
    - Tissue blotting/printing
    - Immunospecific electron microscopy

- **Nucleic acid-based methods**
  - dsRNA / DNA analysis
  - DNA/RNA probes- radio active, non radioactive molecular beacons
  - Nucleic acid hybridization assays on solid supports
  - *In situ* hybridization
  - Microarray / macroarray
  - PCR-based methods
    - PCR
    - Immuno Capture-PCR
    - Reverse Transcription-PCR
    - Multiplex-PCR
    - Print Capture-PCR
    - Spot Capture-PCR
    - PCR-ELISA
    - Isothermal Multiplex Aplidet RNA System
    - RT-PCR-ELOSA (Enzyme-linked oligosorbent assay)
    - Real time PCR
    - Real time RT-PCR

- **Bioelectric recognition assay (BERA)**

A2. Common Conversions

Weight conversions

1 μg = 10^{-6} g
1 ng = 10^{-9} g
1 pg=10^{-12} g
1 fg = 10^{-15} g

Spectrophotometric conversions

1 OD at A_{260nm} double-stranded DNA	=	50 μg/ml concentration
1 OD at A_{260nm} single-stranded DNA	=	33 μg/ml concentration
1 OD at A_{260nm} single-stranded RNA	=	40 μg/ml concentration

SI Unit prefixed

Prefix	Symbol	Factor
Exa	E	10^{18}
Penta	P	10^{15}
Tera	T	10^{12}
Giga	G	10^{9}
Mega	M	10^{6}
Kilo	K	10^{3}
Milli	M	10^{-3}
Micro	μ	10^{-6}
Nano	N	10^{-9}
Pico	P	10^{-12}
Femto	F	10^{-15}
Atto	A	10^{-18}

A3. Requirements for Establishing ELISA and PCR-based Diagnostic Facility

Sl. No.	Item	Cat. #	Cost (US$)	Manufacturer/Supplier
1	Thermal cycler (PCR machine)	5891M95	7950	Techne TC-512 Thermal cycler
2	Power pack	4314C15	729	Power Station 200
3	Horizontal electrophoresis unit	4266J35	592	Gator Electrophoresis system A-2 (20x25 cm)
4	UV-trans illuminator	6284D87	1241	UVP-White/UV Transilluminator
5	Tabletop centrifuge	2508Y60 2508Y75	1875 90	Spectrafuge 24D Gray SNAP-ON Strip Adaptor
6	Hot water bath	9844Y07	695	Waterbath W/Cover, 14 L Analog
7	Vortex shaker	8294G23	210	Labnet
8	Gel documentation unit*	6284F01	3032	DIGIDOC-IT Imaging system
9	Micro Pipettes 0.5 to 10 µl 5-50 µl 20-200 µl 100-1000 µl	 7733V06 7733V08 7733V14 7733V18	 235 235 235 235	Finnpipette
10	Mortar & pestles			
11	UV protective goggles	1233T84	7	Royale UV50 Goggle Clear, EA
12	Glass trays			
13	Magnetic stirrer	1235A25	481	Thomas Hotplate Stirrer
14	96 Well ELISA plate reader, with 405 nm and 620 nm filter		7000	
15	Micro pipette tips Select to suite model and volume of item # 9			
16	Microfuge tubes (0.5 ml, 1.5 ml and 2.0 ml)			
17	PCR tubes (0.2 ml)			
18	96 Well ELISA plate (flat bottom)			

Note: All items listed here can be verified in Thomas scientific (www.thomassci.com). Other models also available and they can be selected as per the local/user needs. Several other agencies also sell these items. Cost is an approximation. Depending on the model and year, price may change. Other general lab requirements such as, Weighing balance; Water distiller; Incubator; Refrigerator (4 to -20°C); gloves; pH Meter; Autoclave, reagent storage bottles, etc., required

Chemicals for DNA extraction and agarose gel electrophoresis*

Item**	**Item****
Ethanol	Electrophoresis grade agarose
SDS	Tris
Potassium acetate	Borate
Tris-buffer saturated phenol	Sodium acetate
Chloroform	Ethidium bromide
Iso-propanol	EDTA

* Reagent/chemical list depends on the protocols. This list covers most but not all.
**Molecular biology grade (high quality reagents).

Note: These chemicals can be purchased from SIGMA®, Merck/BDH®, Thomas Scientific® or any other chemical suppliers. Reagents for PCR not listed as it depends on the user.

A4. Glossary

Abiotic stress: Outside (nonliving) factors, which can cause harmful effects to plants, such as soil conditions, drought, extreme temperatures.

Absorbance (optical density): This is a measure of the amount of light absorbed by a suspension of bacterial cells or a solution of an organic molecule; it is measured by a colorimeter or spectrophotometer. Absorbance values are used to plot the growth of bacteria in suspension cultures and to gauge the purity and concentration of molecules (such as proteins) in solution. Absorbance is defined as a logarithmic function of the percent transmission of a wavelength of light through a liquid.

Accession or entry: A population or line in a breeding programme or germplasm collection; also an individual sample in a germplasm bank. A sample of a crop variety collected at a specific location and time; may be of any size.

Adenine (A): A nitrogenous base, one member of the base pair AT (adenine-thymine).

Agarose gel electrophoresis: A matrix composed of a highly purified form of agar that is used to separate larger DNA and RNA molecules ranging 20,000 nucleotides.

Alternate host: One of two kinds of plants on which a parasitic fungus (e.g., a rust) must develop to complete its life cycle.

Alternative host: A plant other than the main host that a virus can infect.

Amino acid: Any of 20 basic building blocks of proteins--composed of a free amino (NH2) end, a free carboxyl (COOH) end, and a side group (R).

Amplification: An increase in the number of copies of a specific DNA fragment; can be in vivo or in vitro.

Amplify: To increase the number of copies of a DNA sequence, in vivo by inserting into a cloning vector that replicates within a host cell, or in vitro by polymerase chain reaction (PCR).

Anion: A negatively charged molecule

Anode: A positive electrode in an electrolytic cell toward which anions migrate.

Anneal: The pairing of complementary DNA or RNA sequences, via hydrogen bonding, to form a double-stranded polynucleotide. Most often used to describe the binding of a short primer or probe.

Antibody: An immunoglobulin protein produced by B-lymphocytes of the immune system that binds to a specific antigen molecule.

Antigen (Immunogen): Any foreign substance, such as a virus, bacterium, or protein that elicits an immune response by stimulating the production of antibodies.

Antigenic determinant: A surface feature of a microorganism or macromolecule, such as a glycoprotein, that elicits an immune response.

Antiserum: The serum from a vertebrate that has been exposed to an antigen and which contains antibodies that react specifically with the antigen.

Antisense: Nucleic acid that has a sequence exactly opposite to an mRNA molecule made by the body; binds to the mRNA molecule to prevent a protein from being made.

Antisense RNA: A complementary RNA sequence that binds to a naturally occurring (sense) mRNA molecule, thus blocking its translation.

Asymptomatic: Without signs or symptoms of disease.

AT content: The percentage of nitrogenous bases on a DNA molecule which are either adenine (A) or thymine (T) (from a possibility of four different ones, also including cytosine (C) and guanine (G)).

AT/GC ratio: The ratio of adenine-thymine base pairs to guanine-cytosine base pairs on a DNA molecule.

Avirulent: Not exhibiting virulence; nonpathogenic.

Base: one of the four chemical units (nucleotides) arranged along the DNA or RNA molecule.

Base composition: The relative proportions fo the four respectrive nucleotides in a given sequence of DNA or RNA.

Base pair (bp): A pair of complementary nitrogenous bases in a DNA molecule--adenine-thymine and guanine-cytosine. Also, the unit of measurement for DNA sequences.

Base sequence: The order of nucleotide bases in a DNA molecule; determines structure of proteins encoded by that DNA.

Bioassay: The measurement of infective virus concentration in plant extracts.

Biological control: The deliberate use by humans of one species of organism to eliminate or control another.

Biodiversity: The variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems.

Biotechnology: The scientific manipulation of living organisms, especially at the molecular genetic level, to produce useful products. Gene splicing and use of recombinant DNA (rDNA) are major techniques used.

Biotic stress: Living organisms, which can harm plants, such as viruses, fungi, and bacteria, and harmful insects.

Biotype: A subspecies of organism morphologically similar to but physiologically different from other members of the species.

Blotting: Following electrophoresis: the transfer of nucleic acids and/or proteins from a gel strip to a specialized, chemically reactive matrix on which the nucleic acids, etc. may become covalently bound in a pattern similar to that present in the original gel.

Breeding line: Genetic lines of particular significance to plant or animal breeders that provides the basis for modern varieties.

Buffer solution: Is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. It has the property that the pH of the solution changes very little when a small amount of acid or base is added to it. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications.

Carrier: Organism that carries a virus either in form of an infection or while it is in incubation.

Cation: A positively charged ion.

Causal agent of disease: That which is capable of causing disease.

cDNA: DNA synthesized from an RNA template using reverse transcriptase.

cDNA library: A library composed of complementary copies of cellular mRNAs.

Chlorosis: The loss of chlorophyll from the tissues of a plant, resulting from microbial infection, viral infection, the action of certain phytotoxins, the lack of light, to magnesium or iron deficiency, etc. Chlorotic tissues commonly appear yellowish.

Central dogma: Francis Crick's seminal concept that in nature genetic information generally flows from DNA to RNA to protein.

Circulative transmission: Virus transmission characterized by a long period of acquisition of the virus by a vector, a latent period of several hours before the vector is able to transmit the virus, and retention of the virus by the vector for a long period, usually several days. (Also termed **persistent transmission**)

Cistron: A DNA sequence that codes for a specific polypeptide; a gene.

Clone: An exact genetic replica of a specific gene or an entire organism.

Cloning: The mitotic division of a progenitor cell to give rise to a population of identical daughter cells or clones.

Coalesce: To merge or grow together into a similar but larger structure.

Coat protein (capsid): The coating of a protein that enclosed the nucleic acid core of a virus.

Codon: A group of three nucleotides that specifies addition of one of the 20 amino acids during translation of an mRNA into a polypeptide. Strings of codons form genes and strings of genes form chromosomes.

Complementary DNA or RNA: The matching strand of a DNA or RNA molecule to which its bases pair.

Complementary nucleotides: Members of the pairs adenine-thymine, adenine-uracil, and guaninecytosine that have the ability to hydrogen bond to one another.

Control: Economic reduction of crop losses caused by plant diseases.

Cross-hybridization: The hydrogen bonding of a single-stranded DNA sequence that is partially but not entirely complementary to a singlestranded substrate. Often, this involves hybridizing a DNA probe for a specific DNA sequence to the homologous sequences of different species.

Cross-pollination: Fertilization of a plant from a plant with a different genetic makeup.

Crop rotation: The practice of growing a sequence of different crops on the same land in successive years or seasons; done to replenish the soil, curb pests, etc.

Cross-protection: The protection conferred on a host by infection with one strain of a virus that prevents infection by a closely related strain.

Cultivar: A cultivated variety (genetic strain) of a domesticated crop plant. A cultivated plant variety or cultural selection. International term denoting certain cultivated plants that are clearly distinguishable from others by one or more characteristics and that when reproduced retain their distinguishing characteristics. In the United States, 'variety' is considered to be synonymous with cultivar (derived from 'cultivated variety').

Dalton: A unit of measurement equal to the mass of a hydrogen atom, 1.67 x 10E-24 gram/L (Avogadro's number).

Degenerate primers: Oligonucleotides designed to include a mixture of different sequences to allow for variation at particular nucleotide positions in a target sequence.

Denature: To induce structural alterations that disrupt the biological activity of a molecule. Often refers to breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce in single-stranded polynucleotides or altering the secondary and tertiary structure of a protein, destroying its activity.

Density gradient centrifugation: High-speed centrifugation in which molecules "float" at a point where their density equals that in a gradient of cesium chloride or sucrose.

Diagnostic: A distinguishing characteristic important in the identification of a disease or other disorder.

Diagnosis: The evaluation of symptoms and laboratory tests which confirms or establishes the nature/origin of a disease.

Differential host: A plant host that on the basis of disease symptoms serves to distinguish between various strains or races of a given plant pathogen.

Diploid: A full set of genetic material, consisting of paired chromosomes one chromosome from each parental set.

Disease: An abnormal condition of a plant in which its physiology, morphology, and/or development is altered under the continuous influence of a pathogen.

DNA (Deoxyribonucleic acid): An organic acid and polymer composed of four nitrogenous bases-adenine, thymine, cytosine, and guanine linked via intervening units of phosphate and the pentose sugar deoxyribose. DNA is the genetic material of most organisms and usually exists as a double-stranded molecule in which two antiparallel strands are held together by hydrogen bonds between adeninethymine and cytosine-guanine.

DNA diagnosis: The use of DNA polymorphisms to detect the presence of a disease gene.

DNA fingerprint: The unique pattern of DNA fragments identified by Southern hybridization (using a probe that binds to a polymorphic region of DNA) or by polymerase chain reaction (using primers flanking the polymorphic region).

DNA probe: a fragment of DNA used to recognize a specific complementary DNA sequence, or gene(s). Probes can be employed, for example, to bind to the genetic material of microbes for purposes of detection, identification, or, in some cases, inactivation.

DNA sequencing: Procedures for determining the nucleotide sequence of a DNA fragment.

Downstream: The region extending in a 3' direction from a gene.

Ecology: The study of the interactions of organisms with their environment and with each other.

Electron Microscopy: An imaging method, which uses a focused beam of electrons to enlarge the image of an object on a screen or photographic plate.

Electrophoresis: The technique of separating charged molecules in a matrix to which is applied an electrical field.

Encapsidation: Process by which a virus' nucleic acid is enclosed in a capsid.

Endemic: Restricted to specified region

Enveloped: possessing an outer (bounding) lipoprotein bilayer membrane.

Enzymes: Proteins that control the various steps in all chemical (metabolic) reactions.

Enzyme-linked immunosorbent assay (ELISA): a sensitive, inexpensive assay technique involving the use of antibodies coupled with indicators (e.g., enzymes linked to dyes) to detect the presence of specific substances, such as enzymes, viruses, or bacteria.

Epidemic: A change in the amount of disease in a population in time and space.

Epidemiology: The science concerned with the determination of the specific causes of a disease or the interrelation between various factors determining a disease, as well as disease trends in a specific region.

Epitope: The region of antigen that triggers and interacts with antibody.

Eradication. Control of plant disease by eliminating the pathogen after it is established or by eliminating the plants that carry the pathogen.

Escape: Failure of inherently susceptible plants to become diseased, even though disease is prevalent.

Etiology: The study or theory of factors which cause disease.

Exon: A DNA sequence that is ultimately translated into protein.

Express: To translate a gene's message into a molecular product.

Flanking region: The DNA sequences extending on either side of a specific locus or gene.

GxE interaction: Genotype by Environment interaction. Phenomenon that two (or more) varieties will react differently to a change of environment.

Gene: A locus on a chromosome that encodes a specific protein or several related proteins. It is considered the functional unit of heredity.

Genetic code: The three-letter code that translates nucleic acid sequence into protein sequence. The relationships between the nucleotide base-pair triplets of a messenger RNA molecule and the 20 amino acids that are the building blocks of proteins.

Genetic disease: A disease that has its origin in changes to the genetic material, DNA. Usually refers to diseases that are inherited in a Mendelian fashion, although noninherited forms of cancer also result from DNA mutation.

Genetic engineering: The manipulation of an organism's genetic endowment by introducing or eliminating specific genes through modern molecular biology techniques. A broad definition of genetic engineering also includes selective breeding and other means of artificial selection.

Genome: The genetic complement contained in the chromosomes of a given organism, usually the haploid chromosome state.

Genomic library: A library composed of fragments of genomic DNA.

Genotype: The structure of DNA that determines the expression of a trait. Genetic constitution of the organism distinguished by physical appearance.

Glycoprotein: A protein molecule coated with carbohydrates.

Hapten: A small chemicals coupled to larger protein molecules (carriers). Small chemicals (hapten) serve as epitopes for binding to the antibodies on the B-cell surface.

Haploid: A single set of chromosomes (half the full set of genetic material), present in the egg and sperm cells of animals and in the egg and pollen cells of plants.

Heredity: The handing down of certain traits from parents to their offspring. The process of heredity occurs through the genes.

Heterozygosity: The presence of different alleles at one or more loci on homologous chromosomes.

Heteroduplex: A double-stranded DNA molecule or DNA-RNA hybrid, where each strand is of a different origin.

Histopathology: The study of pathology of cells and tissues; the microscopic changes characteristic of disease.

Horizontal resistance: In a given cultivar: the existence of similar levels of resistance to each of the races of a given pathogen.

Host: An organism that contains another organism.

Hybrid: An individual produced from genetically different parents. The term is often reserved by plant breeders for cases where the parents differ in several important respects. Hybrid are often more vigorous than either parent, but cannot breed true.

Hybridization: The hydrogen bonding of complementary DNA and/or RNA sequences to form a duplex molecule.

Hybridoma: A hybrid cell, composed of a B lymphocyte fused to a tumor cell, which grows indefinitely in tissue culture and is selected for the secretion of a specific antibody of interest.

Hydrogen bond: A relatively weak bond formed between a hydrogen atom (which is covalently bound to a nitrogen or oxygen atom) and a nitrogen or oxygen with an unshared electron pair.

Hypersensitive: The state of being abnormally sensitive. It often refers to an extreme reaction to a pathogen (e.g., the formation of local lesions by a virus or the necrotic response of a leaf to bacterial infection).

Immune: Cannot be infected by a given pathogen.

Immunity: A natural or acquired resistance to a specific disease.

Inbred line: Genetically (nearly) homozygous population, derived through

several cycles of selfing (see below), also used for hybrid seed production.

Incubation period: The period of time between penetration of a host by a pathogen and the first appearance of symptoms on the host.

Indexing: A procedure to determine whether a given plant is infected by a virus. It involves the transfer of a bud, scion, sap etc. from one plant to one or more kinds of indicator plants sensitive to the virus.

Indicator host: A plant species that gives characteristic symptoms to a specific virus. Used in virus diagnosis.

Infection: Condition in which virulent organisms are able to multiply within the cell and cause a response. Infection may or may not lead to visible symptoms.

Infectious: Capable of being transmitted by infection, with or without actual contact.

Inoculate: To introduce a microorganism into an environment suitable for its growth; to bring a parasite into contact with a host.

Inoculation: The act of inoculating; the placement of microorganisms or viruses at a site where infection is possible (the infection court).

Inoculum: The population of microorganisms introduced in an inoculation; the units of a parasite capable of initiating an infection

In situ: Refers to performing assays or manipulations with intact tissues.

In vitro: (Literally "in glass".) Cultivated in an artificial, non-living environment.

In vivo: Refers to biological processes that take place within a living organism or cell.

Initiation codon: The mRNA sequence AUG, coding for methionine, which initiates translation of mRNA.

Intergenic regions: DNA sequences located between genes that comprise a large percentage of the human genome with no known function.

Intron: A noncoding DNA sequence within a gene that is initially transcribed into messenger RNA but is later snipped out.

Ion: A charged particle.

Isolate: In plant pathology: a culture or subpopulation of a microorganism separated from its parent population and maintained in some sort of controlled circumstance; also, to effect such separation and control, for example to isolate a pathogen from diseased plant tissue.

Isotope: One of two or more forms of an element that have the same number of protons (atomic number) but differing numbers of neutrons (mass numbers). Radioactive isotopes are commonly used to make DNA probes and metabolic tracers.

Land race: Primitive or antique variety usually associated with traditional agriculture. Often highly adapted to local conditions.

Legume: A member of the pea family that possesses root nodules containing nitrogen-fixing bacteria.

Local infection: An infection affecting a limited part of a plant.

Local lesion: A localized spot produced on a leaf upon mechanical inoculation with a virus.

Lyophilization: Rapid freezing of a material at low temperature followed by rapid dehydration by sublimation in a high vacuum. A method used to preserve biological specimens or to concentrate macromolecules with little or no loss of activity. (Also freeze-drying)

Masked symptoms: Virus-induced plant symptoms that are absent under some environmental conditions but appear when the host is exposed to certain conditions of light and temperature.

Mechanical inoculation: Of plant viruses, a method of experimentally transmitting the pathogen from plant to plant; juice from diseased plants is rubbed on test-plant leaves that usually have been dusted with carborundum or some other abrasive material.

Mass selection: Selection of individual plants from a population. Mass selection may be positive and negative selection. Seeds from mass selection form the next generation.

Messenger RNA (mRNA): The class of RNA molecules that copies the genetic information from DNA, in the nucleus, and carries it to ribosomes, in the cytoplasm, where it is translated into protein.

Molecular biology: The study of the biochemical and molecular interactions within living cells.

Molecular cloning: The biological amplification of a specific DNA sequence through mitotic division of a host cell into which it has been transformed or transfected.

Monoclonal antibodies: Immunoglobulin molecules of single-epitope specificity that are secreted by a clone of B cells.

Monoculture: The agricultural practice of cultivating crops consisting of genetically similar organisms.

Monogenic resistance: Resistance determined by a single gene.

Mosaic: A common symptom induced in leaves by many plant virus infections in which there is a pattern of dark green, light green and sometimes chlorotic areas. This pattern is often associated with the distribution of veins in the leaf. In monocotyledonous leaves it shows as stripes.

Mottle: A diffuse form of the mosaic symptom in plant leaves in which the dark and light green are less sharply defined. This term is frequently used interchangeably with mosaic.

Multicomponent virus: A virus in which the genome needed for full infection is divided between two or more particles (e.g., cowpea mosaic virus, brome mosaic virus, cucumber mosaic virus).

Necrosis. Localized death of cells or tissues (necrotic. Dead)

Negative sense (= minus strand); for RNA or DNA: The negative strand is the strand with base sequence complementary to the positive-sense strand.

Nitrocellulose: A membrane used to immobilize DNA, RNA, or protein, which can then be probed with a labeled sequence or antibody.

Nitrogen fixation: The conversion of atmospheric nitrogen to biologically usable nitrates.

Nitrogenous bases: The purines (adenine and guanine) and pyrimidines (thymine, cytosine, and uracil) that comprise DNA and RNA molecules.

Nodule: The enlargement or swelling on roots of nitrogen-fixing plants. The nodules contain symbiotic nitrogen-fixing bacteria.

Nomenclature: A system of names, or naming, as applied to the subjects or study in any art or science.

Noncirculative transmission: Virus transmission characterized by a very short period of acquisition of the virus by a vector (e.g., an aphid), no latent period before the vector can transmit the virus, and a short period of retention by the vector after acquisition. (Also termed non-persistent transmission.)

Nontarget organism: An organism which is affected by an interaction for which it was not the intended recipient.

Northern hybridization: (Northern blotting). A procedure in which RNA fragments are transferred from an agarose gel to a nitrocellulose filter, where the RNA is then hybridized to a radioactive probe.

Nuclease: A class of enzymes that degrades DNA and/or RNA molecules by cleaving the phosphodiester bonds that link adjacent nucleotides. In deoxyribonuclease (DNase), the substrate is DNA. In endonuclease, it cleaves at internal sites in the substrate molecule. Exonuclease progressively cleaves from the end of the substrate molecule. In ribonuclease (RNase), the substrate is RNA. In the S1 nuclease, the substrate is single-stranded DNA or RNA.

Nucleic acids: The two nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are made up of long chains of molecules called nucleotides.

Nucleoprotein: A compound of nucleic acid and protein.

Nucleoside: A building block of DNA and RNA, consisting of a nitrogenous base linked to a five carbon sugar.

Nucleoside analog: A synthetic molecule that resembles a naturally occuring nucleoside, but that lacks a bond site needed to link it to an adjacent nucleotide.

Nucleotide: A building block of DNA and RNA, consisting of a nitrogenous base, a five-carbon sugar, and a phosphate group. Together, the nucleotides form codons, which when strung together form genes, which in turn link to form chromosomes.

Oligonucleotide: A short DNA polymer composed of only a few nucleotides.

Open pollination: Pollination by wind, insects, or other natural mechanisms.

Open reading frame: A long DNA sequence that is uninterrupted by a stop codon and encodes part or all of a protein.

Organelle: A cell structure that carries out a specialized function in the life of a cell.

Parasitism: The close association of two or more dissimilar organisms where the association is harmful to at least one.

Pathogen: Organism which can cause disease in another organism.

Pathotype: An infrasubspecific classification of a pathogen distinguished from others of the species by its pathogenicity on a specific host(s).

Pellet: The material concentrated at the bottom of a centrifuge tube after centrifugation.

Pesticide: A substance that kills harmful organisms (for example, an insecticide or fungicide or acaricide).

pH: a measure of the acidity or basicity of a solution.

Phenotype: The observable characteristics of an organism, the expression of gene alleles (genotype) as an observable physical or biochemical trait.

Phosphodiester bond: A bond in which a phosphate group joins adjacent carbons through ester linkages. A condensation reaction between adjacent nucleotides results in a phosphodiester bond between 3' and 5' carbons in DNA and RNA.

Plasmid (p): A circular DNA molecule, capable of autonomous replication, which typically carries one or more genes encoding antibiotic resistance proteins. Plasmids can transfer genes between bacteria and are important tools of transformation for genetic engineers.

Polycyclic: Of a disease or pathogen: Producing many generations of inoculum and many cycles of infection during a single growing season.

Polyetic: Of plant disease epidemics: Continuing from one growing season to the next.

Polygenic: A character controlled by many genes.

Polymorphism: Difference in DNA sequence among individuals. Genetic variations occurring in more than 1% of a population would be considered useful polymorphisms for genetic linkage analysis. Compare mutation.

Polyacrylamide gel electrophoresis: Electrophoresis through a matrix composed of a synthetic polymer, used to separate proteins, small DNA, or RNA molecules of up to 1000 nucleotides. Used in DNA sequencing.

Polyclonal antibodies: A mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope.

Polymerase (DNA): Synthesizes a double-stranded DNA molecule using a primer and DNA as a template.

Polymerase chain reaction (PCR): A procedure that enzymatically amplifies a DNA polymerase.

Polypeptide (protein): A polymer composed of multiple amino acid units linked by peptide bonds.

Primer: A short DNA or RNA fragment annealed to single-stranded DNA, to initiate synthesis of DNA by a DNA Polymerase or reverse transcriptase which extends a new DNA strand to produce a duplex molecule.

Probe: *(1)* A sequence of DNA or RNA, labeled or marked with a radioactive isotope, used to detect the presence of complementary nucleotide sequences. *(2)* A single-stranded DNA that has been radioactively labeled and is used to identify complementary sequences in genes or DNA fragments of interest.

Propagative virus: A circulative virus that replicates in its insect vector. Such a virus is said to be propagatively transmitted (e.g., potato yellow dwarf virus).

Protein: A polymer of amino acids linked via peptide bonds and which may be composed of two or more polypeptide chains.

Positive-sense (= plus strand, message strand) RNA: The RNA strand that contains the coding triplets that are translated by ribosomes

Positive-sense DNA: the strand that contains the same base sequence as the mRNA. However, mRNAs of some dsDNA viruses are transcribed from both strands and the transcribed regions may overlap. For such viruses this definition is inappropriate.

Pseudotypes (pseudo-virus): enveloped virus particles in which the envelope is derived from one virus and the internal constituents from another.

Purine: A nitrogen- containing, single- ring, basic compound that occurs in nucleic acids. The purines in DNA and RNA are adenine and guanine.

Pyrimidine: A nitrogen- containing, double-ring, basic compound that occurs in nucleic acids. The pyrimidines in DNA are cytosine and thymine; in RNA, cytosine and uracil.

Race: A subspecies group of pathogens that infect a given set of plant varieties.

Recessive: Moving back and out of view. In genetics, a recessive gene is a gene that does not express its instructions when paired with a dominant gene.

Recombinant: A cell that results from recombination of genes.

Recombinant DNA: The process of cutting and recombining DNA fragments from different sources as a means to isolate genes or to alter their structure and function.

Recombinant DNA technology: a broad term referring to molecular cloning as well as techniques for making recombinant DNA or using it for specific purposes.

Renature: The reannealing (hydrogen bonding) of single-stranded DNA and/or RNA to form a duplex molecule.

Resistance: The ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor.

Resistant: Possessing resistance.

Response: The change produced in an organism by a stimulus.

Reverse transcriptase (RNA-dependent DNA polymerase): An enzyme isolated from retrovirus-infected cells that synthesizes a complementary (c)DNA strand from an RNA template.

Ringspot: A type of local lesion consisting of single or concentric rings of discoloration or necrosis, the regions between the concentric rings being green. The center of the lesion may be chlorotic or necrotic.

RNA (ribonucleic acid): An organic acid composed of repeating nucleotide units of adenine, guanine, cytosine, and uracil, whose ribose components are linked by phosphodiester bonds.

RNA-dependent RNA polymerase (viral RNA polymerase): Enzyme with replicase and transcriptase activity (viral RNA polymerase with no distinction between replication and transcription functions).

RNA polymerase: Transcribes RNA from a DNA template.

RNA replicase: Enzyme synthesizing progeny viral strands of plus and minus polarity.

RNA transcriptase: Enzyme involved in messenger RNA synthesis; (virion associated polymerases). *[Note, for some viruses it has yet to be established whether or not the replicase and transcriptase activities reflect distinct enzymes rather than alternative activities of a single enzyme]*

Rouging: The removal of diseased plants from a crop in order to prevent the spread of the disease.

Rosette: An abnormal condition in which the leaves form a radial cluster on the stem.

Rugose: Wrinkled.

Satellite RNA (viroids): A small, self-splicing RNA molecule that accompanies several plant viruses, including *Tobacco ringspot virus*.

Satellite virus: A defective virus requiring a helper virus to provide functions necessary for replication. It may code for its own coat protein or various other products.

Secondary infection: Any infection caused by inoculum produced as a result of a primary or a subsequent infection; an infection caused by secondary inoculum.

Secondary inoculum: Inoculum produced by infections that took place during the same growing season.

Secondary organism: An organism that multiplies in already diseased tissue but is not the primary pathogen.

Secondary symptom: A symptom of virus infection appearing after the first (primary) symptoms.

Self-pollination: Pollen of one plant is transferred to the female part of the same plant or another plant with the same genetic makeup.

Selection: Natural selection is the differential contribution of offspring to the next generation by various genetic types belonging to the same populations. Artificial selection is the intentional manipulation by man of the fitness of individuals in a population to produce a desired evolutionary response.

Selective breeding: The selection of certain seeds or animals for reproduction in order to influence the traits inherited by the next generation.

Serology: Branch of science dealing with properties and reactions of sera, particularly the use of antibodies in the sera to examine the properties of antigens.

Serotype: A subdivision of virus strains distinguished by protein or a protein component that determines its antigenic specificity.

Southern hybridization (Southern blotting): A procedure in which DNA restriction fragments are transferred from an agarose gel to a nitrocellulose filter, where the denatured DNA is then hybridized to a radioactive probe (blotting).

Species: A classification of related organisms that can freely interbreed.

Spot: A symptom of disease characterized by a limited necrotic area, as on leaves, flowers, and stems.

Stem-pitting: A symptom of some viral diseases characterized by depressions on the stem of the plant.

Stringency: Reaction conditions--notably temperature, salt, and pH--that dictate

the annealing of single-stranded DNA/DNA, DNA/RNA, and RNA/RNA hybrids. At high stringency, duplexes form only between strands with perfect one-to-one complementarity; lower stringency allows annealing between strands with some degree of mismatch between bases.

Substrate: A substance acted upon by an enzyme.

Supernatant: The soluble liquid &action of a sample after centrifugation or precipitation of insoluble solids.

Suppression: A hypoplastic symptom characterized by the failure of plant organs or substances to develop

Surface projections (= spikes, peplomers, knobs): Morphological features, usually consisting of glycoproteins, that protrude from the lipoprotein envelope of many enveloped viruses.

Susceptible: Vulnerable or predisposed to a disease (Lacking the inherent ability to resist disease or attack by a given pathogen; not immune).

Susceptibility: The inability of a plant to resist the effect of a pathogen or other damaging factor.

Symptoms: Any perceptible, subjective change in the organism or its functions that indicates disease or phases of disease.

Symptomatology: The study of symptoms of disease and signs of pathogens for the purpose of diagnosis.

Symptomless carrier: A plant that, although infected with a virus, produces no obvious symptoms.

Systemic: Spreading internally throughout the plant body.

Systemic infection: An infection resulting from the spread of virus from the site of infection to all or most cells of an organism.

***Taq* polymerase:** A heat-stable DNA polymerase isolated from the bacterium *Therrnus aquaticus*, used in PCR.

Taxonomy: Classification based on natural relationships.

Taxon: The named classification unit to which individuals, or sets of species, are assigned. Higher taxa are those above the species level.

Template: An RNA or single-stranded DNA molecule upon which a complementary nucleotide strand is synthesized.

Tolerance: The ability of a plant to sustain the effects of a disease without dying or suffering serious injury or crop loss.

Transcapsidation: The encapcidation of the nucleic acid of one virus with a coat protein of a different virus.

Transmission: The transfer of a pathogen from one plant to another, or from one plant organ to another.

Transcription: The process of creating a complementary RNA copy of DNA.

Transgenic: An organism in which a foreign DNA gene (a transgene) is incorporated into its genome early in development. The transgene is present in both somatic and germ cells, is expressed in one or more tissues, and is inherited by offspring in a Mendelian fashion.

Transgenic organism: an organism formed by the insertion of foreign genetic material into a germ cell.

Transgenic plant: Genetically engineered plant or offspring of genetically engineered plants. The transgenic plant usually contains material from at least one unrelated organisms, such as from a virus, animal, or other plant.

Translation: The process of converting the genetic information of an mRNA on ribosomes into a polypeptide. Transfer RNA molecules carry the appropriate amino acids to the ribosome, where they are joined by peptide bonds.

Upstream: The region extending in a 5' direction from a gene.

Variation: Differences in the frequency of genes and traits among individual organisms within a population.

Variety: An infrasubspecific rank which has no official standing in nomenclature.

Vector: 1. A living agent that transmits a pathogen from an infected plant to an uninfected one. 2. An autonomously replicating DNA molecule into which foreign DNA fragments are inserted and then propagated in a host cell. 3. Also living carriers of genetic material (such as pollen) from plant to plant, such as insects.

Vein banding: A symptom of virus-infected leaves in which tissues along the veins are darker green than other laminar tissue.

Vein clearing: A symptom of virus-infected leaves in which veinal tissue is lighter green than that of healthy plants.

Viroid: A plant pathogen that consists of a naked RNA molecule of approximately 250-350 nucleotides, whose extensive base pairing results in a nearly correct double helix.

Virion: Morphologically complete virus particle; the infectious unit of a virus.

Virology: The study of viruses and viral disease.

Viroplasm (= virus factory, virus inclusion, X-body): A modified region within the infected cell in which virus replication occurs, or is thought to occur.

Virulence: The degree of ability of an organism to cause disease.

Viruliferous: Used to describe a vector containing a virus and capable of transmitting it.

Virus: An infectious particle composed of a protein capsule and a nucleic acid core, which is dependent on a host organism for replication. A double-stranded DNA copy of an RNA virus genome that is integrated into the host chromosome during lysogenic infection.

Weed: An undesirable plant.

Wild relative: Plant species that are taxonomically related to crop species and serve as potential sources for genes in breeding of new varieties of those crops.

Wild species: Organisms captive or living in the wild that have not been subject to breeding to alter them from their native state.

Wilt: A disease (or symptom) characterized by a loss of turgidity in a plant (e.g., vascular wilt).

Witches' broom: An abnormal form of plant growth, most common in woody plants, in which there is a profuse outgrowth of lateral buds to give a "witches' broom" appearance. The shoots may be thickened and may bear abnormal leaves.

Wild type: An organism as found in nature; the organism before it is genetically engineered.

Yellowing: A symptom characterized by the turning yellow of plant tissues that were once green.

Yellows: Any of a wide variety of plant diseases in which a major symptom is a uniform or non-uniform yellowing of leaves and/or other plant components. Yellows may be caused by fungi (e.g., celery yellows), viruses (e.g., sugar beet yellows virus), bacteria, protozoa (e.g., hartrot), spiroplasmas or phytoplasmas (e.g., coconut lethal yellowing).