

Laboratory Manual for the Diagnosis of Cassava Virus Diseases





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Preface

“Laboratory Manual for the Diagnosis of Cassava Virus Diseases” is prepared for the benefit of participants of the regional training for the Disease Objective of Great Lakes Cassava Initiative (GLCI) on ‘Cassava Viruses: Biology, Diagnostics and Management’ held from 28 October – 6 November, 2009 at IITA, Dar es Salaam, Tanzania. The objective of this course is to train the ‘trainers’ from the GLCI Project National Program Partners in Burundi, Democratic Republic of Congo, Kenya, Rwanda, Tanzania and Uganda, in cassava virus disease biology, diagnostics and management to facilitate the capacity building in cassava disease diagnostics and management within the six target countries.

This manual, a modified version of our previous laboratory manual, provides basic principles and offers step-by-step protocols for virus diagnosis using molecular assays for the detection of major viruses infecting cassava in sub-Saharan Africa. The polymerase chain reaction (PCR)-based diagnostic methods described in this manual are based on our experience over the years and involve contributions from many of the past and present 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. Literature pertinent to theoretical and practical aspects of plant virology and disease diagnosis has been provided. These methods can also be used with appropriate modifications for the diagnosis of plant virus infecting other crops.

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Abbreviations

cv	Cultivar
DNA	Deoxyribonucleic acid
dH ₂ O	Distilled water
dNTPs	Deoxynucleotide triphosphates
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
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Scanning electron microscope
TEM	Transmission electron microscope
VLP	Virus-like particles

ACMV	African cassava mosaic virus
CBSD	Cassava brown streak disease
CBSV	Cassava brown streak virus
CMD	Cassava mosaic disease
CMBV	Cassava mosaic begomoviruses
CMGs	Cassava mosaic geminiviruses
EACMV	East African cassava mosaic virus
EACMCV	East African cassava mosaic Cameroon virus
EACMKV	East African cassava mosaic Kenya virus
EACMMV	East African cassava mosaic Malawi virus
EACMV-UG	East African cassava mosaic virus-Uganda
EACMZV	East African cassava mosaic Zanzibar virus
ICMV	Indian cassava mosaic virus
SACMV	South African cassava mosaic virus
SLCMV	Sri Lankan cassava mosaic virus

List of Symbols/Units

A	Absorbance
cm	Centimeter
°C	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

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 presence or absence of virus. For example, cassava brown streak disease is '*diagnosed*' as due to Cassava brown streak virus (CBSV). Methods such as RT-PCR are employed to '*detect*' CBSV 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 (less practiced), and inter-relationships from nucleotide sequence information to determine the virus genomic properties, expression strategy and virus taxonomic status.
- 11) Fulfill Kochs' postulates, especially using purified virus or isolated virus cultures if purified virus preparation loses 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*, family *Nanoviridae*). Few diseases are caused by mixed infections of unrelated viruses. For example, at least 9 virus species belong to the genus *Begomovirus*, are involved in the etiology of cassava mosaic disease, 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). In addition to these, a recombinant strain *East African cassava mosaic virus-Uganda* (EACMV-UG) in East and Central Africa was recognized in CMD etiology in SSA. Mixed infection of one or more of these viruses is common in cassava. All these viruses can cause similar symptoms on their own or in mixed infections.

Some diseases are caused due to mixed infection 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.

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 listed below:

(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 1.1).

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 chapter 3.

Table 1.1: 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

(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 conjunction with other confirmatory tests.

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, ribonucleoproteins) in mammalian systems (rabbit, goat, chicken) can be used to develop variety of serological tests. Serological assays are two types, solid phase assays (ELISA, 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 Hampton 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 off 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 (digoxigenin)] 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°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 1.2.

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 1.2: 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 hemagglutination	20-50 ng/ml
Latex test	5-20 ng/ml
ELISA	1-10 ng/ml
Immunolectron microscope	1-10 ng/ml
Western blotting	1-10 ng/ml
Nucleic acid-based	
Molecular hybridization	<1 pg
PCR/RT-PCR	<1 fg

Table 1.3. Plant virus classification and their major properties*

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

Tospovirus	<i>Tomato spotted wilt virus</i>	Sap, Th (prop)	Env, Icos. 80-100
Unassigned genera			
Ophiovirus	<i>Citrus psorosis virus</i>	Unknown	Thin filaments
<i>Tenuivirus</i>	<i>Rice stripe virus</i>	Ph (prop)	Thin filaments, 3-10
<i>Varicosavirus</i>	<i>Lettuce big-vein virus</i>	Fungus	Rod shaped, 350-360X 18nm.
Single stranded RNA viruses (Positive polarity)			
Family: Bromoviridae			
Bromovirus	Brome mosaic virus	Sap, beetles	Icos, 28-30
<i>Cucumovirus</i>	<i>Cucumber mosaic virus</i>	Sap, Ap (np)	Icos, 28-30
<i>Alfavirus</i>	<i>Alfalfa mosaic virus</i>	Ap (np), seed,	Bacilliform, 4 particles, 30-57X18
<i>Ilarvirus</i>	<i>Tobacco streak virus</i>	pollen	Icos, 3 particles,
<i>Oleavirus</i>	<i>Olive latent virus 2</i>	Unknown	Bacilliform, multipartite
<i>Anulavirus**</i>	<i>Pelargonium zonate spot virus</i>	Seed, pollen	Bacilliform, 35 nm
Family: Comoviridae			
Comovirus	Cowpea mosaic virus	Sap, Bt,	Icos, 28-30
<i>Nepovirus</i>	<i>Tobacco ringspot virus</i>	Nematodes, pollen, seed	Icos
<i>Fabavirus</i>	<i>Broad bean wilt virus 1</i>	Ap (np)	Icos
Family: Closteroviridae			
Closterovirus	Beet yellows virus	Ap (sp), Mb, Wf	flexuous filaments, 1250- 2000, monopartite
<i>Crinivirus</i>	<i>Lettuce infectious yellows virus</i>	Ap (sp), Mb, Wf (sp)	flexuous filaments, bipartite, 700-900 & 650- 850,
<i>Ampelovirus</i>	<i>Grapevine leafroll-associated virus 3</i>	<i>Pseudococcus longispinus,</i> <i>Planococcus ficus</i> (Hemiptera)	Flexuous filaments, 1800-2200nm
Family: Luteoviridae			
<i>Luteovirus</i>	<i>Barley yellow dwarf virus-PAV</i>	Ap (cir, np)	Icos, 25-28
<i>Polerovirus</i>	<i>Potato leafroll virus</i>	Ap (cir, np),	Icos, 24,
<i>Enamovirus</i>	<i>Pea enation mosaic virus-1</i>	Ap	Icos, 25-28
Family: Tymoviridae			
<i>Tymovirus</i>	<i>Turnip yellow mosaic virus</i>	Bt,	Icosahed, 30
<i>Marafivirus</i>	<i>Maize rayadofino virus</i>	Lh	Isom, 28-32
<i>Maculavirus</i>	<i>Grapevine fleck virus</i>		
Family: Sequiviridae			
Sequivirus	Parsnip yellow fleck virus	Aphids (sp, np, cir.) depending on helper virus)	Icos, 30
<i>Waikavirus</i>	<i>Rice tungro spherical virus</i>	Lh (sp)	Icos, 30
Family: Tombusviridae			
Tombusvirus	Tomato bushy stunt virus	few by seed, pollen, few by fungi.	Icos, 32-35
<i>Carmovirus</i>	<i>Carnation mottle virus</i>	Fungi	Icos, 32-35
<i>Necrovirus</i>	<i>Tobacco necrosis virus A</i>	Fung.	Icos, 28
<i>Machlomovirus</i>	<i>Maize chlorotic mottle virus</i>	Seed	Icos, 30
<i>Dianthovirus</i>	<i>Carnation ringspot virus</i>	Soil, no vectors, some times	Icos, 30

<i>Avenavirus</i>	<i>Oat chlorotic stunt virus</i>	nematode Soilborne,	Isom, Icos, 35
<i>Aureusvirus</i>	<i>Pothos latent virus</i>	zoosporic, fungi, Soilborne, no vector	Isom, Icos, 30
<i>Panicovirus</i>	<i>Panicum mosaic virus</i>		Isom, Icos, 30
Family: <i>Potyviridae</i>			
<i>Potyvirus</i>	<i>Potato virus Y</i>	Ap (np), some by seed also	flexuous filaments 680- 900X11-13
<i>Rymovirus</i>	<i>Ryegrass mosaic virus</i>	Mt (per)	Filamentous 690- 720X11-15
<i>Bymovirus</i>	<i>Barley yellow mosaic virus</i>	Fungi	flexuous filaments 250- 300 & 500-600 both are 15 width.
<i>Macluravirus</i>	<i>Maclura mosaic virus</i>	Ap (np)	flexuous filaments 650- 675X13-16
<i>Ipomovirus</i>	<i>Sweet potato mild mottle virus</i>	Wf (np),	flexuous filamentous, 800-950
<i>Tritimovirus</i>	<i>Wheat streak mosaic virus</i>	Mt (per)	flexuous filamentous, 690-700
Family: <i>Flexiviridae</i>			
<i>Potexvirus</i>	<i>Potato virus X</i>	Contact	Slight flexuous rods, 470- 500X13
<i>Carlavirus</i>	<i>Carnation latent virus</i>	Ap (np)	slightly flexuous filaments, 600- 700nmX12-15
<i>Allexivirus</i>	<i>Shallot virus X</i>	Mt	flexuous filaments, 800X12
<i>Capillovirus</i>	<i>Apple stem grooving virus</i>	No vector	flexuous filaments, 640- 700X12
<i>Trichovirus</i>	<i>Apple chlorotic leaf spot virus</i>	Nematodes	Flexuous filaments, 720- 740 nm
<i>Vitivirus</i>	<i>Grapevine virus A</i>	Pseudococcidae	flexuous filaments, 800X12
<i>Foveavirus</i>	<i>Apple stem pitting virus</i>	No vector	flexuous filaments, 800X12
<i>Mandarivirus</i>	<i>Indian citrus ringspot virus</i>		flexuous filaments, 650nm
Unassigned genera (single stranded (ss) RNA genomes with positive polarity)			
<i>Tobravirus</i>	<i>Tobacco rattle virus</i>	Nematodes	Rigid rods, bipartite, L- 180-215 & 48-115
<i>Tobamovirus</i>	<i>Tobacco mosaic virus</i>	contact, no vectors	rigid rods, mono partite, 300-350X18
<i>Hordeivirus</i>	<i>Barley strip mosaic virus</i>	Sap, contact	NE, rigid rods, tri partite, 110-150X20
<i>Furovirus</i>	<i>Soil-borne wheat mosaic virus</i>	Fungus (<i>Polymyxa graminis</i>)	Rod shaped, bipartite, 260-300X20 140-160X20
<i>Pomovirus</i>	<i>Potato mo-top virus</i>	Fungus	Rod shaped, tripartite 1) 290-310 2) 150-160 3) 65-80
<i>Pecluvirus</i>	<i>Peanut clump virus</i>	Fungus	Rod shaped, 2 predominant length, 245 & 190 with dia-21
<i>Benyvirus</i>	<i>Beet necrotic yellow vein virus</i>	Sap, fungus	Filamentous, 4-5 particles-

<i>Sobemovirus</i>	<i>Southern bean mosaic virus</i>	Bt	390,265,100,85X20
<i>Idaeovirus</i>	<i>Raspberry bushy dwarf virus</i>	Pollen , seed	Icos, 30
<i>Ourmiavirus</i>	<i>Ourmia melon virus</i>	Unknown vector, seed transmission	Isom, 33 bacilliform, multipartite, 28 in dia, length of 55, 43, 43, 37
Umbravirus	<i>Carrot mottle virus</i>	No vectors, Helper virus dependent	No specific particles
<i>Cheravirus</i>	<i>Cherry rasp leaf virus</i>	Nematode	Isometric, 30 nm
<i>Sadwavivirus</i>	<i>Satsuma dwarf virus</i>	Seeds (in French bean)	Icosahedral, 26nm

Viroids

Family: *Pospiviroidae*

<i>Pospiviroid</i>	<i>Potato spindle tuber viroid</i>	Contact, seed, pollen, aphids, vegetative propagation	No specific particles
<i>Hostuviroid</i>	<i>Hop stunt viroid</i>	-do-	No specific particles
<i>Cocadviroid</i>	<i>Coconut cadang cadang viroid</i>	-do-	No specific particles
<i>Apscaviroid</i>	<i>Apple scar skin viroid</i>	-do-	No specific particles
<i>Coleviroid</i>	<i>Coleus blumei viroid 1</i>	-do-	No specific particles

Family: *Avsunviroidae*

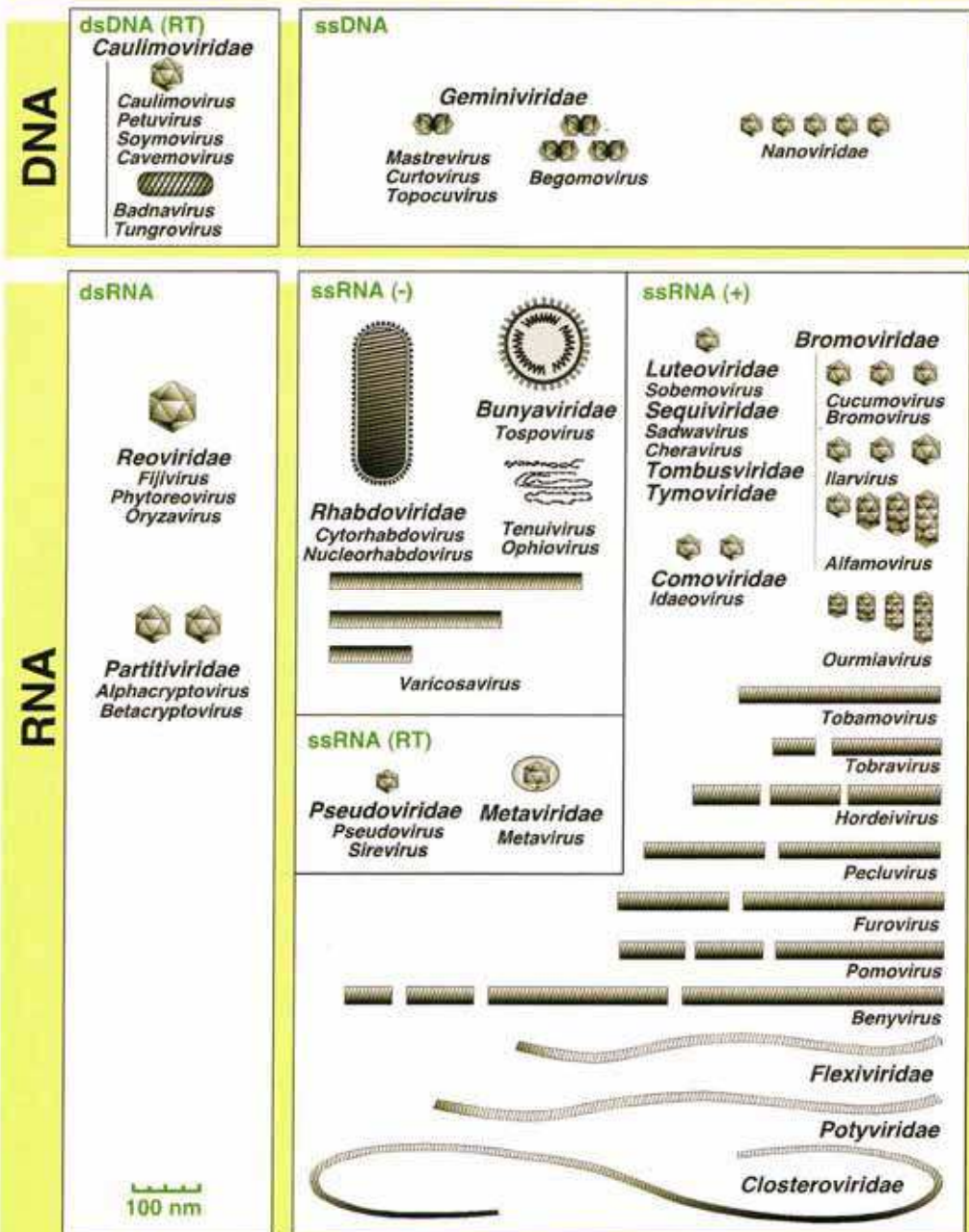
<i>Avsunviroid</i>	<i>Avocado sunblotch viroid</i>	-do-	No specific particles
<i>Pelamoviroid</i>	<i>Peach latent mosaic viroid</i>	-do-	No specific particles

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.

Families and Genera of Viruses Infecting Plants



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 long time 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°C) 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 pyrrolidone 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°C 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, owing 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. Serological and Nucleic Acid-based 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.

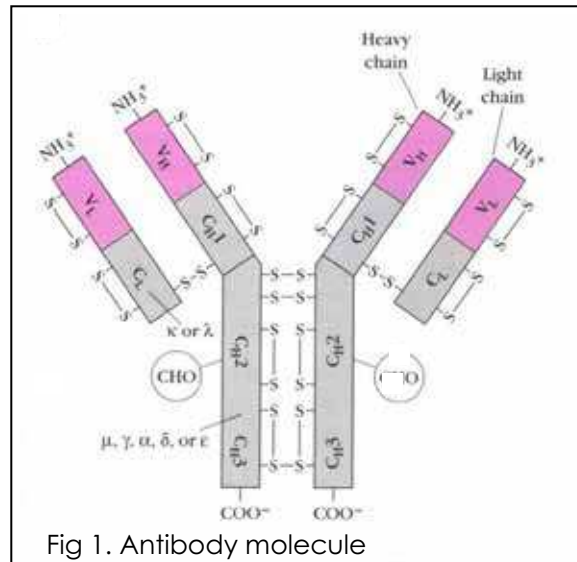


Fig 1. Antibody molecule

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 physico-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. butyricum* 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

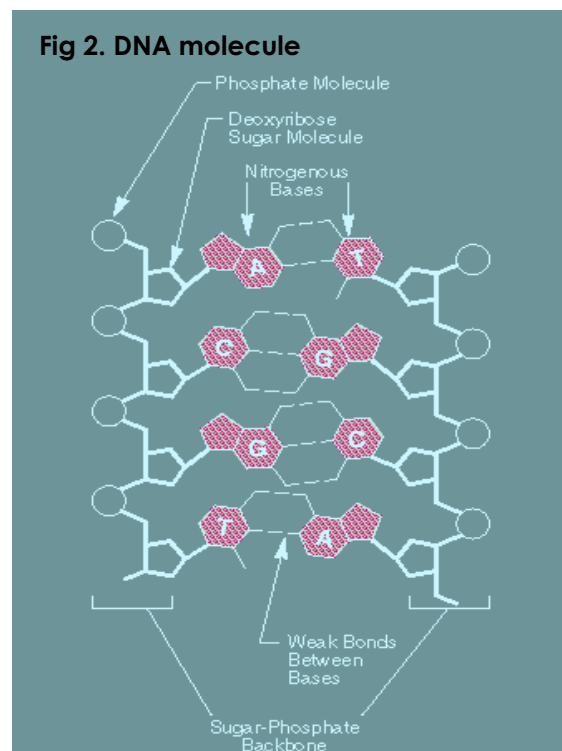
Serological methods have major disadvantage that they are based on the antigenic properties of the virus structural proteins. Thus immunological approaches ignore the rest of the virus genome. It is possible that viruses that are distantly related or not related, as determined by serological methods, 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



groups. The 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 triphosphates (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 $>90^{\circ}\text{C}$ 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 $45\text{-}60^{\circ}\text{C}$. 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 $70\text{-}74^{\circ}\text{C}$. 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 μl 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 μ l 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 μ M final concentration in a 50 μ l 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 amount 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°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 pipetting. 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°C. The maximum recommended temperature for optimum RT enzyme activity is 42°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°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°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°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°C to minimize RNase activity.

4. Screening Germplasm for Virus Resistance

Crop losses caused by plant virus diseases can be prevented in various ways. Over the years three main categories of control measures have been adopted for minimizing virus-induced crop losses. They are (i) removing virus sources, for example by removing volunteer plants or plant remains left from the previous crops; (ii) preventing virus spread usually by killing vectors or interfering with their activity; and (iii) growing the virus-resistant/tolerant varieties of crops. The third option is the most economical for farmers and easily adaptable. Because of this, host resistance has become one of the primary control methods for reducing losses from virus diseases. This form of control is relatively inexpensive for plant producers to implement and is 'eco-friendly'.

The attempts to breed improved crop plants rely on selection, more often intentional, to eliminate the most readily infectible and sensitive types and to select genotypes with superior performance in the field. When the range of genetic variation found in a crop species does not meet the required degree of virus resistance, then related crop species can be screened for the identification of resistance. If the useful source of resistance is identified in cultivated species or closely related and sexually compatible species, it can be used for crossing with a cultivar having desirable agronomic traits. The strategy for breeding depends on the crop species, nature of the reproductive-biology (self-pollinated or self-incompatible), type of cultivar (F1 hybrid, homozygous line or vegetative clone) and inheritance of the resistance (monogenic, oligogenic or polygenic; dominant or recessive). In case of resistant sources available only in related wild species that are difficult or impossible to use in crossing, techniques of interspecific crosses such as *in vitro* culture of immature embryo can be used to introduce resistance.

The basic requirement for successful breeding programs for virus resistance involves selection and crossing appropriate parents, and then making selections from among their progeny, backed, where possible, by knowledge about the genetic control of resistance. This is also possible without detailed knowledge of the genetic mechanism for resistance. The final objective is to combine the resistance with good agronomic traits.

Screening for virus resistance

For any strategy of breeding for virus resistance, good knowledge of the virus and its different strains, and diagnostic tools for their unambiguous detection are essential. The plants to be tested should generally be young and uniform in stage of development. It is essential to use susceptible control plants to ensure that the inoculum used on test plant produces typical symptoms.

Virus transmission onto test plants can be achieved by various means. Mechanically transmissible virus can be inoculated by sap inoculation (eg. *Cowpea mild mottle virus* to soybean). The inoculation can be done manually or using inoculation gun. If the virus is not readily sap transmissible (eg: cassava mosaic begomoviruses; Cassava brown streak virus to cassava), virus vectors (eg: whiteflies) can be used for inoculation purpose. In this case viruliferous vectors need to be reared on infected plants. In case of vegetatively propagated crops such as cassava, graft inoculation can be used. After inoculation the plants should be protected from other viruses to avoid confusions as result other virus infection.

Appearance of symptoms often forms the basis of screening. It is advisable to monitor presence of virus in symptomless plants with sensitive ELISA or PCR-based detection tools. In case where inoculation response is highly variable in the plant population, from complete resistance to partial resistance with different grades of symptom intensities in between, scoring system often denoted by a 'scale' can be used (eg: 1 to 5 rating scale used for cassava mosaic disease).

Large-scale evaluation of genotypes is often carried out under field conditions. This is possible only if the disease recurs at the same area on particular crop every year owing to the presence of vectors and of virus reservoir hosts nearby and there is no risk of mixed infections. Alternatively, growing host plants of the vectors and the virus, inter-spreading the test plants to increase the vector population, allows more consistent disease spread onto test plants evaluated in the field. In any case test plants should be evaluated for presence or absence of virus by diagnostic tools. The screening done under field conditions for 2-3 years takes into account the field resistance. This does not ensure test plants performance against different strains of the virus. The multilocational screening for resistance helps in exposing the genotype to diverse geographic isolates of the

virus. In case of seed transmitted viruses, initial screening of seed material for virus by ELISA is essential. Seed tested positive should be eliminated from the screening trial.

Host response to virus inoculation

Based on the response of the plant to virus inoculation, they can be classified broadly as immune host, infectible (susceptible and resistant) host and tolerant host (see Fig. 1).

Immune host: A host in which virus cannot be detected despite repeated inoculations. This is because cells of immune host lacks surface receptors to facilitate virus particle adsorption and entry, or virus particles may enter into cells, but cell machinery does not support the replication of virus nucleic acid or due to both factors. This reaction typically determines the host range of the virus.

Field immune: A host in which virus cannot be detected under natural virus transmission conditions and under conditions typical to the crop environment. Immunity of such hosts can be overcome by introducing virus through non-convention methods, such as agro-infection.

Infectible: A host which supports virus multiplication. Infectible hosts are two kinds (i) **susceptible host**, which readily supports rapid virus infection, multiplication and invasion; and (ii) **resistant host**, which do not readily support virus infection and multiplication.

Passive resistance: Hosts with resistance to virus entry. If virus enters into the cells, it can multiply and invade as in susceptible host. This kind of response is mainly due to plant resistance to vector (vector resistance); due to lack of surface receptors permitting virus entry or interference with virus adsorption to cells.

Active resistance: This host resistance is against virus replication. Cells do not support virus replication or translation of its products. This response sometimes is influenced by abiotic factors (such as temperature) which can influence cell functions, thus can result in varied host response to virus infection.

Hypersensitive reaction: Severe response of the host plant to minimize the rate and extent of virus invasion. This mainly results in localized necrosis (death of virus infected cells). **Field resistance:** The presence of various forms of resistance separately or in combination minimizing incidence of infection in an infectible plant is termed field resistance.

Tolerant host: Plant is infectible with virus, but it shows only mild symptoms without marked affect on plant growth and vigor or yield. This kind of host response may or may not correlate with virus concentration in the cells. Host may support normal rate of virus multiplication, but show only mild symptoms, such host is susceptible to virus infection, but resistant to disease. If host restricts virus multiplication leading to decrease in virus concentration and show mild symptoms, this host is resistant to virus and also to disease.

Latent host: Virus can infect this host, multiply and invade without causing any effect on the growth, and such plants do not show any symptoms.

Sensitive host: Virus infection leading to conspicuous symptoms markedly affecting the growth pattern and often leading to the plant death. In some cases sensitive reaction depends on the stage at which virus infection occurs.

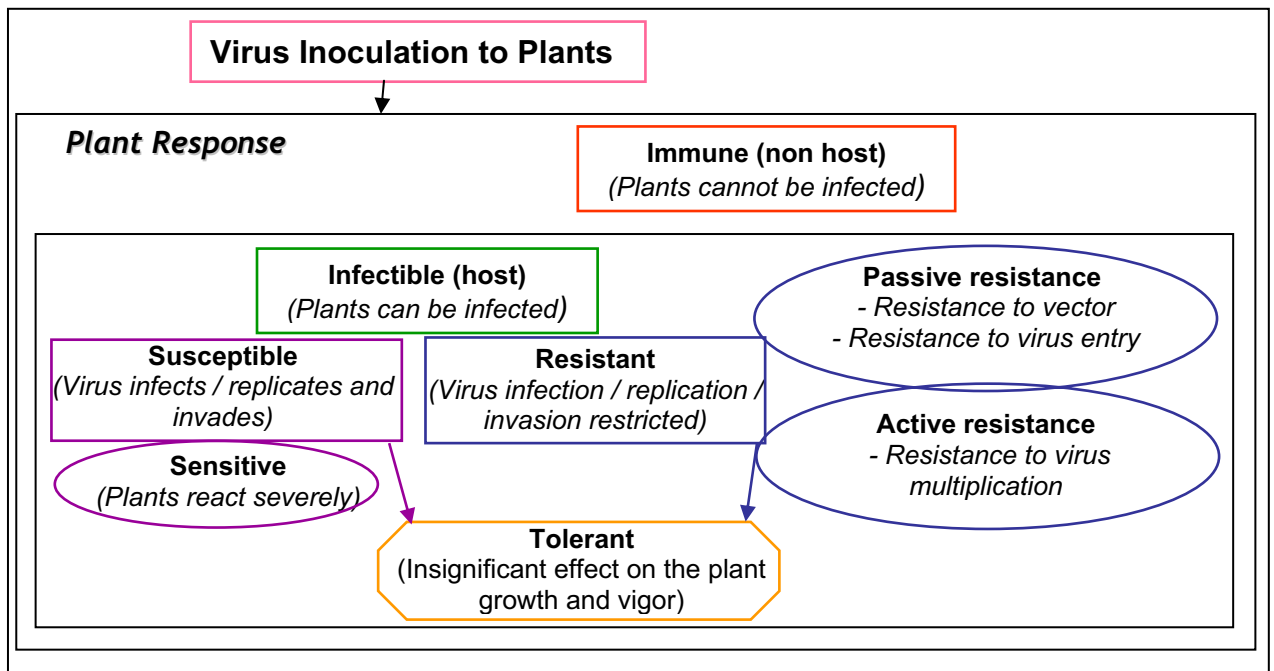


Fig. 1. Different kinds of plant response to virus inoculation (Copper & Jones, 1983)

Laboratory Protocols

5. Nucleic acid-based methods

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. In virology PCR is used for amplification of genome of DNA containing viruses.

Reverse Transcription (RT)-PCR is used for the amplification of viruses containing RNA as their genome. During RT-PCR, the target RNA is first reverse-transcribed to a complementary DNA (cDNA) copy using the enzyme, reverse transcriptase (RT). 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.

In this course, both DNA and RNA viruses will be used as examples. PCR-based assays requires 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. Several 100s of protocols have been described in the literature for the same purpose. Users are advised to make appropriate selection.

5.1. 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 pigeonpea, 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 pigeonpea are being used successfully at ICRISAT for RT-PCR experiments.

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 minicolumn 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 μ l of RLT buffer (supplied with the kit) and 5 μ l of μ -mercaptoethanol (or μ -mercaptoethanol) and mix vigorously (in a vortex shaker).
4. Transfer the lysate into the QIAshredder spin column (supplied with the kit) and centrifuge for 2 min at maximum speed (14,000 rpm) in a microcentrifuge.
5. Transfer flow-through fraction (lysate) from QIAshredder to a new 2 ml tube without disturbing the cell-debris pellet.
6. Add 0.5 volumes (usually 250 μ l) 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-through.
9. Add 700 μ l of RW1 buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
10. Discard the flow-through
11. Add 500 μ l of RPE buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
12. Discard the flow-through.
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 μ l 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°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.
- **0.1 M Tris-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.
- **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.
- **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.
- **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
- **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°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°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 °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 °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 °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 Corporation 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.
- ◇ Discard the supernatant. Air dry the RNA pellet by keeping at 37°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°C for 10 min. Then store sample at -20°C

5.2. 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)
 - 8.5 mM EDTA
 - (Sterilize 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 μ l)
- 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 500 μ l of extraction buffer
2. Transfer contents in to a microfuge tube
3. Add 33 μ l of 20% SDS
4. Vortex briefly and incubate in 65°C water bath for 10 min
5. Allow tubes to cool to room temperature and then add 160 μ l of 5M potassium acetate
6. Vortex and centrifuge at 10,000 g (or 12,000 rpm) for 10 min.
7. Collect supernatant into a separate microfuge tube
8. Add 200 μ l of cold iso-propanol and mix gently and incubate on ice or at 4°C for 20 min.
9. Centrifuge at 10,000 g for 10 min to precipitate DNA
10. Carefully decant the supernatant 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)
11. Add 500 μ l of 70% ethanol into the tubes and centrifuge at 10,000 g for 5 min.
12. 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)
13. Allow the tubes to dry at room temperature or at 37°C to remove final traces of ethanol (it takes about 10-15 min).
14. Resuspend DNA pellets in 50 μ l of TE buffer and store tubes at -20°C until further use.
15. 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.

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

Suitable for preparation of templates for PCR or RT-PCR reactions directly from the leaf tissues of cassava, Musa, soybean, yam, cowpea, maize and other crops, and also from FTA cards.

GEB buffer, pH 9.6

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
PVP-40 (2%)	20 g
BSA (0.2%)	2 g
Tween-20 (0.05%)	0.5 ml
Sterile distilled water to	1 Liter

Sterilize by filtration and store this buffer at 4°C

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 4°C. Prior to use, add 2-mercaptoethanol 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 sterile mortar and pestle for sample grinding). Use this extract immediately or distribute into aliquots and store at -20/-80°C for subsequent use. (Note: Repeated freezing and thawing of this extract will result in poor result).

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 the sample and heat denature at 95°C 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.

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 -20°C for subsequent use. (Note: FTA card piece processed similar to the leaf tissue procedure also resulted in good result in our labs).

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 95°C 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.

6. 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 (Fig. 2). The result is exponential accumulation of the specific target DNA. Here PCR for the detection of *African cassava mosaic virus* (ACMV) is described. Similar protocols can be applied for the detection of viruses with DNA as its genomes.

Materials

Thermal cycler

Sterile 0.2 ml Eppendorf tubes

Primes (see table 2)

Taq Polymerase (Cat.# M3008, Promega, UK)

Four deoxynucleotide triphosphates, 100 mM stock

(dATP, dGTP, dCTP, dGTP)

Mineral oil (optional)

Note: Taq enzyme from other commercial companies can also be used.

Solutions

dNTP mixture

Mix 10 μ l of each dATP, dCTP, dGTP and dTTP from a 100 mM stock. The final concentration of each dNTP in this mixture is 10 mM.

25 mM MgCl₂

Dissolve 0.508 g of MgCl₂.6H₂O in 100 ml distilled water. Sterilize by autoclaving and store at -20 °C. (most commercial Taq enzymes are accompanied with this solution).

6.1. PCR procedure

PCR reaction mixture

Add the following in a sterile 0.2 ml Eppendorf tube:

Add the following for a 12.5 μ l reaction tube (Go Taq DNA polymerase (Cat No: M3008)

2.5 μ l of 5 x Green reaction buffer

0.06 μ l of Go Taq DNA polymerase

0.075 μ l of 10 mM of dNTP mix

0.25 μ l of 10 pmols forward Primer -1 [ACMV-AL1/F 5'-GCGGAATCCCTAACATTATC-3']

0.25 μ l of 10 pmols reverse Primer -2 [ACMV-AR0/R: 5'-GCGGAATCCCTAACATTATC-3']

7.19 μ l of sterile distilled water

1 to 2 μ l of diluted DNA (Usually 1:100 dilution)

PCR programme cycle 1

Perform PCR amplification in a thermal cycler using the following parameters:

94 °C for 1 min: One cycle

94 °C for 1 min; 52 °C for 1 min; 72 °C for 1.30 min: 35 cycles

72 °C for 5 min: One cycle

Note: Mineral oil overlay on the reaction mixture is not necessary if the thermal cycler is provided with the heated lid. Default ramp rate is 100% in GeneAmp PCR System 9700 (PE Applied Biosystems, USA).

Analysis of PCR products

Analyse 8-12 μ l of PCR products in a 1.5% agarose gel as described.

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).

6.2. Reverse Transcription (RT)-PCR

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 μ l, 1-40 μ l, 40-200 μ l and 200-1000 μ l single channel pipettes).
- Microfuge

Solutions

RNase free water

Treat distilled water with 0.1% diethylpyrocarbonate (DEPC; Sigma) for 12 h at 37 °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 MgCl₂

Usually supplied with *Taq* enzyme by the manufacturer.

If necessary, prepare by dissolving 0.508 g of MgCl₂.6H₂O in 100 ml RNase-free water. Sterilize by autoclaving, aliquot and store at -20 °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 °C

6.3. Two Steps RT-PCR reaction

First strand cDNA synthesis (RT reaction)

1. Add the following reagents in a sterile 0.2 ml (or 0.5 ml depending on the thermal cycler) Eppendorf tubes. Keep the tubes in crushed ice during setting up of the reaction: (composition given is for one reaction).

5x MMLV RT buffer (supplied with the enzyme)	4 µl
25 mM MgCl ₂	2 µl
0.1 M DTT	2 µl
10 mM dNTP mixture	0.5 µl
Primer – 1	0.5 µl (10 pico moles)
Primer – 2	0.5 µl (10 pico moles)
RNasin	10 Units
MMLV RT	100 Units
Total RNA	1-4 µl
Sterile dH ₂ O	to 20 µl
Total volume	20 µl

2. Incubate the reaction at 42°C for 45 min.

3. Terminate RT reaction by heating tubes at 94 °C for 5 min.

PCR reaction

1. Add the following in a sterile 0.2 ml (or 0.5 ml depending on thermal cycler) tubes. (composition given is for one reaction)

10x <i>Taq</i> buffer (supplied with the enzyme)	5 µl
25 mM MgCl ₂	3 µl
10 mM dNTPs	0.5 µl
Primer 1	0.5 µl
Primer 2	0.5 µl
Sterile distilled water	20 µl
<i>Taq</i> polymerase	0.2 U
First strand reaction	20 µl
Total volume	50 µl

Note: Mineral oil overlay on the reaction mixture is not necessary if the thermal cycler is provided with a heated coverlid. For machines without heated coverlid, overlay PCR reaction with 10 µl of mineral oil to prevent evaporation.

2. Place the PCR tubes in the thermal cycler and use the following PCR programme for the amplification.

RT-PCR programme:

Perform PCR amplification in a thermal cycler using the following parameters: one cycle of denaturation for 5 min at 94 °C, followed by 35 cycles of amplification by denaturation at 92 °C for 45 sec, primer annealing at 55 °C for 45 sec and primer extension at 72 °C for 90 sec and finally incubate at 72 °C for 5 min for extension.

Analysis of RT-PCR products

Analyze 30-40 µl of PCR products in a 1% agarose gel.

6.4. One step RT-PCR

This example described one step RT-PCR using Yam mosaic virus in yam. Isolate total RNA using any of the methods described for RNA extraction. Add the following in the 0.2 ml PCR tubes.

Sterile distilled water	8.38 µl
10x Taq buffer (supplied with the enzyme)	2.5 µl
10 mM dNTPs	0.25 µl
Taq polymerase	0.06 µl
MMLV-RT enzyme	0.06 µl
25 mM MgCl ₂	0.75 µl
Primer 1	0.25 µl (25 pmol)
Primer 2	0.25 µl (25 pmol)
Total volume	12.5 µl

RT-PCR programme cycle 1

42 °C for 30 min: One cycle

94 °C for 1 min; 52 °C for 2 min; and 72 °C for 3 min: One cycle

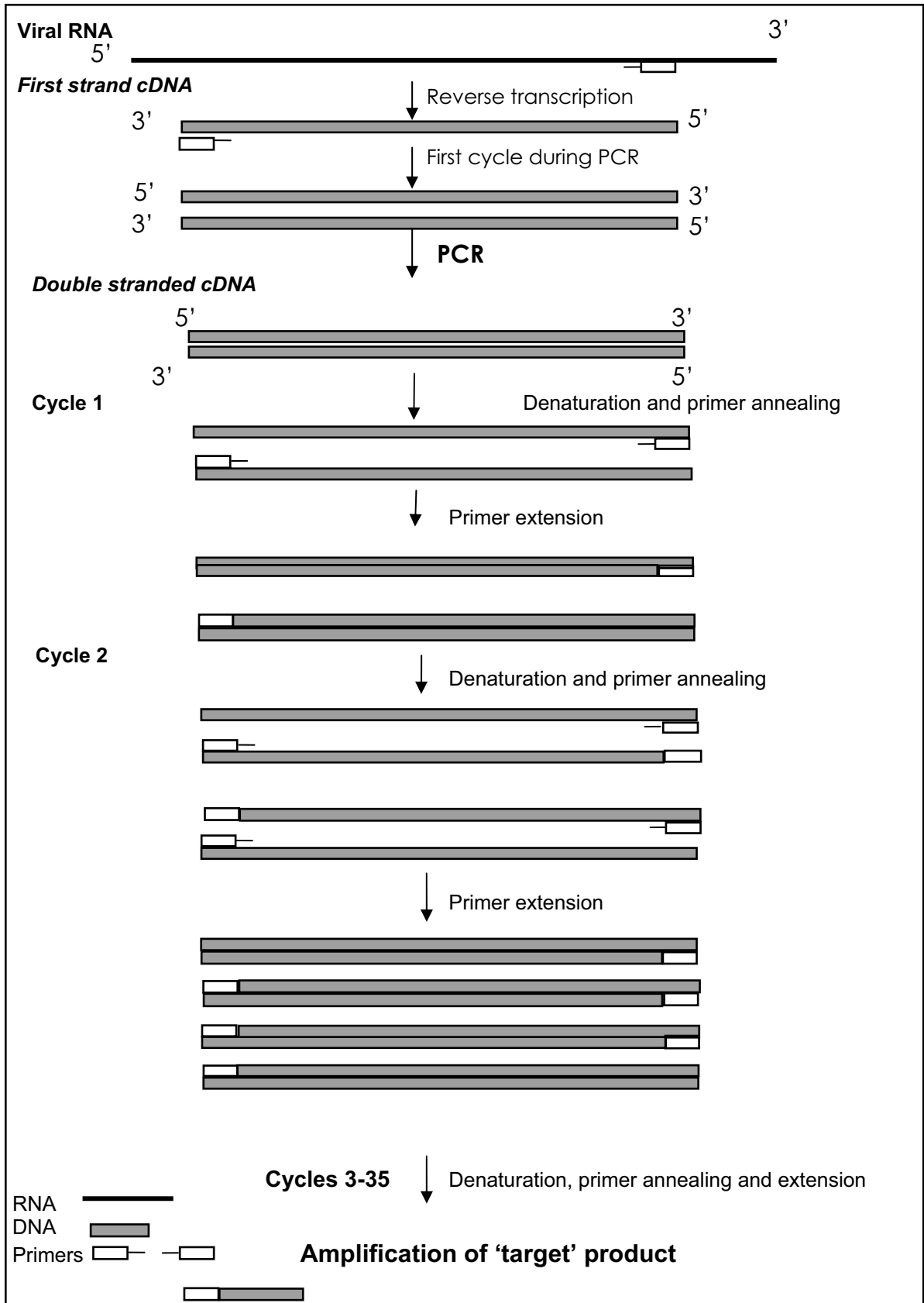
94 °C for 1 min; 52 °C for 1 min; 72 °C for 1 min: 35 cycles

72 °C for 5 min: One cycle

Analysis of RT-PCR products

Analyze 8-12 µl of PCR products in a 1% agarose gel.

Fig. 2
Schematic representation of various steps involved during
the first few rounds of RT-PCR



7. 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 to 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.

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 °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.

Note: Different types of agarose gel electrophoresis units are available in the market. Gel casting procedure depends on the type of the unit. Users are advised to follow the accompanying guidelines. Procedure given below is for casting in a basic model. All other parameters are generally applicable to all units.

Procedure

1. Prepare agarose at the desired concentration (w/v) in 1x TBE buffer (for 1.5% gel, dissolve 1.5 g agarose in 100 ml 1x TBE buffer) and boil in a microwave oven or in a hot water bath, with intermittent shaking until all the agarose is completely dissolved. Replace evaporation loss with distilled water. Add ethidium bromide directly into molten agarose [8 µl (0.05µg/ml) /100 ml solution.
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 °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.
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. Observe the gel on 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.
9. Discard the gel in a separate canister and dispose safely following the procedures recommended for discarding ethidium bromide.

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.

8. Multiplex PCR/RT-PCR for the simultaneous detection of Cassava mosaic begomoviruses and *Cassava brown streak virus*

Mixed infection cassava mosaic begomoviruses [*African cassava mosaic virus* (ACMV) and the *East African cassava mosaic virus* (EACMV) complex (genus, *Begomovirus*; family, *Geminiviridae*)] and *Cassava brown streak virus* (CBSV; Genus, *Ipomovirus*; family, *Potyviridae*) are common in eastern and southern African countries of Tanzania, Kenya, Uganda, Malawi and Mozambique. Separate polymerase chain reaction (PCR) and reverse transcription (RT)-PCR based assays are being used for the detection of viruses in CMD and CBSV-affected plants, respectively. A single tube assay that combines RT-PCR for the detection of CBSV and PCR for the detection of all these viruses is described below. This procedure is useful with total nucleic acid extracts (RNeasy and conventional methods) or even leaf sap from CBSV and/or CMD infected plants. One to three fragments of unique size specific to ACMV, EACMV-like viruses and CBSV is simultaneously amplified and they were identified based on their specific molecular sizes in agarose gel electrophoresis (~230 bp for CBSV; ~328 bp for ACMV and ~640 bp for EACMV-like viruses). The assay has a detection limit of 10^{-3} in leaf sap extract dilutions.

Note that the primers used for CMD is suitable for the detection of ACMV and all EACMV-like viruses (EACMV, EACMCV, EACMKV, EACMMV and EACMV-UG) prevalent in Africa, except *East African cassava mosaic Zanzibar virus* (EACMZV). However, this assay cannot distinguish various EACMV-like viruses. A separate PCR assays are necessary to confirm the exact identify of the EACMV-like virus using specific primers presented in Table 8.3. Primers described for CBSV has been validated so far on isolates in Dar es Salaam, Tanzania and Numulonge in Uganda. It may detect other CBSV isolates also. [Note: CBSV diagnostics are rapidly evolving. Users are advised to contact authors for latest update].

I. Sample Preparation

GEB buffer, pH 9.6

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
PVP-40 (2%)	20 g
BSA (0.2%)	2 g
Tween-20 (0.05%)	0.5 ml
Sterile distilled water to 1 Liter	
Sterilize by filtration and store this buffer at 4°C	

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 4°C. Prior to use, add 2-mercaptoethanol to a final concentration of 1% (v/v).

Procedure for preparation of samples for PCR/RT-PCR:

Step 1: Grind 100 mg leaf tissue in 2 ml buffer [1:20 w/v] in GEB buffer. Use this extract immediately or distribute into aliquots and store at -80/-20°C 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 95°C 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. Do not store this extract.

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 -20°C 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 95°C 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.

II. Multiplex RT-PCR for CMBV and CBSV

Total nucleic acid extracted from plant tissue or leaf sap extract described above can be used as template for performing this multiplex PCR protocol.

Primers

This PCR consists of 5 primers. Primers 1,2,3 listed in the table 8.1 are specific for cassava mosaic begomoviruses (CMBV); whereas primer 4a, 4b are specific to CBSV, each amplifies a separate target. **Note:** Several oligonucleotide primers have been described for the detection and discrimination of cassava mosaic begomoviruses as well as Cassava brown streak virus. The primers listed in Table 8.1. are useful for the simultaneous detection of ACMV and EACMV-like viruses and CBSV in a single reaction.

Table 8.1. Details of multiplex PCR primers used for the detection of CMBV and CBSV

	Primer name	Primer sequence (5' → 3')
1	CMBRep-F	CRTCAATGACGTTGTACCA
2	ACMVRep-R	CAGCGGMAGTAAGTCMGA
3	EACMVRep-R	GGTTTGACAGAACTACATC
4a	CBSV-L/F	CGGGGTACCCAGAATAGTGTGCTGCAG
4b	CBSV-L/R	CGGGAATTCTACATTATTATCATCTCC

PCR reaction mixture

All the PCR/RT-PCR reagents presented in the table are from Promega

Table 8.2. Multiplex reaction mixture

	Components	Volume (µl) for one reaction
1	5x GoTaq Flexi Green PCR Reaction buffer (MgCl ₂ free; supplied by the manufacturer along with the enzyme)	5
2	25 mM MgCl ₂ (supplied by the manufacturer along with the enzyme)	2.4
3	10 mM dNTP mix (Promega)	0.15
4	10 pM CMBRep-F	0.5
5	10 pM ACMVRep-R	0.5
5	10 pM EACMVRep-R	0.5
6	10 pM CBSV-L/F	0.5
7	10 pM CBSV-LR	0.5
8	Taq polymerase (Cat. # M3008) (0.8 units per reaction)	0.12
9	M-MLV RT (24 units per reaction)	0.12
10	Leaf sap extract prepared in section I [Note: Total nucleic acid extract can also be used as template. usually 1:100 dilution works best; if necessary adjust dilution accordingly]	4
11	sterile distilled water	to 25 µl
Total reaction volume		25

Note: Regents from other commercial companies can also be used. But users are advised to test on a few samples to assess the performance of the protocol. Make sure to adhere to the MgCl₂ concentration given in the table.

Thermal cycle conditions

42°C, 30 min: One cycle

94°C, 1 min; 52°C, 2 min; and 72°C, 3 min: One Cycle

94°C, 1 min; 52°C, 1 min; and 72°C, 1 min: 35 cycles

72°C for 5 min: One cycle

After completing PCR program, resolve amplified products in 1.5% agarose gel as described in section 7.

An example of CMBV and CBSV multiplex PCR gel

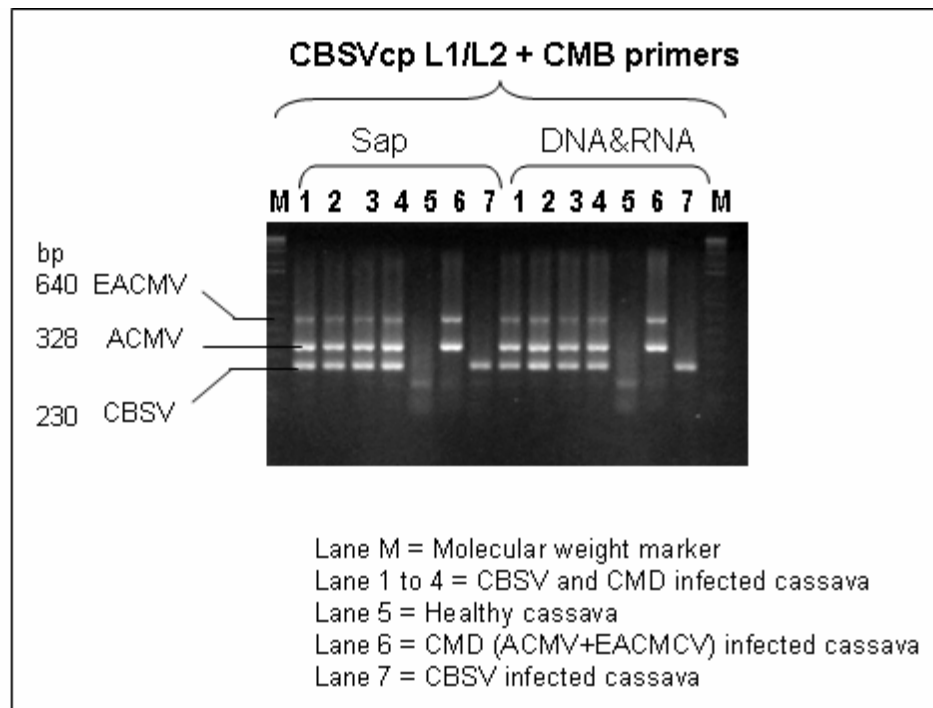


Table 8.3. Details of specific primers for the amplification of various cassava mosaic begomoviruses*

Virus Abbreviation	Virus name	Primer name	Primer sequence (5' → 3')
1. Begomovirus	Amplifies most of the begomoviruses	Bego 1	TAATATTACCKGWKGVCCSC
		Bego 2	TGGACYTTRCAWGGBCCTTCACA
2. EACMV-UG2	East African cassava mosaic virus-Uganda	UV-AL1/F1	TGTCTTCTGGGACTTGTGTG
		ACMV-CP/R3	TGCCTCCTGATGATTATATGTC
3. ACMV	African cassava mosaic virus	ACMV-AL1/F	GCGGAATCCCTAACATTATC
		ACMV ARO/R	GCTCGTATGTATCCTCTAAGGCCTG
4. EACMV	East African cassava mosaic virus	UV-AL3/F	TACACATGCCTCRAATCCTG
		UV-AL1/R2	CTCCGCCACAACTTACGTT
5. ICMV	Indian cassava mosaic virus	ICMV-F	TTCTCTCTCCTCAATCGGTA
		ICMV-R	ACTCAGGGAACCTCGTTTAGT
6. SACMV	Southern African cassava mosaic virus	SACMPCP3	CCTTTATTAATTTGTCCTGC
		SACMVCP5	GCTGTCCCCATTGTCCARGGN
7. EACMZV	East African cassava mosaic Zanzibar virus	EACMZan-F	GATCCATTGTAAACGATTTCCCTGA
		EACMZan-R	CCACATGTTGACGCGCTCCACTACTT
8. EACMMV	East African cassava mosaic Malawi virus	EACMAL-L3-F	TACGCATGCCTCTAATCCAG
		EACMAL-L1-R	TTCCGCCACAACCTTATGTA
9 EACMCV	East African cassava mosaic Cameroon virus	VNF031	GGATACAGATAGGGTTCCAC
		VNF032	GACGAGGACAAGAATTCCAAT

*Several primer sets are available in the literature. The list provided here are commonly used in our labs.

Thermal cycler conditions: 1 cycle of 94°C [5 min]; 35 cycles of 94°C [1 min], 58°C [1 min], 72°C [1 min]; 1 cycle of 72°C [5 min]; 4°C [infinity]

Longitudes west of Ghana, Burkina Faso and Mali are negative, these 3 countries can have both positive and negative longitude values.

If other coordinate formats are needed for local purposes (eg UTM meter coordinates), it is easy and fast to convert decimal degrees into any other coordinate system. It is complicated and time consuming to convert from most other systems into decimal degrees, unless we receive a spatial dataset already.

In any case make sure that you **receive** (if data is taken by non IITA partners) and **send** us the **whole** information related to the dataset. Eg **coordinate format** (decimal degrees, degrees minutes and seconds, degrees minutes, meters), the **map datum** (eg WGS 84, UTM zone, other local datum and projections). If names of villages or lowest available admin units (districts, communes, LGAs, postos, provinces, states etc) are added in the excel files we receive, it helps us to check if errors exist or if at least points are in the admin unit they are supposed to be.

If you have received coordinates in degree s minutes and seconds or degrees minutes formats (see examples below) please have them entered into excel in the following way (fig 2), not like this: 07°29'56.00" N; 003°54'25.60" E. Entering the whole string in one or two excel cells causes a lot of additional work, both for the people entering the data and the people creating the spatial datasets and can cause further errors. If the degrees, minutes and seconds are split in columns we can easily convert to decimal degrees and proceed.

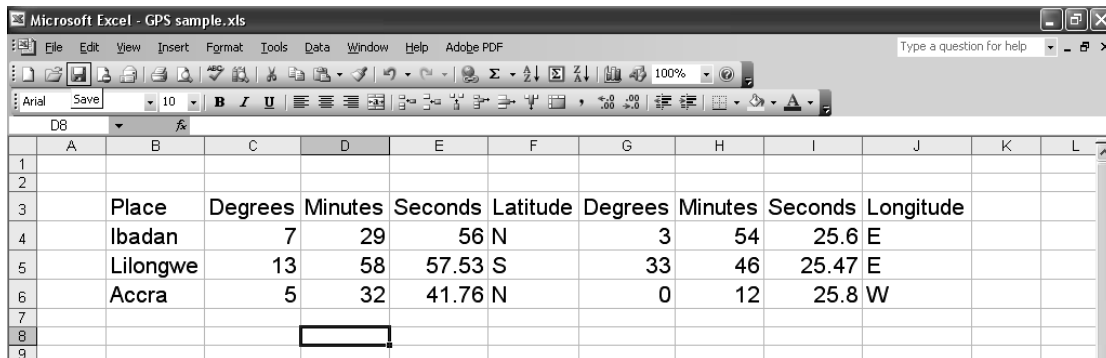


Fig 2. Example for entering coordinates in degrees minutes and seconds

Examples: Some locations in different coordinate systems

Place	Latitude/Northing	Longitude/Easting
Ibadan, Nigeria		
Decimal degrees	7.49889° N	3.90711° E
Degrees minutes and seconds	7°29'56.00" N	3°54'25.60" E
Degrees minutes	7°29.9334' N	3°54.4266' E
UTM Zone 31N	0829003	600085
Lilongwe, Malawi		
Decimal degrees	-13.982646° S	33.773741° E
Degrees minutes and seconds	13°58'57.53" S	33°46'25.47" E
Degrees minutes	13°58.9588' N	33°46.4245' E
UTM Zone 36L	8454057	583565
Accra, Ghana		
Decimal degrees	5.544932° N	-0.207167 W
Degrees minutes and seconds	5°32'41.76" N	000°12'25.80"
Degrees minutes	05°32.6959' N	000°12.4300' W
UTM Zone 30N	0613630	809449

10. Protocols for whitefly identification, rearing, virus transmission and silver leaf testing in laboratory conditions

Rearing insect vectors in artificial conditions and the ability to generate large numbers, when required, is an essential part of conducting controlled experiments. Below outlined is an example protocol for whitefly (*Bemisa tabaci* (Fig. 1), Homoptera, Aleyrodidae) identification, collection, rearing and generating large numbers in laboratory conditions.

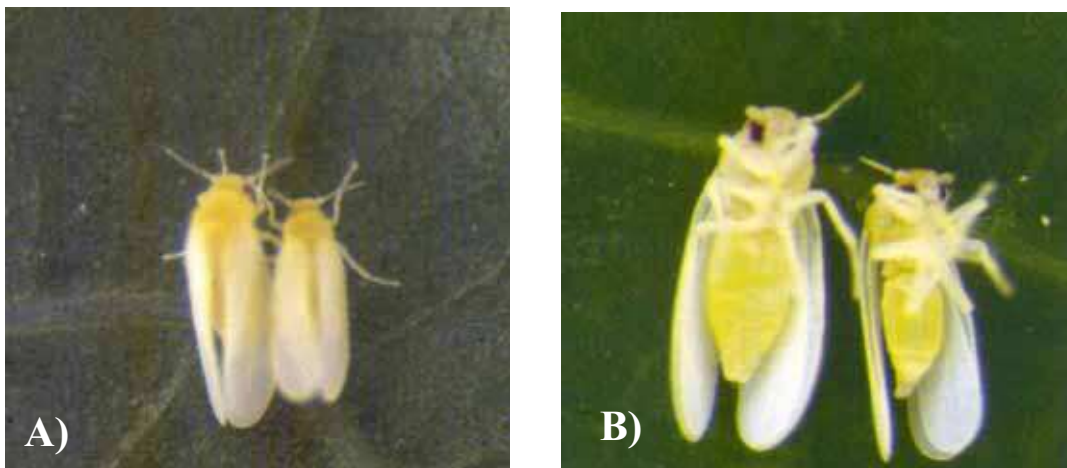


Figure 1. A) *Bemisa tabaci* adults (bigger size female on the left, smaller size male on the right) in copulation on a plant (approximate size X 500 times). B) Differentiation of *Bemisa tabaci* adults based on abdominal size and shape; big, blunt abdomen of a female insect on the left, small pointed abdomen of a male insect on the right (approximate size X 750 times).

Field collection of whiteflies:

Materials required

- 1) Plastic/glass jars (10 cm wide, 10 cm deep) with the possibility for ventilation through holes in lids
 - 2) Nylon mesh, size 40 (40 holes per square inch)
 - 3) Aspirator for the collection of insects
 - 4) Hand-held magnifying lens
 - 5) Sticky tapes to secure jar lids (cello tape or masking tape)
 - 6) Labelling kit (labels, marker pens, etc.)
 - 7) Licence to import, keep and move invertebrates to exotic locations (if required)
1. Identify crop plants/fields which have been planted for two months or more, and plan collection early in the day. *B. tabaci* becomes more active with the increase in temperature and are difficult to catch later in the day. Collection is also difficult on a windy day.
 2. Identify 1-2 leaves with many 3rd or 4th instar *B. tabaci* nymphs. Remove it from the plant and transfer it inside the jar. Older leaves at the base of the plant will have many nymphs, which serve as a source of insects when adults emerge from nymphs.
 3. *B. tabaci* adults tend to feed on the underside of young leaves usually on the upper part of the plant. Slowly turn the leaf over to find feeding insects.
 4. Collect about 15-20 insects per plant using an aspirator by sucking gently. The aspirator is designed such a way that sucking of insects into the mouth is prevented by using two layers of nylon mesh.
 5. Transfer insects into jars through the holes provided in the lids or by opening the lids slightly and blowing gently through the aspirator.
 6. Repeat the collection of adults from at least five plants from each field in order to have a representative collection. *B. tabaci* is host-specific therefore collection from a single plant species can be pooled in a single jar while the collection from different plant species should not be mixed.
 7. Label the jars with date, location, host-plant and the name collector.

Importing and transport of whiteflies:

Introduction of certain biotypes of *B. tabaci* to new countries have caused unprecedented economic losses during the past two decades hence their importation is strictly regulated. Licenses and special permissions must be obtained with relevant authorities prior to the importation of live insects. Utmost care therefore should be taken to transport whiteflies from one country to another securely by placing the jars in insect-proof bags.

Establishing whitefly colonies and maintenance in the insectary:

Materials required:

- 1) Quarantine-regulated insectary facilities for the importation of whiteflies from another country or region
- 2) Facilities to grow healthy plants
- 3) Insect rearing Perspex screen cages, sides covered with nylon mesh for ventilation (Example dimensions; medium size cage - 110H x 40W x 40D cm or small cage - 75H x 32W x 40D cm)

Procedure

1. Grow healthy plants of the same species (for example, grow cassava if whiteflies were to be collected on cassava in the field) or universal host such as eggplant prior to importing whiteflies so they will be ready to initiate the colonies.
2. Set up a cage with two plants placed inside a plastic tray for regular watering (Fig. 2). Cage should be set up in a designated insect-proof area.
3. Remove lids of the jars containing *B. tabaci* adults and the leaves containing nymphs and place jars inside the cage with two healthy plants.
4. Label the cages and maintain the colony in controlled environment facilities (if possible) or at constant conditions around 28°C, 60% R.H. and L12:D12 hours.
5. *Establishment of virus-free colony*; Transfer about 100 adults onto healthy eggplants in a



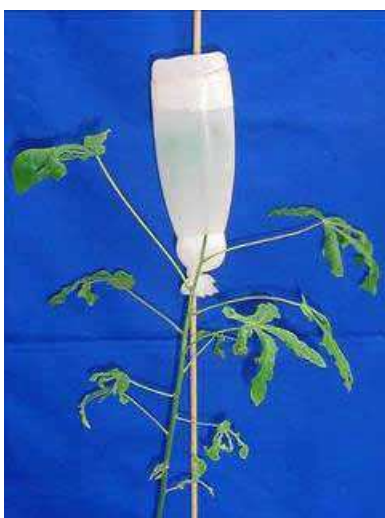
Figure 2. A screen cage with two young cassava plants ready for setting up a live colony of whiteflies.

6. separate cage about two months after initiating the colony. Field-collected whiteflies are likely to carry viruses in them and therefore needs to be cleaned. Eggplant is considered to be a non-host for whitefly-transmitted viruses hence the new generation of whiteflies will be free of viruses.
7. After about another two months initiate a virus-free colony from adults collected from the eggplant.
8. Confirm the purity of the colony by observing for no symptoms of virus infection and also testing by virus diagnostic protocols (ELISA, PCR etc), if possible.
9. Once a month replace the old plants in cages with young fresh plants for colony maintenance
10. If large numbers of whiteflies are required for experimental purposes, introduce 4-5 young plants into the cages depending on the size of the cage. A medium size cage can supply up to 1000 adults in a week, if more insects are required set up more cages.

Below is an example protocol for the transmission of cassava mosaic viruses using *B. tabaci*, but the general features apply equally well to the other virus-vector systems.

Material required:

- 1) Virus-infected plants
 - 2) Facilities to grow virus-free plants
 - 3) Aspirator, clip-cages, plastic bottles
 - 4) Insect rearing cages
 - 5) Insecticides to kill insects after inoculation of viruses
-
1. Grow virus-infected cassava plants two months before the start of experiments. When required for transmission experiments they should be placed in an insect-proof container for transport to the designated laboratory or controlled environment room where the experiments are to be conducted.
 2. Collect adult whiteflies of either sex from the appropriate colony using an aspirator.
 3. Anaesthetise the whiteflies using CO₂ and place into plastic bottles (with muslin bottoms for aeration and to prevent condensation). For routine transmissions 100-200 insects/bottle is sufficient.
 4. Place a bottle of insects onto the first leaf or apex of a suitable virus source plant (e.g. one-month-old cassava plant showing disease symptoms) in a suitable insect escape-proof container.
 5. Allow the insects to acquire virus for 48 h.
 6. After the acquisition period, collect the insects using an aspirator, and place batches (usually 30 insects) into clip cages.
 7. Prepare healthy test plants (e.g. propagate cuttings of cassava in the non-quarantine glasshouse) and acclimatise in the designated laboratory or controlled environment room where transmission experiments will be conducted.
 8. Place test plants in an insect escape-proof screen cage and clip a single clip-cage of viruliferous whiteflies onto the apex of each plant and allow inoculation feeding for 48 h.
 9. After the inoculation period remove the whiteflies from the test plants using an aspirator and kill the whiteflies by placing in a freezer at -20°C for 48 h and then autoclaving.
 10. Depending on available space, the inoculated test plants may either be kept in the cage or may be enclosed in perforated polythene bags and maintained in a controlled environment room for the expression of disease symptoms and pathogen testing.
 11. Prune the plants that show no symptoms at the end of 12 weeks and record any symptoms on the new growth.
 12. At the end of the experiment, the plants and soil and perforated plastic bags should be destroyed by freezing at -20 C for 48 h and subsequent incineration or autoclaving. Insect escape-proof screen cages should be thoroughly cleaned and checked for damage before and after each experiment.



Virus source plant



Virus target plant

Figure 3. A diagrammatic representation of the transmission of cassava mosaic virus using whiteflies.

Silver leaf method for the detection B-biotype:

Materials required:

- 1) Colony of silver leaf producing whiteflies (B-biotype)
- 2) Silver leaf sensitive crop plants; Example Squash var. Long Green, pumpkin var. Big
- 3) Aspirator, clip-cages, plastic bottles
- 4) Insect rearing cages
- 5) Insecticides

1. Establish a colony of B-biotype as described above.
2. Grow squash or pumpkin plants in insect-free cages.
3. Collect about 10 B-biotype adults from the cage using an aspirator.



nymphs.

4. Release the whiteflies onto two weeks old plants enclosed in perforated plastic bags.
5. After 24 hours remove the whiteflies from the test plants using an aspirator
6. Keep the inoculated plants in an insect-free area for up to six weeks for the expression silvering symptoms (Fig. 4)

Figure 4. Silver leaf symptoms (left leaf) developed on pumpkin plant var. Big upon feeding by B-biotype

11. Protocol for Cassava Pest and Disease Monitoring

The following protocol is routinely used by the scientists at IITA to provide data on all of the major diseases and pests of cassava that will allow for statistically meaningful comparisons to be made between different sampled regions in the same year and between 'districts' from one year to the next.

Methods

i) Sampling 'domain'

Surveys will be conducted in project target 'districts' (or equivalent) of target countries. The number of sampled 'districts' and their area will vary from country to country and depends on user plan.

ii) Sampling timing

There is no single ideal period for sampling in the six GLCI countries. Different pests and diseases are more effectively assessed at different times of the year. The best compromise is the third quarter of the year (June to September), when young crops are available, CBSV symptoms in both leaves and roots are readily seen and attack from some of the most important pests is also clearly evident. Sampling timing will be consistent for each country throughout the project to ensure that valid year-on-year comparisons can be made.

iii) Field selection

Farmers' fields: Fields will be sampled along motorable roads running through target districts. Fields will be selected at regular intervals, determined by dividing the length of the route to be covered through the district by the number of sites to be sampled per district. Sampling sites will be selected according to the intervals determined for the district and where 3-6 months old cassava crops are seen. These crops comprise the 'young' sampled field and will be sampled for the full range of cassava major pests and diseases. Mature crops (more than 10 months after planting) neighbouring these fields, and referred to as the 'old' sampled field, will be identified and sampled only for CBSV because in such fields it is possible to check for the necrosis symptoms that affect the tuberous roots. For every three 'young' fields, two 'old' fields will be sampled.

iv) Field background information

For each sampled field, details of location are recorded on the sampling data sheet. Separate sheets are provided for the 'young' and the 'old' fields. These include administrative level identifiers for the site, longitude/latitude and altitude recorded using a GPS and basic information about the cassava variety being sampled and the field environment (see Appendices 1 and 2). 'Neighbouring cassava fields' is the number of cassava fields that can be seen readily from the cassava field being sampled. Other crop plants being grown together with the sampled cassava are indicated under 'intercrop'. The approximate size of the sampled field is estimated.

v) Sampling approach

In both 'young' and 'old' fields, only the predominant variety is sampled, although other varieties are recorded (Sseruwagi *et al.*, 2004). This follows customary practice for recent surveys in the region, and ensures that data obtained for each variety can be compared statistically with data obtained for the same variety in other locations or for other varieties. The predominant variety is the variety that occurs most frequently in the selected field. In the 'young' field, 30 plants are sampled at regular intervals along an 'X' transect. In the 'old' field, 10 plants are sampled at regular intervals along an 'X' transect.

vi) Data recorded in the 'young' field

Severity and damage scores are as set out in the standardized IITA pest/disease scoring table provided as Appendix 3. Specific details for each of the major pests and diseases to be assessed under GLCI surveillance surveys are provided below:

Cassava mosaic disease (CMD)

The parameters taken for CMD will be symptom severity and infection type. Severity is scored on a scale of 1-5 where 1 represents no symptoms and 5 the most severe symptoms. Infection types are categorized as "C" (cutting-borne infection), "W" (whitefly-borne infection) or "H" healthy for

uninfected plants. Where the lower first-formed leaves show symptoms, infection is assumed to be cutting-borne, whilst where only upper leaves show symptoms, infection is considered to be whitefly-borne. When assessing severity, only the infected portion of the plant is considered.

Whitefly abundance

Adult whitefly (*Bemisia tabaci*) are counted on the top five apical leaves and nymphs are counted on the 14th leaf of the tallest shoot for 5 of the 30 plants sampled per field and the totals of individual counts are recorded separately.

Cassava bacterial blight (CBB)

Cassava bacterial blight (CBB) severity is assessed by scoring severity of the disease on the 30 sampled plants using a scale of 1-5, where 1 represents no symptoms and 5 the most severe symptoms.

Cassava brown streak disease (CBSD)

Leaf and shoot symptoms of CBSD are assessed for each of the thirty sampled plants using a severity scale of 1-5, where 1 represents no symptoms and 5 the most severe symptoms that include stem streaking and shoot tip die-back. In addition, in the 'stem' column of the datasheet, the presence or absence of CBSD stem symptoms is noted for each plant with '+' (for present) and '-' (for absent).

Sooty mould and whitefly physical damage assessments

Sooty mould and physical damage on leaves caused by the feeding effects of whitefly are assessed using a scale of 1-5 on every second plant along the sampling transect. In these assessments, the effect on the whole plant is considered.

Cassava green mite (CGM) and cassava mealybug (CM) damage assessment

The severities of CGM and CM are assessed on a scale of 1-5, where '1' represents no symptoms and '5' the most severe symptoms.

***Typhlodromalus aripo* (T.aripo), predatory mite of CGM**

The occurrence is assessed by carefully opening the shoot tip of the tallest shoot of every third plant (10 in total) and indicating presence by '+' and absence by '-'

Other observations

It is important that the GLCI project is aware of the potential for occurrence and spread of completely novel (and possibly exotic) pests or diseases. As such, where any unusual pest, disease or apparent disease symptom is noted, a written note will be made on the field's datasheet and a picture should be taken. A rough assessment of the importance of the 'other' pest/disease should be made, where *** indicates severe and present on most or all sampled plants, ** indicates moderate and present on more than half of the sampled plants and * indicates mild and present on less than half of the sampled plants.

CMD/CBSD in other varieties

If the CMD or CBSD status in varieties that are NOT the predominant and sampled variety is greatly different to the predominant, sampled variety, a note should be made in the appropriate line at the bottom of the datasheet. For example, if the predominant variety that was sampled is CMD-resistant improved material, and the unsampled local variety has a high incidence of severe CMD, this should be noted in one sentence on the line 'CMD in other varieties'. A similar approach should be used for CBSD.

vii) *Data recorded in the 'old' field*

Cassava brown streak disease (CBSD)

Ten plants are examined along an 'X' transect of the 'old' field. For each of these plants, leaf and shoot symptoms are assessed as for the 'young' field, and the presence/absence of stem symptoms is noted. However, with the permission of the farmer, and coupled with the payment of an appropriate level of compensation (equivalent to the local market value of the fresh roots), the ten plants are dug up for assessment of root symptoms. All roots are then assessed by making five cross-section cuts with a knife or cutlass for each root. Each of the five cut sections is then

scored separately using the pictorial severity scale provided as a laminated card (Appendix 4). Consequently, five scores are obtained for each of the sampled plants' roots. Images of unusual symptom types should be recorded using both written descriptions and by taking photos. Remarks should be added for non-sampled varieties where their CMD or CBSV disease status (leaf/stem symptoms) contrasts strongly with that of the predominant sampled variety.

viii) *Sample collection*

Cassava mosaic disease (CMD)

One CMD-diseased plant expressing symptoms typical of CMD in the 'young' field should be selected for leaf sampling. One leaflet of the topmost expanded leaf showing clear CMD symptoms is picked and rubbed, using the base of a microfuge tube, onto a single sample spot on a sheet of FTA paper. A piece of 'parafilm' is placed between the microfuge base and the leaf during the rubbing. If very unusual CMD symptoms are seen, an 'extra' sample may be collected on a separate FTA paper (kept separate from the main survey sample series and used ONLY for small numbers of unusual samples), making sure to clearly indicate the field and plant number next to the spot where the sample was rubbed onto the FTA card.

Cassava brown streak disease (CBSV)

For samples to be collected for CBSV diagnostics, the frequency of sampling will vary depending on whether or not a multiplication site being tested for CBSV is present in the district or not. For districts where a CBSV testing multiplication site is present, two out of every three sampled plants in the 'young' field will be used for CBSV sample collection (making two sets of ten composite samples). For every three plants along the 'X' transect, the second and third plant will be used. For districts where multiplication sites are not being tested for CBSV, every third plant in the 'young' field will be used for CBSV sample collection (making one set of ten composite samples). For each of the plants sampled for CBSV diagnostics, the central leaflet is picked from the second fully-expanded leaf (counting from the shoot apex) and this leaflet is stuck onto a sheet of blank newsprint using masking tape. Newsprint sheets are labeled at the top with site details (country, district, field number) and the plant number is written next to each leaflet after it is stuck in. This number should correspond with the plant number of the 'young field' datasheet, and a tick should be entered into the relevant space under the CBSV 'sample' column to indicate that that plant was sampled for CBSV diagnostics. Ten leaflets obtained from ten plants will be placed on a single blank newsprint sheet. Where 20 leaflets are collected from a single field (for districts with CBSV testing of multiplication sites), two sheets of blank newsprint will be used. Following the completion of sampling, sheets of newsprint with attached leaflets will be placed into a herbarium press for storage through the duration of the survey. In the whole process of sampling and storage, moisture must be avoided to ensure good quality samples. Samples will need to be stored in a freezer (ideally -80°C) on arrival at the laboratory prior to testing. For countries where CBSV testing is not possible, samples should be sent by courier together with all necessary phytosanitary certification to the designated central testing laboratory.

Whiteflies

Bemisia tabaci whitefly adults are collected from one in five 'young' fields using an aspirator. Care must be taken to distinguish between *B. tabaci* and *B. afer* adults based on the characteristic morphological distinguishing features. At least 10 adults should be collected per field, although the target should be more than thirty, with a maximum of 100. Collected whitefly adults are killed by adding 80% ethanol to the aspirator vessel and these are then transferred to a 2ml polythene sample tube to which a pencil-written label is added. The tube is then labeled externally with a permanent marker and sealed with parafilm. On the pencil-written label placed inside the tube, the following should be indicated: country (short-form e.g. Tz for Tanzania, Ug Uganda etc), field number, the species identity (in this case '*B. tabaci*'), and the date. On the outside of the tube, country and field number should be written BOTH on the top of the tube's lid, as well as on the side of the tube, using the permanent marker, before the tube is sealed with parafilm.

ix) *Data recording, collation and analysis*

Data will initially be recorded in the field using forms such as that appended to this document as Appendices 1 and 2. In year 2 and subsequently, a switch will be made to field data recording using hand-held PC devices pre-loaded with excel spreadsheets designed to handle data entry

and allow simple data processing. Ideally, these should also have integrated GPS capability. A target will be to provide summarized data 'in real time' through uploading field-collected data to the Project web site at the end of each sampling day. Mapping will be done using these real-time collated data to provide up-to-date visual representations of the distributions and incidence/severity levels of each of the major pests and diseases.

Averaged disease and pest data for the district or equivalent level will allow for statistical comparisons to be made between districts within the same year and between districts from year to year. These data will enable assessments to be made of rates of disease change and will facilitate the forecasting of future patterns of pest spread or disease epidemic development. The complete dataset will provide the basis for sub-regional determinations of the epidemiology of the two main virus diseases, CMD and CBSD.

References

Legg, J. P., Owor, B., Sseruwagi, P. and Ndunguru, J. 2006. Cassava mosaic virus disease in East and Central Africa: Epidemiology and management of a regional pandemic. *Advances in Virus Research* 67, 355-418.

Sseruwagi, P., Sserubombwe, W.S., Legg, J.P., Ndunguru, J. and Thresh, J.M. 2004. Methods of surveying the incidence and severity of cassava mosaic disease and whitefly vector populations on cassava in Africa: a review. *Virus Research* 100, 129-142.

Appendix 1 GLCI DISEASE SURVEY DATA SHEET - Young Field

Country							Field Size (m ²)						
Dist./Prov./Territoire							Age (months)						
Village/Sous-Colline							Cassava Vars.						
Latitude							Sampled Var.						
Longitude							Intercrop(s)						
Altitude (m)							Neighbouring Cassava Fields						
Date/Time							CM sev.		CMD		CBSD		CBB sev.
Researcher(s)							inf.		sev.		sev.	stem	sample
Adult Whitefly	Whitefly Nymphs	Whitefly Damage	Sooty Mould	CGM sev.	<i>T. aripo</i>	CM sev.	inf.	sev.	sev.	stem	sample	CBB sev.	
Plt. 1													
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													
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28													
29													
30													

Other Observations: _____

CMD in other varieties: _____
CBSD in other varieties: _____

Appendix 2 GLCI DISEASE SURVEY DATA SHEET - Old Field

Country		Field Size (m ²)	
Dist./Prov./Territoire		Age (months)	
Village/Sous-Colline		Cassava Vars.	
Latitude		Sampled Var.	
Longitude		Intercrop(s)	
Altitude (m)		Neighbouring Cassava Fields	
Date/Time			
Researcher(s)			

	Plant 1					Plant 2					Plant 3					Plant 4					Plant 5				
Leaf																									
Stem																									
Root #																									
Rootsev	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Root 1																									
Root 2																									
Root 3																									
Root 4																									
Root 5																									
Root 6																									
Root 7																									
Root 8																									
Root 9																									
Root 10																									

	Plant 6					Plant 7					Plant 8					Plant 9					Plant 10				
Leaf																									
Stem																									
Root #																									
Rootsev	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Root 1																									
Root 2																									
Root 3																									
Root 4																									
Root 5																									
Root 6																									
Root 7																									
Root 8																									
Root 9																									
Root 10																									

Other Observations: _____

CMD in other varieties: _____

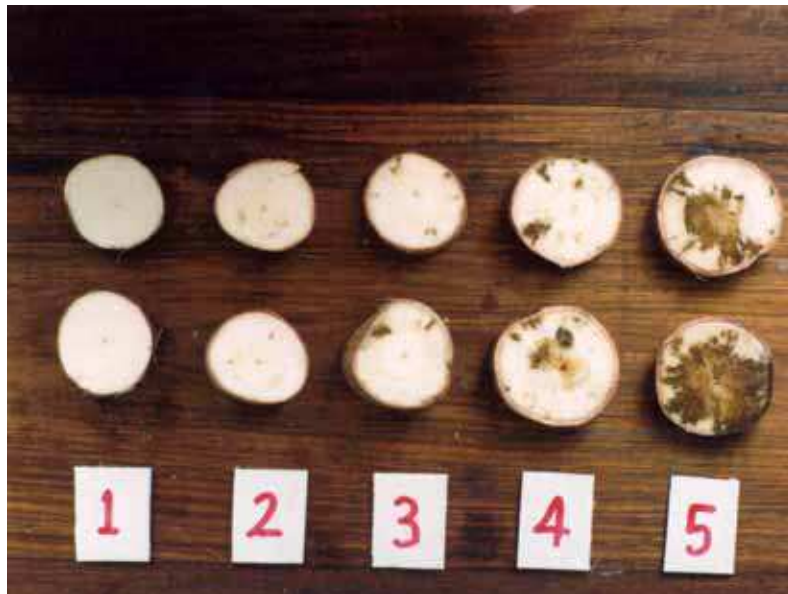
CBSD in other varieties: _____

Appendix 3. Pest and Disease abundance classes and damage scores

Pest	Abundance classes and damage scores				
	1	2	3	4	5
1. CMD	<ul style="list-style-type: none"> • no symptoms 	<ul style="list-style-type: none"> • mild chlorotic mosaic on leaves • little distortion of leaf shape 	<ul style="list-style-type: none"> • moderate chlorotic mosaic on leaves • moderate distortion of leaf shape with cupping 	<ul style="list-style-type: none"> • bright yellow chlorosis covering much of leaf area • severe distortion of leaf shape with reduced size • down-turned petioles 	<ul style="list-style-type: none"> • bright yellow chlorosis affecting much of leaf area • severe distortion of leaf shape with reduced size • down-turned petioles with leaf drop • plant stunted
2. CBSD	<ul style="list-style-type: none"> • no symptoms on leaves or stems 	<ul style="list-style-type: none"> • mild/slight vein yellowing or chlorotic blotches on leaves • no brown streaks/lesions on green stem portions 	<ul style="list-style-type: none"> • mild/slight vein yellowing or chlorotic blotches on leaves • mild brown streaks/lesions on green stem portions 	<ul style="list-style-type: none"> • severe/extensive vein yellowing or chlorotic blotches on leaves • severe brown streaks/lesions on green stem portions • no defoliation, stem dieback or stunting 	<ul style="list-style-type: none"> • severe/extensive vein yellowing or chlorotic blotches on leaves • severe brown streaks/lesions on green stem portions • defoliation, stem dieback and stunting
3. CBB	<ul style="list-style-type: none"> • no symptoms 	<ul style="list-style-type: none"> • angular leaf spotting only 	<ul style="list-style-type: none"> • wilting • angular leaf spots enlarged • leaf blight • defoliation • gum exudates on stem/petioles 	<ul style="list-style-type: none"> • wilting • blighting • defoliation • gum exudation • shoot tip die-back 	<ul style="list-style-type: none"> • wilting and blighting • defoliation and gum exudation • abortive lateral shoot formation • stunting • complete die-back
4. Cassava green mite	<ul style="list-style-type: none"> • no damage 	<ul style="list-style-type: none"> • <5% chlorotic 	<ul style="list-style-type: none"> • >5%, <50% chlorotic 	<ul style="list-style-type: none"> • >50% chlorotic 	<ul style="list-style-type: none"> • dead leaf, leaf drop
5. Cassava mealybug	<ul style="list-style-type: none"> • no damage 	<ul style="list-style-type: none"> • margins curling 	<ul style="list-style-type: none"> • slight bunchy top 	<ul style="list-style-type: none"> • strong bunchy top 	<ul style="list-style-type: none"> • complete defoliation
6. Whitefly abundance	<ul style="list-style-type: none"> • actual count 	<ul style="list-style-type: none"> • N/A 	<ul style="list-style-type: none"> • N/A 	<ul style="list-style-type: none"> • N/A 	<ul style="list-style-type: none"> • N/A

7. Whitefly direct damage	<ul style="list-style-type: none"> • none 	<ul style="list-style-type: none"> • mild chlorotic blotches on < 10% of leaves 	<ul style="list-style-type: none"> • moderate chlorotic blotches on 10-30% of leaves 	<ul style="list-style-type: none"> • general yellowing of upper leaves • yellowing or chlorosis on 30-50% of leaves 	<ul style="list-style-type: none"> • yellowing and deformation of upper leaves • chlorosis on > 50% of leaves • plant stunting
8. Whitefly-induced sooty mould	<ul style="list-style-type: none"> • none 	<ul style="list-style-type: none"> • mild sooty mould on < 5% of leaves 	<ul style="list-style-type: none"> • mild sooty mould on 5-20% of leaves 	<ul style="list-style-type: none"> • moderate sooty mould on > 20% of leaves • mild leaf curling 	<ul style="list-style-type: none"> • heavy sooty mould on 20-50% of leaves • strong leaf curling of lower leaves • stunted growth of bottom half of plant

Fig. 1. CBSD root severity scoring sheet



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
 - Latex agglutination assay
 - 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
 - PCR-based methods
 - Immuno Capture-PCR
 - Reverse Transcription-PCR
 - Multiplex-PCR
 - Print Capture-PCR
 - Spot Capture-PCR
 - PCR-ELISA
 - Isothermal Multiplex Aptidet RNA System
 - RT-PCR-ELOSA (Enzyme-linked oligosorbent assay)
- **Bioelectric recognition assay (BERA)**

A2. Common Conversions

Weight conversions

$$1 \mu\text{g} = 10^{-6} \text{ g}$$

$$1 \text{ ng} = 10^{-9} \text{ g}$$

$$1 \text{ pg} = 10^{-12} \text{ g}$$

$$1 \text{ fg} = 10^{-15} \text{ g}$$

Spectrophotometric conversions

$$1 \text{ OD at } A_{260\text{nm}} \text{ double-stranded DNA} = 50 \mu\text{g/ml concentration}$$

$$1 \text{ OD at } A_{260\text{nm}} \text{ single-stranded DNA} = 33 \mu\text{g/ml concentration}$$

$$1 \text{ OD at } A_{260\text{nm}} \text{ single-stranded RNA} = 40 \mu\text{g/ml concentration}$$

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. Basic 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. Useful Virology Resources

Virology text books

Agrios, G.N. Plant Pathology 5th Edition. Academic Press.

Astoer, A.; npiu, K., Maury, Y., Robaglia, C. and Lecoq, H. 2007. Principles of Plant Virology (Genome, Pathogenicity, Virus Ecology). Science Publishers, Jersey. ISBN 978-1-57808-316-9.

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J. and Watson, L. (eds.) (1996). *Viruses of Plants: Descriptions and Lists from the VIDE Database*. CAB International. UK

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (eds.) (1996). *Plant Viruses Online: Descriptions and Lists from the VIDE Database (1996 onwards)*. Version: 20th August 1996. URL <http://biology.anu.edu.au/groups/MES/vid e/>

Fauquet, C., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. 2005. Virus Taxonomy. 8th Report of the International Committee on Taxonomy of Viruses. Elsevier Publication.

Foster, G.D., Johansen, I.E., Hong, Y. And Nagy, P.D. Plant Virology Protocols. Second Edition. Methods in Molecular Biology # 451. Humana Press. ISBN 978-1-58829-827-0.

Hull R. 2002. Matthews' Plant Virology, 4th Edition. Academic Press, New York.

Hadidi, A., Khetarpal, R.K., and H. Koganezawa, H. (editors). 1998. Plant Virus Disease Control. APS Press.

Kumar P L, Jones A T, Reddy D V R. 2002. *Pigeonpea sterility mosaic virus: Detection and screening for resistance - Methods manual (Version 2)*. Patancheru 502 324, A.P, India. ICRISAT, 65pp

Madden, L.V., Hughes, G. and van den Bosch, F. The Study of Plant Disease Epidemics. APS Publication. ISBN:978-0-89054-354-2.

Mandhar, C.L. (editor). 1999. Molecular biology of plant viruses. Kluwer Academic Publishers, Boston.

Matthews, R.E.F. 1991. Plant Virology, 3rd Edition, Academic Press, New York.

Matthews, R.E.F. (editor) 1993. Diagnosis of Plant Virus Diseases. CRC Press, Boca Raton.

Mahy, B.W.J. 2009. The Dictionary of Virology (fourth ed.), Academic Press/Elsevier, Burlington, MA, USA ISBN 9780123737328.

Schumann, G.L. and D'Arcy, C.J. 2006. Essential Plant Pathology. APS Press. ISBN-13:978-089054-342-9.

Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E., Estes, M., Lemon, S., Maniloff, J., Mayo, M.A., McGeoch, D., Pringle, C.R., and Wickner, R.B. 2000. Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, New York.

Web resources:

All the virology on WWW.

(<http://www.tulare.edu/~dmsander/garry-favweb.html>)

British Society of Plant Pathology www.bspp.org.uk

Collection of digital images of crop diseases. American Phytopathological Society (APS), Publication on CD-ROM (<http://www.shopapspress.org>)

Descriptions of plant viruses (<http://www.dpvweb.net/>)

Disease Diagnostics on-line (www.idia.iita.org)

Virus Taxonomy on-line: (<http://www.virustaxonomyonline.com>)

Plant diseases On-line (www.apsnet.org)

National Center for Biotechnology Information. (www.ncbi.nlm.nih.gov)

International Committee on Virus Taxonomy on line. (www.ICTVonline.org)

AGORA (free on-line journal access for developing countries. (<http://www.aginternetwork.net/whalecom> www.aginternetwork.org/whalecom0/en/journals)

FAO Statistics (<http://faostat.fao.org/>)

New Disease Reports (www.bspp.org.uk/ndr)

Phytosanitary issues (<https://www.ippc.int/IPP/En/default.jsp>)

Wikipedia: free encyclopedia on-line <http://en.wikipedia.org/>

Molecular biology & serology text books:

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 1994. Current Protocols in Molecular Biology.

Crowther, J.R. (editor). 1995. ELISA. Methods in Molecular Biology 42. Humana Press, New Jersey.

- Harlow, A., and Lane, D. 1998. *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
- Hampton, R., Ball, E., and De Boer, S. 1990. *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*. APS Press.
- Harwood, A.J. (editor). 1996. *Basic DNA and RNA Protocols. Methods in Molecular Biology 58*. Humana Press, New Jersey.
- Sambrook, J., and Russell, D .W. 2000. *Molecular cloning - a laboratory manual, 3rd ed.* Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
- Sambrook J, Fritsch, E F, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual 2nd ed.* Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
- White, B.A. (editor). 1993. *PCR Protocols. Current Methods and Applications. Methods in Molecular Biology 15*. Humana Press, New Jersey.

Some international journals:

Advances in Virus Research
 African Journal of Biotechnology
 Annals of Applied Biology
 Annual Reviews in Plant Pathology
 Archives of Virology
 Crop Protection
 Crop Science
 Integrated Pest Management
 Journal of General Virology
 Journal of Phytopathology
 Journal of Virology
 Journal of Virological Methods
 Molecular Plant Pathology
 Molecular Plant Microbe Interactions
 Plant Disease
 Plant Pathology
 Phytopathology
 Virus Genes
 Virology Journal
 Virus Research

A5. Glossary of Common Terms in Virology and Diagnostics

- 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 for the four respective 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 guanine-cytosine 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, 1.67×10^{-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.

Dagnosis: 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 adenine-thymine 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 occurring 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 *Thermus 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 encapsidation 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).

A6. Training Course Program



Regional Training for the Disease Objective of GLCI Cassava Viruses: Biology, Diagnostics and Management

28 October to 6 November 2009, IITA, Dar es Salaam, Tanzania

WEEK 1 – THEORETICAL PERSPECTIVES

Wednesday October 28, 2009

- Am Opening remarks (Manyong/Kanju)
Introductions
Test to establish baseline knowledge (Legg/Kumar)
Overview of cassava pests and diseases – new perspectives for 2009 (Legg)
The biology of cassava mosaic geminiviruses (Thresh)
The biology of cassava brown streak virus (Maruthi)
- Pm Plant virus epidemiology (Thresh)
Managing the health of cassava in the environment – quality assurance systems (Smith)

Thursday October 29, 2009

- Am Cassava pest/disease diagnostics – symptom recognition test (Legg)
Virus diagnostics – what are the options? (Kumar)
- Pm Diagnostics for cassava mosaic geminiviruses – theory (Maruthi)
Diagnostics for cassava brown streak viruses – theory (Kumar)
a) RNA extraction
b) RT-PCR and multiplex tests
c) Real-time PCR
d) LAMP-LFD and future approaches

Friday October 30, 2009

- Am Management of plant viruses – general principles (Thresh)
Host plant resistance for cassava virus control (Kanju)
- Pm Question and Answer Session – Cassava Viruses
Training Assignment – Week 1
Results of the symptom recognition test

WEEK 2 – PUTTING THEORY INTO PRACTISE

Monday November 2, 2009

- Am Practical – Sample collection techniques for CMGs and CBSV
Theory – Survey approaches – refining current methods (Legg)
Theory – Sample collection and storage (Kumar)
- Pm Practical – Extraction of DNA (CMGs) and RNA (CBSV)

Tuesday November 3, 2009

- Am Practical – Specific primer PCR for CMG detection and identification
Theory – How does PCR work? (Kumar)
- Pm Practical – Specific primer RT-PCR for CBSV detection

Wednesday November 4, 2009

- Am Practical – Completion of specific primer PCR for CMGs
Theory – What are the outputs of PCR and how can they be used? (Kumar)
- Pm Practical – Completion of specific primer PCR for CBSV
Assessment of results and group discussion

Thursday November 5, 2009

- Am Practical – Multiplex PCR for the detection of CMGs and CBSV
Theory – Sequencing for virus characterization (Kumar)
- Pm Practical – Completion of multiplex PCR
Assessment of results and group discussion

Friday November 6

- Am Test to establish progress made through training (Legg/Kumar)
Training in novel strategies: DEWN, 'Smart' surveillance, Epidemiology and Virus challenging (Legg/Ndyetabula/Kanju)
- Pm Results of training progress test (Legg)
Group discussion – how to apply the knowledge, further training needs
Certification presentation and closing (Manyong)

A7. Contact address of participants and resource persons

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A8. Group Photograph

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GLCI
Great Lakes Cassava Initiative

**Regional Training for the Disease Objective of GLCI
Cassava Viruses: Biology, Diagnostics and Management**
28 October – 6 November 2009, IITA, Dar es Salaam, Tanzania

www.iita.org

A9. Publications on cassava mosaic viruses in Africa and plant virus epidemiology extracted from the IITA Proceedings on Plant Virology in Sub Saharan Africa

Cassava virus diseases in Africa

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Abstract

Cassava plays a key role in the food security of sub-Saharan Africa, but as a vegetatively propagated crop, it is particularly vulnerable to the effects of virus diseases and these therefore represent a major threat to the livelihoods of millions of Africans. Nine viruses have been isolated from African cassava, but only cassava mosaic geminiviruses (CMGs) and *Cassava brown streak virus* (CBSV) cause diseases of major economic significance. In recent years, both CMGs and CBSV have come under increasing research scrutiny, because of the devastating losses they cause. Molecular and field studies of CMGs have revealed a hitherto unrecognized level of complexity in biodiversity, interactions, and epidemiology, most notably in association with the pandemic of severe CMD which has impacted large areas of East and Central Africa. Comprehensive surveys of cassava in coastal East Africa have revealed cassava brown streak virus disease (CBSD) to be the major constraint to production in that zone, with the greatest effects of the disease being reported from northern Mozambique. This review describes recent progress in research on CMGs and CBSV in sub-Saharan Africa, examines the dynamic nature of their status, and provides insights into the major control initiatives that are required to tackle them.

Résumé

Le manioc joue un rôle clé dans la sécurité alimentaire en Afrique subsaharienne. Culture à propagation végétative, elle est particulièrement vulnérable à l'effet des maladies virales, qui constituent une menace importante pour des millions d'Africains. 9 virus ont été isolés à partir du manioc africain, mais seuls deux d'entre eux, notamment le geminivirus de la mosaïque du manioc (CMG) et le virus de la striure brune du manioc (CBSV) causent des maladies d'une importance économique significative. Au cours des dernières années, ces deux affections ont fait l'objet de recherches poussées du fait des pertes importantes signalées. Des études moléculaires sur le terrain sur

la CMG ont révélé un niveau de complexité dans la biodiversité, les interactions et l'épidémiologie insoupçonné jusqu'ici, surtout par rapport à la pandémie de la CMD qui a considérablement affecté l'Afrique de l'Est et centrale. Des études approfondies sur le manioc cultivé en zone côtière en Afrique de l'est indiquent que le virus de la striure brune (CBSD) est la principale contrainte à la production dans cette zone et des effets les plus ravageurs ont été signalés au Mozambique. La présente revue fait le point sur les récents progrès réalisés en matière de recherche sur les CMG et CBSV en Afrique subsaharienne, analyse le dynamisme des affections et décrit les principales initiatives de lutte nécessaires pour contrôler ces affections dans les années à venir.

Cassava in Africa and constraints to production

Although cassava has a relatively recent history in Africa, having been introduced from Latin America by the Portuguese in the 16th Century, it has become one of the most important crops grown in the continent. Currently cassava is cultivated on almost 17 million hectares (FAO 2003) from the Islands of Cape Verde just off Senegal in the west to Madagascar off the southeastern tip of the continent. Much of its success may be attributed to its adaptability, its capacity to provide acceptable yields under marginal farming conditions, and its tolerance to drought. As such, it has become the continent's most important food security crop.

Cassava production in Africa makes up 54% of the world total, with the other two major producing continents being South America and Asia (FAO 2003). This 54%, however, is cultivated on an estimated 65% of the total area under cassava. The reason for this apparent mismatch is that yields in Africa, averaging 8.9 t/ha, are only 70% of those in South America and 61% of those in Asia. Poor yields in Africa can be attributed to a range of factors, but one of the most important is loss due to pests and diseases. Although there is much less diversity among the pests and diseases of cassava in Africa compared with those of Latin America, those that are present are particularly damaging. Economically important among the pests are the cassava green mite, *Mononychellus tanajoa* (Bondar) and the cassava mealybug, *Phenacoccus manihoti* Matt.-Ferr., both of which were introduced inadvertently to the continent from South America in the early 1970s, and both of which are now under effective management following the successful implementation of classical biological control programs. The most important diseases include cassava bacterial blight, *Xanthomonas campestris* f.sp. *manihoti*, also introduced from South America in the 1970s, and two virus diseases, cassava mosaic disease (CMD) and cassava brown streak disease (CBSD), both of which are thought to have risen from infection of cassava by viruses already present in the indigenous

African flora. Following the success of the cassava mealybug and cassava green mite biological control programs, CMD and CBSD have become prominent in research and management initiatives, and CMD is now commonly considered to be the most damaging pest or disease constraint to cassava production in Africa.

The viruses of cassava

Cassava is a vegetatively propagated crop, and diseases from viruses cause particular problems as they are carried from one crop cycle to the next through the cuttings used as planting material. Without intervention, infection can therefore readily build up from one crop cycle to the next, particularly where there is also a significant level of vector transmission. Despite the relatively recent arrival of cassava in Africa, there is almost as much diversity among viruses of cassava in Africa as there is in the South/Central American region of origin (Table 1). Four virus genera are represented among the taxa that have been described. However, only two of these are of economic significance, namely: *Cassava brown streak virus* (CBSV) (*Potyviridae: Ipomovirus*) and the group of cassava-infecting geminiviruses (*Geminiviridae: Begomovirus*) (Table 2). This review will therefore be restricted to a consideration of CBSV and CMGs. Tables 3 and 4 summarize research milestones.

Table 1. The viruses of cassava (Calvert and Thresh 2002).

Africa

Cassava mosaic geminiviruses (Geminiviridae: Begomovirus)#
Cassava brown streak virus (Potyviridae: Ipomovirus)
*Cassava Ivorian Bacilliform virus** (unassigned)
 Cassava Kumi viruses A and B*
 Cassava "Q" virus*
Cassava common mosaic virus (Potexvirus)*

South/Central America

Cassava common mosaic virus (Potexvirus)
*Cassava virus X (Potexvirus)**
Cassava vein mosaic virus (Caulimoviridae)
*Cassava Colombian symptomless virus (Potexvirus)**
*Cassava American latent virus (Comoviridae: Nepovirus)**
 Cassava frogskin "virus"

Asia/Pacific

Cassava common mosaic virus (Potexvirus)*
Indian cassava mosaic virus (Geminiviridae: Begomovirus)
Sri Lankan cassava mosaic virus (Geminiviridae: Begomovirus)
Cassava green mottle virus (Comoviridae: Nepovirus)*

Viruses with names in italics are recognized species.

The cassava mosaic geminiviruses have recently been reclassified. See Table 2.

* Viruses with localized distributions and not of economic significance.

Table 2. Cassava mosaic geminivirus species and strains (Fauquet et al. 2003).

Species	Accession number	Acronym
<i>African cassava mosaic virus</i> (Cassava latent virus)		ACMV
African cassava mosaic virus – [Cameroon-DO2]	AF366902, AF112353	ACMV-[CM/DO2]
African cassava mosaic virus – [Cameroon]	AF112352, AF112353	ACMV-[CM]
African cassava mosaic virus – [Ghana]		ACMV-[GH]
African cassava mosaic virus – [Côte d'Ivoire]	AF259894, AF259895	ACMV-[IC]
African cassava mosaic virus – [Kenya]	J02057, J02058	ACMV-[KE]
African cassava mosaic virus – [Nigeria]	X17095, X17096	ACMV-[NG]
African cassava mosaic virus – [Nigeria-Ogo]	AJ427910, AJ427911	ACMV-[Nig-Ogo]
African cassava mosaic virus – [Uganda]	Z83252, Z83253	ACMV-[UG]
African cassava mosaic virus – Uganda Mild	AF126800, AF126801	ACMV-UGMid
African cassava mosaic virus – Uganda Severe	AF126802, AF126803	ACMV-UGSvr
<i>East African cassava mosaic Cameroon virus</i>		EACMCV
East African cassava mosaic Cameroon virus – Cameroon	AF112354, AF112355	EACMCV-CM
East African cassava mosaic Cameroon virus – Cameroon [Côte d'Ivoire]	AF259896, AF259897	EACMCV-CM[CI]
<i>East African cassava mosaic Malawi virus</i>		EACMMV
East African cassava mosaic virus – Malawi, EACMV-MW		EACMMV-MW[K]
East African cassava mosaic Malawi virus – Malawi [K]	AJ006460	EACMMV-MW[MH]
East African cassava mosaic Malawi virus – Malawi [MH]	AJ006459	EACMV
<i>East African cassava mosaic virus</i>		EACMV
East African cassava mosaic virus – [Kenya – k2B]	Z83258	EACMV-[KE-k2B]
East African cassava mosaic virus – [Tanzania]	Z83256	EACMV-[TZ]
East African cassava mosaic virus – [Uganda1]	AF230375	EACMV-[UG1]
East African cassava mosaic virus – Uganda2 (Uganda variant)	Z83257	EACMV-UG2
East African cassava mosaic virus – Uganda2 Mild	AF126804	EACMV-UG2Mid
East African cassava mosaic virus – Uganda2 Severe	AF126806	EACMV-UG2Svr
East African cassava mosaic virus – Uganda3 Mild	AF126805	EACMV-UG3Mid
East African cassava mosaic virus – Uganda3 Severe	AF126807	EACMV-UG3Svr
<i>East African cassava mosaic Zanzibar virus</i>		EACMZV
East African cassava mosaic Zanzibar virus	AF422174, AF422175	EACMZV
<i>South African cassava mosaic virus</i>		SACMV
South African cassava mosaic virus	AF155807, AF155806	SACMV
South African cassava mosaic virus – [M12]	AJ422132	SACMV-[M12]

Table 3. Summary of CMD research milestones.

1894	First reported from Tanzania
1926	First recorded from West Africa
1932	First whitefly transmission (Congo)
1930s–1960	Comprehensive studies and resistance developed from inter-specific crosses
1971–present	IITA breeding program
1983	Etiology determined
1970s/80s	Epidemiology characterized (Kenya/Côte d'Ivoire)
1994	First distribution map of CMGs (Africa)
1990s	Pandemic of severe CMD recorded (Uganda)
1997	Novel recombinant CMG described (UK)
1997	Mixed infections first recorded (UK)
1996–2000	Increased diversity observed (Africa)
1995–present	Spread of CMD pandemic through East and Central Africa recorded
1990s–present	Major deployment of host-plant resistance
2001	New single dominant CMD resistance gene identified from local landraces
2003	Geminiviruses reclassified; six CMG species recorded for Africa

Table 4. Summary of CBSD research milestones.

1936	First recognized: Tanzania. Distinguished from cassava mosaic disease. Graft-transmissible; assumed to be viral.
1940	Reported in Zanzibar
1950	Reported from Uganda (assumed to have been introduced)
1940s–50s	Resistance breeding (Tanzania)
1950	Reported in southern Malawi. Detailed descriptions of symptoms. Effects of temperature/altitude recognized. "Natural control" reported
1959	Successful sap transmissions (to/from cassava and herbaceous hosts)
1970s/80s	Virus studied in Kenya and UK
1990	"Rediscovered" in Tanzania
1992	"Rediscovered" in Malawi
1994	"Rediscovered" in Uganda
1993/94	First countrywide survey (Tanzania)
1996	Report in Zambia
1999	First identification from Mozambique
1999–2000	Mozambique surveys: Nampula/Zambezia
2000	CBSD/CMD survey in coastal Kenya
1995–2000	Epidemiology studies (Tanzania)
2001	Cassava brown streak virus characterized
2001	RT-PCR based diagnostic protocol developed
1999–present	Control programs (Mozambique, Tanzania)

Cassava mosaic disease

Historical background

Cassava mosaic disease (CMD) was first described from what is now Tanzania towards the end of the 19th Century (Warburg 1894) although its etiology remained unclear for many years. Although early studies (Zimmerman 1906) suggested a viral cause, it was not until the 1930s that a more concerted effort to study the disease was initiated at the Amani Research Station in northeastern Tanzania. Storey and colleagues (Storey 1936, 1938; Storey and Nichols 1938) were able to demonstrate the graft transmissibility of CMD, and confirmed earlier experiments (Kufferath and Ghesquière 1932) showing that a *Bemisia* whitefly species was the vector. They also recorded the occurrence and properties of mild and severe virus strains, and carried out the first epidemiological experiments recording seasonal differences in rates of spread. This work provided the base for a breeding program which lasted from the late 1930s to the early 1960s. A broad-based multigenic resistance was successfully introgressed into cultivated cassava from the wild relative, *Manihot glaziovii* Muell.-Arg (Jennings 1957). Parental material developed through this work, notably clone 58308 that was selected in Nigeria, became the base material for the later strategic breeding program of the International Institute of Tropical Agriculture (IITA) which started at the beginning of the 1970s (Jennings 1994).

Studies of both the etiology and epidemiology of CMD continued in both East (Kenya) and West Africa (Côte d'Ivoire) in the 1970s and 1980s with the Overseas Development Administration/Kenya Agricultural Research Institute (ODA/KARI) and Institut français de recherche scientifique pour le développement en coopération (ORSTOM) programs. In the Kenya-based ODA Plant Virology Project, it was finally proved that CMD was caused by a cassava mosaic geminivirus following the demonstration of Koch's postulates (Bock and Woods 1983). The patterns of disease spread were described and evidence was adduced for the occurrence in Africa of two distinct CMD-causing viruses (Bock et al. 1981). These were distinguished by both serological (Swanson and Harrison 1994) and molecular (Hong et al. 1993) methods and referred to as *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV). In Côte d'Ivoire, Fauquet, Fargette, and colleagues provided detailed quantitative information on the epidemiology of CMD, demonstrating the occurrence of environmental gradients of spread (Fargette et al. 1985), and regional differences in epidemiology (Fauquet et al. 1988), and providing evidence that external sources of inoculum were more important than within field sources (Fargette et al. 1990). Vital information was also provided on the effects of CMD on cassava yield, and an attempt

was made to assess the continent-wide impact of the disease on cassava production (Fargette et al. 1988). However, the estimate of a 30 million tonnes loss based on total production of 50 million tonnes was made on the erroneous assumption that all plants in Africa are infected.

This pioneering work was influential in raising the profile of the principal virus disease of cassava, a crop which has been and still continues to be widely regarded as a neglected “orphan”. However, the appearance and spread of an epidemic of an unusually severe form of CMD, first recorded in Uganda during the late 1980s (Otim-Nape et al. 1994), was to have an even more profound effect on the course of research on and attitudes to CMD and its causal viruses, as discussed in subsequent sections.

Cassava mosaic geminiviruses—structure

Viruses of the family *Geminiviridae* comprise “twin” particles which together are *c.* 30 by 20 nm in size. The protein subunits, which are arranged in an icosahedral array with 22 pentamers, are *c.* 30 kDa in size and enclose a bipartite genome of single-stranded circular DNA (Bock et al. 1977; Harrison et al. 1977). The two DNA components, designated DNA-A and DNA-B, are each 2700–2800 base pairs long. DNA-A contains four genes that code for the proteins required for DNA replication (AC1) and production and encapsidation of the coat protein (AV1) (Stanley 1983), while DNA-B contains two genes which code for the proteins required for virus movement with the plant (Etessami et al. 1988). There is a short (*c.* 200 bp) conserved intergenic or common region to both DNA-A and DNA-B. This includes sequences that are essential for the correct function of DNA replication and transcription (Chatterji et al. 1999). The genome arrangement is illustrated in linear diagrams in Figure 1.

Symptoms and diagnostic methods

Cassava plants infected with CMGs express a range of symptoms which depend on the virus species/strain, environmental conditions, and the sensitivity of the cassava host. The most typical symptoms consist of a yellow or pale green chlorotic mosaic of leaves, commonly accompanied by distortion and crumpling. Symptoms are readily distinguished from those of mineral deficiency or cassava green mite damage as the virus-induced chlorosis and malformation of leaflets is asymmetrical about the midrib. Where symptoms are severe, the plant becomes generally stunted and petioles immediately below the shoot tip may be angled downwards and occasionally may become necrotic, shrivel, and absciss. Where the virus or virus strain is mild or the cassava variety is tolerant, leaf chlorosis may be patchy and absent on some leaves, and there is little or no leaf distortion or malformation and little effect on overall plant vigor.

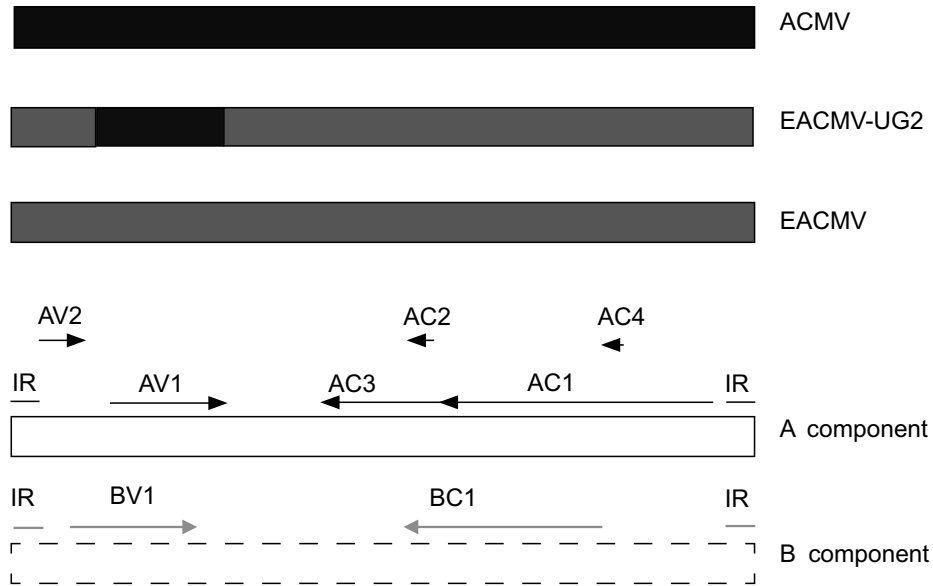


Figure 1. Genome arrangement of cassava mosaic geminiviruses (CMGs) and diagrammatic illustration of the DNA-A of the CMG recombinant, EACMV-UG.

Visual assessments of the presence/absence of CMGs in field-grown cassava plants are normally reliable. Infected plants usually express symptoms, unless the plant has only recently been infected (symptoms typically appear 3–5 weeks after infection) or conditions are unfavorable, as during periods of drought when most leaves are shed. Resistant varieties that are infected may “recover” i.e., begin to produce symptomless leaves during the later stages of crop growth and particularly during hot, dry weather. But even so, careful observation usually detects symptoms on lower leaves.

The serological techniques of DAS-ELISA (Sequeira and Harrison 1982) and TAS-ELISA (Thomas et al. 1986) have been used successfully to detect and distinguish between CMGs, and panels of monoclonal antibodies. They were subsequently developed to facilitate the discrimination between ACMV and EACMV (Swanson and Harrison 1994). This technique was used to produce the first CMG distribution map for Africa (Swanson and Harrison 1994) (Fig. 2a), and a similar approach was used for more detailed distribution mapping of ACMV and EACMV in East and Southern Africa (Ogbe et al. 1996; Ogbe et al. 1997). Evidence for the occurrence of recombinant CMGs (Zhou et al. 1997), however, made it clear that DNA-based diagnostic techniques were required if reliable diagnoses were to be made, and PCR-based techniques are now being more widely practiced (Fondong et al. 1998; Offei et al. 1999; Ogbe et al. 1999; Legg et al. 2001; Neuenschwander et al. 2002). Much of this diagnostic and detection work makes use of specific PCR primers designed from full length sequences of DNA-A (Zhou et al. 1997; Fondong et al. 2000; Berrie et al. 2001; Pita et al. 2001a).

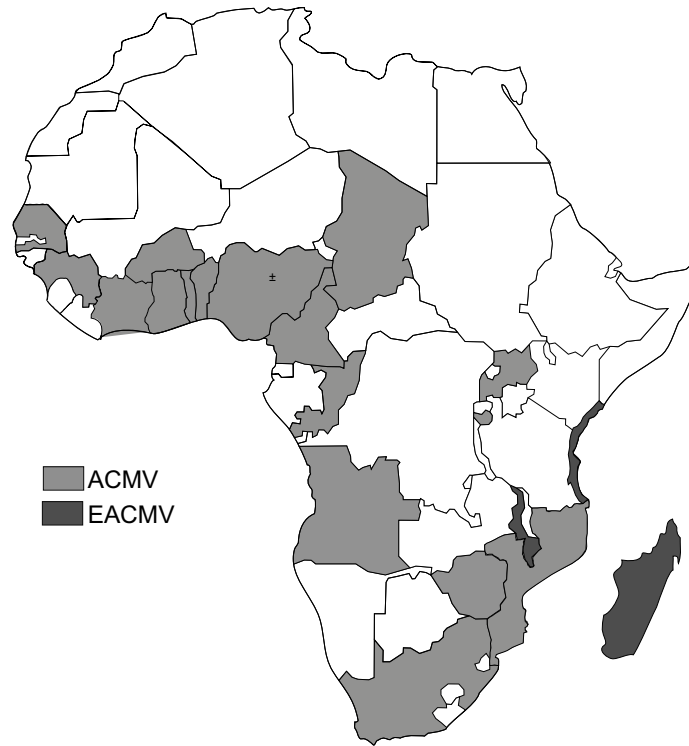


Figure 2a. Known distribution of cassava mosaic geminiviruses (CMGs) in Africa: 1994 (Swanson and Harrison 1994).

An alternative molecular approach currently being used both for CMG diagnostics and variability studies is PCR with restriction fragment length polymorphism (RFLP) analysis. In this method, universal and abutting CMG DNA-A primers (Bridson et al. 1993) are used to amplify near full-length DNA-A fragments from whole plant DNA (Fig. 3a). Amplified full-length DNA-A products are then digested with restriction enzymes (commonly *EcoRV* and *mluI*) and the digests run on an agarose gel. Depending on the virus sequence and, therefore, the sites at which the restriction enzymes cut, one or more DNA fragments are generated providing a distinctive pattern of bands on the gel. *EcoRV* provides a consistent distinction between ACMV and EACMV in Uganda, based on the characteristic banding patterns produced for each (Fig. 3b). Fragments produced by *mluI* are much more variable (Fig. 3c), particularly for EACMV. This enzyme, therefore, provides useful information on virus variability and the possible occurrence of strains. Clearly as more sequence information becomes available, further refinements of the RFLP approach will be possible, and it is likely to become an even more useful and widely used approach for virus detection, identification, and diversity studies.

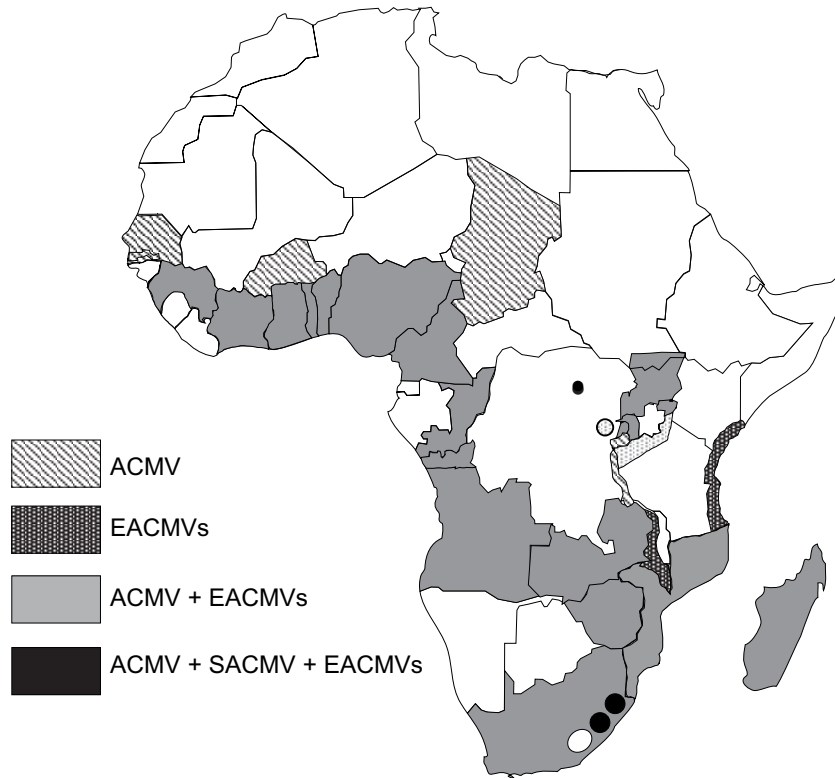


Figure 2b. Known distribution of cassava mosaic geminiviruses (CMGs) in Africa: 2002.

Characterization and variability

Application of the modern techniques of DNA manipulation and study, coupled with an increased interest in CMD in Africa, has resulted in much new information on the character and diversity of CMGs affecting cassava in Africa. Arguably, the most important breakthroughs came from studies at the Scottish Crop Research Institute (SCRI) on material collected from Uganda, which showed first that a novel virus had risen through a recombination event between ACMV and EACMV (Zhou et al. 1997), and that mixed virus infections were both frequent and resulted in a synergistic interaction (Harrison et al. 1997). The novel virus, designated the Uganda variant (UgV) (Zhou et al. 1997) or EACMV-UG (Deng et al. 1997) was associated with the epidemic of severe CMD in Uganda, and showed a large portion of the DNA coding for the ACMV coat protein gene spliced into an otherwise EACMV-like DNA-A. Consequently, earlier serological tests erroneously recognized the virus as ACMV. This finding stimulated much additional detailed study of CMGs across Africa, with the result that recombination in both DNA-A and DNA-B was found to be a common phenomenon within EACMVs (Zhou et al. 1998; Fondong et al. 2000; Pita et al. 2001a) and was also recorded within the DNA-A

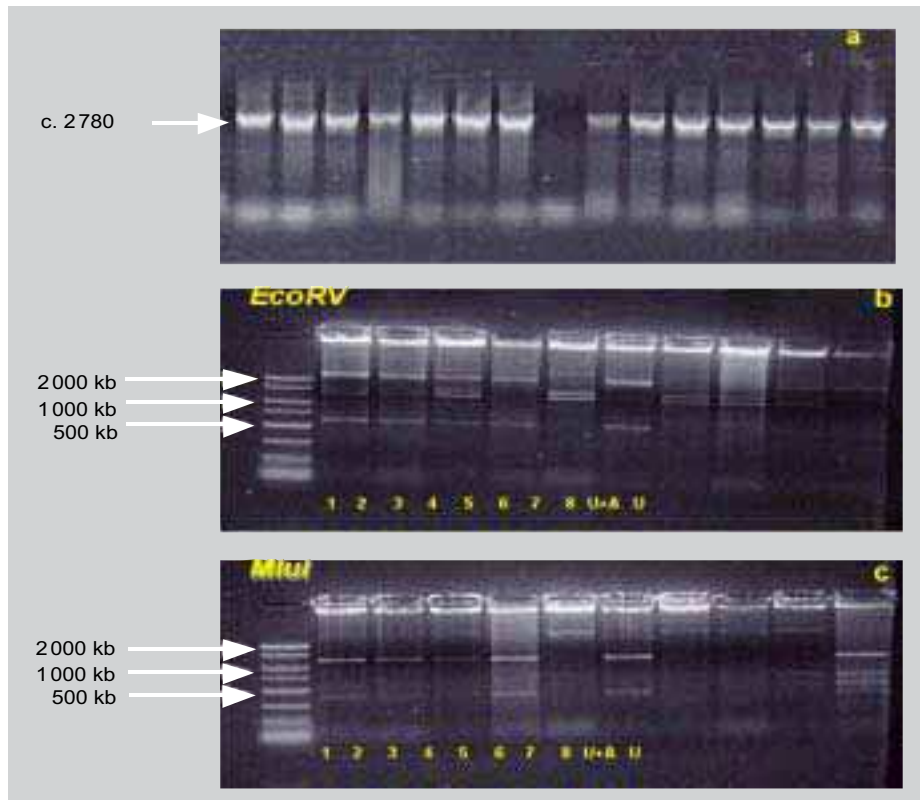


Figure 3. RFLP-based diagnostic method. Gels showing:
a) Near full-length DNA-A fragments from virus-diseased samples amplified with abutting universal primers.
b) DNA-A fragments cut with *EcoRV*. [AIEACMVs (1,2,3,4,6,U+A,U) produce bands of c. 2,195 bp and 585 bp; ACMV produces bands of c. 1480 bp and 1285 bp (1,2,3,5,7,8,U+A)].
c) DNA-A fragments cut with *MluI*. [ACMV single uncut fragment c. 2765, same samples as b); EACMVs variable, producing either two (1,2,3,4,and 6) or four (U+A, U) bands].

of the *South African cassava mosaic virus* (SACMV) (Berrie et al. 2001). CMGs were shown also to transreplicate, a process in which the DNA-A of one virus replicates the DNA-B of a heterologous virus (Pita et al. 2001b). Thus in Uganda, severe CMD in the epidemic-affected area is most commonly associated with the concurrence of the DNA-A of EACMV-UG2 and the DNA-B of EACMV-UG3 (Pita et al. 2001a). Synergism has also been widely reported (Fondong et al. 2000; Pita et al. 2001a), although in all known cases, this is between ACMV and EACMV.

The rapid increase in the availability of sequence data for geminiviruses, and the evident taxonomic complexity, recently led to the convening of a study group to review the taxonomy of this family. This resulted in the recognition of six species of CMG in

Africa: ACMV, SACMV, and four EACMVs (Table 2), although with continued interest on this topic, the number seems certain to increase as more material is examined. While this may seem rather academic, recent results highlight the dramatic effects that relatively minor sequence differences and virus–virus interactions can have on the nature of infection and ultimately, the impact on the plant. Good information on the character of the CMG or CMG mixture infecting plants is therefore essential. Such knowledge can be useful for assessing the likely impact of CMG mixtures on production and can inform breeders on how best to manage and manipulate resistant genes to ensure optimal performance and targeting, and how to minimize the possibility of resistance breakdown.

Virus transmission and epidemiology

Definitive studies confirming that the whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) transmits CMGs were carried out by Chant (1958) and Dubern (1979, 1994). Transmission was shown to be persistent, transtadial but not transovarial (Dubern 1994), with minimum times for acquisition, latent period, and inoculation of 3.5 hours and 5–10 minutes, respectively. Reports on transmission efficiency have varied from very low (0.15–1.7% of individuals infective) for field-collected insects (Fargette et al. 1985), to moderate (4–13%) for laboratory-reared insects (Dubern 1994; Maruthi et al. 2002). Recent evidence also suggests that there is only limited coadaptation between CMGs and their vector within Africa, as the frequencies of transmission of different CMGs by *B. tabaci* populations from geographically distant locations in Africa were not significantly different (Maruthi et al. 2002).

An introduction to epidemiological studies has been provided earlier in this review. In more recent work within the last decade, much data have been collected on different responses to infection and patterns of spread for varieties with different resistance levels and the marked differences in patterns of disease progress and overall infection described. Epidemiological studies in Bénin and Cameroon have demonstrated the role and potential benefits of intercrops in reducing rates of CMD spread into initially CMD-free crops (Ahohuendo and Sarkar 1995; Fondong et al. 2002), and similar benefits have been demonstrated for mixtures of resistant and susceptible varieties in Uganda (Sserubombwe et al. 2001). An important finding from further epidemiological work in Uganda, in which a range of resistant and susceptible varieties was tested in contrasting agroecological zones, was that final CMD incidence for the susceptible varieties could be related to estimates of inoculum pressure in the immediate surrounds of the test plot and recording the number of adult *B. tabaci* in the test plot for the first two weeks after sprouting (Legg et al. 1997). Significantly, while both CMD inoculum

availability within a 250 m radius of the test plot and *B. tabaci* adult numbers within the test plot contributed significantly to variability in final CMD incidence, inoculum availability explained more of this variation. This highlights the importance of nearby CMD-affected fields in influencing the amount and pattern of disease development in any newly planted plot. Many of the findings of the epidemiological studies described have important practical implications, some of which are discussed further in the section on management.

Economic impact of CMD

Trials to assess the effect of CMD on cassava yield have provided differing results ranging from virtually no loss to almost total loss (Thresh et al. 1994a). Results from such experiments depend on a series of factors, some of the most important of which are the susceptibility of the variety, the stage of crop growth at which infection occurred, the severity of the virus or virus mixture causing the infection, and the abiotic environmental conditions (Fargette et al. 1988; Fauquet and Fargette 1990; Spittel and van Huis 2000). In some of the most recent work, losses attributable to specific viruses and virus mixtures have been quantified for a single CMD-susceptible variety (Owor 2003). Thus, while a mild strain of EACMV-UG2 gave only minor yield reductions in comparison with healthy controls, losses of up to 87% were recorded for mixed ACMV and EAMCV-UG2 infections.

The overall impact of CMD clearly depends on both the loss attributed to infection and the incidence of infected plants. Fargette et al. (1988) estimated all continent-wide losses based on an average yield loss figure of 37% for a local variety in Côte d'Ivoire. This assumed, however, that all plants are infected, a deficiency highlighted by Thresh et al. (1997) in their conservative estimate. They assumed an overall incidence of 50–60%, with an accompanying average yield loss for infected plants of 30–40%. On this basis, Africa-wide losses were put at 15–24%. Since then, additional incidence and yield loss data have become available, most notably for each of the top 10 cassava producing countries of Africa excluding Angola. Table 5 provides recent CMD incidence data for 16 of the main cassava-producing countries of Africa, with 90% of total production in the Africa. Using FAO production estimates, CMD incidence and the 30–40% range of yield loss used by Thresh et al. (1997), estimates of “lost” production have been calculated for each of the 16 countries. For the remaining 10% of production for which incidence figures are not available, the average incidence of 50% for the other 16 countries has been used. Based on these assumptions, the losses attributable to CMD are 19–27 million tonnes, based on the current (CMD affected) production total of 97 million tonnes (FAO 2003). This value is very close to the earlier figure of Thresh et al. (1997), relative to the respective total production figures for the different dates, and

Table 5. Surveys of the incidence of cassava mosaic disease (CMD) in 18 African countries.

Country	Organization (reference)	Year	CMD incidence (%)	Production 2002 m/t	Estimated loss (30–40%)
Uganda	NARO (1)	1990–1992	57		
Uganda	NARO (2)	1994	65		
Uganda	NAROMITA (14)	1997	68	5.27	1.4–2.0
Chad	USAID (3)	1992	40	0.31	0.04–0.06
Malawi	NARS (4)	1992	21		
Malawi	NARS/IITA (15)	1998	42	1.54	0.22–0.31
Tanzania	NARS/NRI (5)	1993	26		
Tanzania	NARS/IITA (16)	1998	34	5.65	0.64–0.089
Ghana	ESCaPP (6)	1993–1994	72		
Ghana	NARS/IITA (17)	1998	71	8.97	2.43–3.56
Bénin	ESCaPP (6)	1994	53		
Bénin	NARS/IITA (18)	1998	36	2.45	0.30–0.41
Cameroon	ESCaPP (6)	1994	67		
Cameroon	NARS/IITA (19)	1998	62	1.70	0.39–0.56
Nigeria	IITA (7)	1994	55		
Nigeria	ESCaPP (6)	1994	82		
Nigeria	NARS/IITA (20)	1998	56	33.56	6.78–9.69
Zambia	NARS/SARRNET	1995–1996	41	0.95	0.13–0.19
Zanzibar	(12)	1998	71	NA	
South Africa	NARS/NRI (8)	1998	31	< 0.01	
Madagascar	NARS (9)	1998	47	2.23	0.37–0.52
Mozambique	NARS/IITA (21)	1999–2000	20	5.36	0.34–0.47
Rwanda	NARS/NRI (10/12)	2001	30	0.69	0.07–0.09
DRC	NARS/IITA (22)	2002	60	14.93	3.28–4.71
Congo Rep.	NARS/IITA (23)	2002	79	0.85	0.26–0.39
Guinea-	NARS/IITA (24)	2003	63	1.00	0.23–0.34
Conakry	NARS/IITA	1993	20		
Kenya	KARI/NRI (13)	1996	56		
(Western)	KARI/NRI (13)	1998	84		
“ “	KARIESARC (13)	2000	58		
“ “	NARS/NRI (11)	1998	51	0.95	0.17–0.24
Kenya (Coastal)	NARS/IITA (25)		Est. 50	10.60	1.87–2.65
Kenya					
*Others			50	97.01	18.87–27.05
Total					

References

- | | |
|---|------------------------------------|
| 1 Otim-Nape et al. 1998 | 14 Legg et al. 1999 |
| 2 Otim-Nape et al. 2001 | 15 Theu et al. 2003 |
| 3 Johnson 1992 | 16 Ndunguru et al 2003 |
| 4 Nyirenda et al. 1993 | 17 Cudjoe et al 2003 |
| 5 Legg and Raya 1998 | 18 Gbaguidi et al. 2003 |
| 6 Yaninek et al. 1994; Wydra and Msikita 1997 | 19 Ntonifor et al. 2003 |
| 7 L.C. Dempster (unpublished) | 20 Echendu et al. 2003 |
| 8 Thresh and Mbwana 1998 | 21 Ranomenjanahary et al. 2003 |
| 9 Jericho et al. 1999 | 22 Sseruwagi, unpublished data |
| 10 Hillocks et al. 2002 | 23 W. Tata-Hangy, unpublished data |
| 11 Munga and Thresh 2002 | 24 Ntawuruhunga, unpublished data |
| 12 Thresh and Hillocks 2003 | 25 Kamau et al. 2003. |
| 13 Muimba-Kankolongo et al. 1999 | |

again emphasizes the magnitude of the problem that this disease continues to pose to all those with a stake in cassava in Africa.

The African CMD pandemic

Until the 1990s, CMD was given little attention in East Africa, and greater emphasis was placed on bacterial blight. CMD was considered as something of a benign presence, and there had been no reports of major CMD-associated problems since the Ugandan epidemics of the 1930s and 1940s (Jameson 1964). In the late 1980s, however, reports were received from northern central Uganda of a severe form of CMD that was devastating the crop (Otim-Nape et al. 1994). Subsequent studies revealed that there was an epidemic of the severe CMD (moving southwards across much of Uganda at 20–30 km per year) which was characterized by rapid spread and unusually large whitefly population densities (Gibson et al. 1996; Otim-Nape et al. 1997; Legg and Ogwal 1998). The epidemic had a devastating effect on cassava production throughout the main cassava growing regions of the country, and for some time, many farmers abandoned the crop altogether (Thresh et al. 1994b; Otim-Nape et al. 1997). As previously indicated, the epidemic was associated with a novel recombinant CMG, EACMV-Ug, which elicited more severe symptoms in cassava than the originally occurring ACMV, and which commonly spread in synergistically enhanced mixed infections together with ACMV (Harrison et al. 1997).

In 1995, the first report of the spread of the epidemic beyond the borders of Uganda into western areas of neighboring Kenya (Gibson 1996), was received by similar reports of EACMV-Ug in Sudan (Harrison et al. 1997), and spread to Tanzania (Legg and Okao-Okuja 1999) and Rwanda (Legg et al. 2001). The “pandemic” as it became designated could readily be mapped in a defined and contiguous zone of East and Central Africa (Legg 1999). However, reports were also received of severe spread of CMD from the Republic of Congo (ROC) and the western part of the Democratic Republic of Congo (DRC) in the late 1990s, and diagnostic tests revealed the occurrence of EACMV-Ug2 in both areas (Neuenschwander et al. 2002). While it appeared that the pandemic now straddled the central equatorial zone of Africa, and could rightly be referred to as the African CMD pandemic (Legg et al. 2002a), the gap in knowledge that existed for the vast Congo Basin area in the center of war-affected DRC meant that it was difficult to be entirely certain about this.

Current work is focused on confirming the sequence homology of the DNA-A of putative EACMV-Ug2 from western DRC and ROC with that of EACMV-Ug2 from East Africa. Evidence for the widespread occurrence of EACMV-Ug2 in southern African countries (Berry and Rey 2001), in the absence of any apparent CMD epidemic, would seem to raise questions about the primary importance of the virus in “driving” the

pandemic in East Africa. This has led researchers to examine the role of the whitefly vector and the significance of the increased vector populations that are a feature of the pandemic in East Africa. There is evidence to suggest that *B. tabaci* populations collected from the epidemic-affected area have a similar biology, have comparable virus transmission capabilities, and interbreed with populations collected from within the affected area (Maruthi et al. 2001, 2002). RAPD PCR techniques failed to detect genetic differences between the two populations, but a comparison of the rate of population growth of Ugandan *B. tabaci* on CMD-free versus CMD-diseased plants of a common local variety revealed a strong synergistic interaction between CMD and *B. tabaci* (Colvin et al. 1999). By contrast, in another study of *B. tabaci* populations collected along three transects straddling the epidemic front in Uganda, sequences of the mitochondrial cytochrome oxidase gene provided a clear indication for the occurrence of a distinct and rapidly expanding population of *B. tabaci* associated with the epidemic (Legg et al. 2002b). These two sets of data, while appearing to be contradictory, may in fact be complementary, although a substantial amount of research remains to be carried out to elucidate the role of the whitefly vector in the CMD pandemic.

Management

Two major approaches have been used in attempts to control CMD in Africa: the maintenance of a CMD-free crop through phytosanitation and the development and deployment of host plant resistance (Thresh and Otim-Nape 1994). Phytosanitation involves the removal of diseased plants from the field (or roguing) to prevent further spread and/or the selection of CMD-free stems at the end of each growing cycle in order to plant new fields with “clean” material. Both roguing and selection have been widely practiced within official multiplication blocks of CMD-resistant varieties (Thresh et al. 1998a), and have been rigorously implemented in open quarantine sites in Kenya and Tanzania (Legg et al. 1999), through which germplasm has been introduced as stem cuttings from Uganda for use in neighboring countries. However, both techniques proved difficult for farmers to use. There are several reasons for this. First, farmers have small plots of land, so even if they try to maintain a “clean” crop, it may become infected from external inoculum sources in neighbors’ fields. Secondly, farmers are often unwilling to remove growing plants that might contribute to some yield. The reluctance is even greater when there is a high rate of disease spread leading to the infection of a substantial proportion, if not all, of the plants. Finally, regarding selection, either there may be an insufficient number of disease-free plants remaining at the end of the growing season from which to select, or, if there are sufficient plants, the conditions at harvest time may be unfavorable for symptom development, as can

occur during hot, dry periods when many of the symptom-bearing leaves absciss. All of these difficulties complicate the implementation of phytosanitation. Current research aims to develop simple guidelines on the most appropriate method or combination of methods of phytosanitation to adopt under different conditions of inoculum pressure and with varieties differing in resistance.

Programs to develop host plant resistance to CMD began in the 1930s in both Tanzania (Nichols 1947) and Madagascar (Cours 1951). Both were successful, but the Amani program in Tanzania was crucial in providing seed to Nigeria where clone 58308 was selected and later used widely in the IITA germplasm development program, based at Ibadan, Nigeria. Breeding for CMD resistance became one of the main objectives of this program from its initiation in 1970 (Hahn et al. 1980). The first resistant varieties that were developed retained the broad-based multigenic resistance derived from *M. glaziovii*. The main features were low susceptibility to infection, low symptom intensity, low virus content, and high levels of recovery (symptom remission in new growth) (Fargette et al. 1996; Thresh et al. 1998b). Some of the early CMD-resistant varieties developed at IITA were introduced to Uganda in the 1980s, and formed the basis for the CMD pandemic management program which began in the early 1990s (Otim-Nape et al. 1994, 1997; Thresh et al. 1994b). Three of these varieties were released officially in 1993, namely: TMS 60142 (released as Nase 1), TMS 30337 (Nase 2), and TMS 30572 (Nase 3). These varieties are substantially more resistant to CMD infection than local cultivars (Otim-Nape 1993), although over a sequence of cropping cycles, it is common for a large proportion of Nase 1 and Nase 2 plants to become diseased. Significantly, however, they are largely tolerant of CMD infection, incurring little or no yield loss (Osiru et al. 1999). All three have been widely adopted in Uganda by farmers affected by the crisis, although acceptance is greatest in the eastern part of the country where cassava is mainly processed into flour and the effects of the epidemic had been greatest as a result of earlier, almost total reliance on a single highly CMD-susceptible local variety, "Ebwanateraka". Recent data collected from surveys of six districts in central and southeastern Uganda indicate that more than a quarter of all the cassava grown now are now of CMD-resistant varieties (Legg unpublished data).

The breeding program at IITA, Ibadan, Nigeria has since its inception used local Nigerian landraces in crosses either directly with 58308 originating from East Africa, or with resistant progeny derived from this clone. This work finally led to the development of near immune clones in the 1990s, and detailed examinations of the genetics behind this conducted in collaboration with CIAT, showed that certain landraces have a single dominant gene, designated CMD2, which confers resistance to CMD. This provides the opportunity to strengthen the resistance still further, and to do this more rapidly than

was possible previously, using marker-assisted selection (Fregene et al. 2000). Material produced from crosses combining the original multigenic resistance with CMD2 has been advanced through IITA's regional germplasm development program based in Serere, Uganda, albeit fortuitously before the nature of the resistance was determined. These materials have since 1997 been distributed to neighboring countries through open quarantine arrangements. As a result, significant success has been realized in identifying, deploying, and multiplying farmer-acceptable CMD-resistant varieties in Burundi, Kenya, Rwanda, and Tanzania. Major programs for the introduction, evaluation, and dissemination of these varieties are also currently underway in DRC and ROC. These materials are having a significant impact on CMD management in areas affected by the pandemic, but also offer great potential for more general dissemination across sub-Saharan Africa as an effective CMD control measure since they combine high levels of resistance to CMD, cassava mealybug, and cassava green mite, together with many of the farmer-preferred qualities of local landraces.

In areas where CMD-resistant varieties have not been widely accepted, alternative management approaches have been sought. It has been observed that local CMD-susceptible varieties infected with mild strains of EACMV-Ug2 in Uganda remain mildly diseased when neighboring plants of the same variety that sprouted disease-free become much more severely diseased. Preliminary investigations show that mild EACMV-UgG2 strains do indeed restrict the transmission of more severe strains of the same virus and when grown in the field, may yield substantially more than initially "healthy" plants (Owor 2003). Current work aims to identify the molecular mechanisms behind this phenomenon and seeks to enhance the effect and develop an approach to its practical application.

Little attention has been given to the possible control of CMD through managing populations of its whitefly vector. This is partly due to the facts that there seems to be no clear correlation between populations of whiteflies supported by a given variety and the spread of CMD (Fargette et al. 1996), difficulty in controlling whitefly populations on cassava throughout its period of growth, and because of the mobility of the vector. However, some of the newly-released CMD-resistant varieties being used to tackle the CMD pandemic appear to be particularly favorable hosts to *B. tabaci*, supporting populations sufficiently large to cause physical damage. This raises two important concerns. Firstly, direct damage may have an economically important effect on the productivity of these new varieties, and secondly, the increased number of vector-virus-host "contacts" that occur may increase the probability of resistance breakdown. In order to address this problem, efforts are currently underway to investigate options

for the identification and deployment of whitefly resistance, and for managing *B. tabaci* populations using biological control approaches (Legg et al. 2003; Otim 2003).

The challenge of developing a comprehensive integrated pest management (IPM) approach for CMD remains unmet, not least since host plant resistance has been so successful, particularly in the management of the CMD pandemic in East Africa. New concerns about whitefly populations highlighted here do mean, however, that efforts are needed to extend and improve the control options available for farmers, and this, together with the incorporation of genetic transformation-based control methods, will be the challenge for all those working on CMD in the next decades.

Cassava brown streak disease

Historical background

Cassava brown streak disease (CBSD) was first reported at the time Storey and colleagues began work on virus diseases of cassava and other crops at the Amani Station in Tanzania in the 1930s (Storey 1936). The symptoms were described and it was assumed that the causal agent was a virus since no pathogen was visible in infected tissues and the disease was transmissible by grafting. Efforts to study CBSD continued after the Second World War (1939–1945) with the detailed work of Nichols (1950). Comprehensive descriptions of the symptoms were provided and it was noted that CBSD was endemic throughout Tanzania, occurring right up to the borders with Kenya to the north and Mozambique to the south. There were also records of CBSD in Malawi along the Lakeshore, throughout Zanzibar, and in Uganda where it was thought to have been introduced in cutting material from Amani in 1934 (Jameson 1964). A major campaign was launched in Uganda to eradicate CBSD, with mixed results during the 1940s, and Nichols (1950) reported observations in Uganda on the natural spread of CBSD at both Serere and Kaberamaido in the northeastern part of the country. Following this initial occurrence, however, there appears to have been no further report of CBSD in Uganda until an isolated sighting near Entebbe many years later (Thresh et al. 1994c).

Nichols (1950) observed that CBSD symptoms became more apparent in cool dry conditions and plants developing from infected cuttings planted at altitudes greater than 1000 m a.s.l. typically had very severe die-back symptoms and often died. Although symptoms were exaggerated at these altitudes, CBSD appeared not to spread naturally. Some years after this key early work, Lister (1959) was successful in transmitting the viral agent causing CBSD to a range of herbaceous indicator plants through mechanical inoculation. He was then able to transmit the putative causal virus back to cassava

seedlings by mechanically inoculating them with sap from leaves of infected cassava or of infected herbaceous indicator plants.

The next research effort on CBSD began almost 20 years later through the ODA/Kenya Plant Virology Project. Further sap transmissions to test plants were made (Bock 1994) and the first electron microscopy studies conducted in UK revealed the presence of filamentous particles *c.* 650 nm long. It was considered at the time that these could possibly be carlaviruses, although low concentrations and the absence of particles in many preparations made it difficult to draw firm conclusions. *Nicotiana debneyi* was found to be a particularly good herbaceous test host and allowed the recognition of two isolates that produced distinct symptoms. Further detailed studies of the causal virus of CBSD were carried out at the Scottish Crop Research Institute, Dundee. The occurrence of filamentous particles in infected plants again suggested the presence of a carlavirus, a conclusion that was supported by positive reactions of extracts from infected leaves in serological tests using antibodies made against the carlavirus, *Cowpea mild mottle virus* (Lennon et al. 1986). However, the presence of pin-wheel inclusions was also noted in electron microscopy preparations, structures that at the time had only been observed in association with potyvirus infections.

After 50 years of sporadic research into CBSD, a substantial body of information had been developed on the disease, but critically, the etiology had not been fully determined, the causal virus had not been characterized, no vector was known, and there was little information on temporal or spatial patterns of disease spread. This unsatisfactory situation was at least partially addressed by further research done in the next two decades and which is summarized in the following sections.

Cassava brown streak virus—etiology, structure, and properties

Critical studies on the etiology of CBSD, the nature of the causal virus, and methods to detect it were made through a series of projects starting in the late 1990s at the University of Bristol, UK. These projects pursued a molecular approach to the study of the virus, beginning with partial virus purifications from CBSD-affected cassava material collected from Tanzania (Monger et al. 2001a). Total RNA was extracted from these purifications and converted to double-stranded cDNA using commercial kits. DNA was amplified with polymerase chain reaction (PCR) and the fragments of DNA produced were sequenced. The longest sequence generated was 1114 bp, and based on this, primers were designed for a reverse transcription (RT)-PCR procedure which subsequently was able to detect the sequence in extracts from CBSD-affected leaves. The sequence was compared with known viruses thought to be closely related, including examples from the *Ipomovirus*, *Bymovirus*, *Tritimovirus*, *Macluravirus*, and

Potyvirus genera of the family *Potyviridae*. Closest sequence identity was observed with *Sweetpotato mild mottle virus*, an *Ipomovirus*, and the deduced amino acid sequence appeared to include the coat protein, as judged by comparison with coat protein sequences of related viruses. Universal primers for the four genera of the *Potyviridae* failed to give any product in RT-PCR with RNA from CBSV-affected samples. These evidences pointed to the designation of CBSV as an *Ipomovirus*, a conclusion that was further supported by more recent results (Foster unpublished data) indicating an even closer homology (76.3% in the deduced amino acid sequence) with *Cucumber vein yellowing virus* (CVYV), another newly described member of the *Ipomovirus* genus (Lecoq et al. 2000). The protein subunits, c. 45 kDa in size, were also shown to be larger than those typical for members of the *Potyviridae*, but similar to those of the described ipomoviruses. Although these studies were entirely molecular, and no attempt was made to fulfil Koch's postulates, it was concluded that the virus detected was an *Ipomovirus*, and further, that it was *Cassava brown streak virus*, the causal agent of CBSV.

Symptoms and diagnostic methods

As mentioned earlier, Nichols (1950) provided the first detailed description of the symptoms of CBSV from his work at Amani in northeastern Tanzania. A key facet of the symptoms highlighted by Nichols was their extreme variability, both from one variety to another, and from season to season. Symptoms were noted on the leaves, stems, fruits, and tuberous roots. In sensitive varieties, symptoms may be present on all plant parts, while for more tolerant types, there may be only one symptom, commonly on the leaves.

Nichols (1950) recognized two types of symptoms on the leaves: the first was a yellow chlorosis associated with the secondary and tertiary veins, and the second, more common type, was a general blotchy chlorotic mottle. In both cases, these were more prominent on lower leaves, and the yellowing can be readily distinguished from senescence by the presence of patches of green that occur in symptomatic leaves of CBSV-affected plants. Unlike CMD, there is no leaf distortion and no size reduction.

The brown necrotic streaks that occur on the green portions of stems of CBSV-sensitive varieties gave the disease its name. However, this is perhaps inappropriate, since stem symptoms are less frequent than either root or leaf symptoms. Leaf scars may show sepia to brown necrotic lesions and in sensitive varieties, during periods of cool dry weather, shoot dieback may occur, with the upper portion of the stem becoming necrotic, then drying out. In extreme cases, this may destroy the entire plant.

By far, the most important symptom produced by CBSV is the development of dry, sepia to brown, corky, and necrotic lesions in the root tissue. This was confirmed to be

associated with aboveground symptoms following an extensive survey-based study in Tanzania (Hillocks et al. 1996). Moreover, roots of affected plants may be malformed and have constrictions. Symptoms in roots become more intense as the crop matures, particularly “beyond” physiological maturity at about 12 months after planting (Nichols 1950; Hillocks et al. 2001). Symptomatic roots also appear to be more susceptible to secondary soil-borne pathogens, and plants are often affected by soft rots.

In view of the ephemeral and variable nature of CBSD symptoms, diagnosis of infection is less certain than for CMD. Visual inspection is not reliable, and there is a need for a sensitive and reliable diagnostic method. For many years, failure to correctly identify and characterize the causal virus impeded efforts to develop such a technique, although an ELISA-based approach was used with limited success on cassava samples in Malawi (Sweetmore 1994). Characterization of CBSV and sequencing of a portion of its RNA genome (Monger et al. 2001a) facilitated the development of an RT-PCR-based diagnostic protocol using specific primers (Monger et al. 2001b). This was tested on samples collected from Tanzania and Mozambique, and most importantly, could detect the virus even in newly emerging and symptomless leaves of otherwise infected plants. This offers great promise as a support tool for both virologists and breeders working with CBSD, although the challenge in the coming years will be to get the technique functioning in strategically placed laboratories in the worst-affected countries.

Characterization and variability

Very little information is currently available on the variability of different isolates of CBSV. The published data on the characterization of CBSV (Monger et al. 2001a) relates to a single isolate collected from coastal Tanzania. During the development of their diagnostic technique, the University of Bristol team examined CBSV isolates from three varieties obtained near Dar es Salaam, Tanzania, and from Zambézia Province, northern Mozambique. The isolates each elicited different symptom types in the herbaceous indicator plants *N. benthamiana* and *N. tabaci*, but comparisons of sequences revealed only *c.* 8% differences in nucleotides and 6% differences in deduced amino acids (Monger et al. 2001b). Additional comparisons with isolates from Nampula in northern Mozambique revealed similar levels of sequence divergence (Foster unpublished information). It must be recognized, however, that to date, few isolates have been examined, and these cannot be considered representative for CBSV in East/Central Africa as a whole. Extensive additional sampling and characterization of isolates collected from the full range of locations and environments within which CBSD occurs will be required before a more comprehensive and accurate assessment of the diversity of CBSV can be made.

Virus transmission and epidemiology

From the earliest period of research on CBSD, it was hypothesized that the whitefly, *Bemisia tabaci* was the most likely vector of the virus, since it was apparent that there was natural field spread and there were no other obvious candidates (Storey 1936; Nichols 1950). Extensive tests on whitefly and aphids within the Kenya Virology Project (Bock 1994) failed to give any positive results, and recent studies undertaken by Tanzanian scientists in collaboration with the UK's Natural Resources Institute (NRI) have also failed to provide any definitive answer on the vector. The finding that CBSV is an ipomovirus is significant, in that all other described members of this genus are vectored by *Bemisia tabaci* (Monger et al. 2001a). Another important observation from transmission studies with related viruses such as CVYV, is that the efficiency of transmission has invariably been very low (Lecoq et al. 2000). This seems to suggest that *B. tabaci* may indeed be the vector, but that conditions used so far in transmission studies have not been appropriate to demonstrate this. Future efforts to demonstrate transmission should therefore continue to focus on *B. tabaci* and *B. afer* while experimenting with a range of vector, test plant, and environmental conditions.

Although it was realized in the 1930s that CBSD spread naturally under field conditions, and these observations were confirmed later by Nichols (1950), no quantitative data were produced on epidemiology prior to the NRI Project with the Root Crops Programs in Tanzania and Mozambique in the 1990s. Experiments conducted in Tanzania with diseased and disease-free planting stocks of a range of local CBSD-susceptible varieties revealed natural infection in all trials but ranging from 2 to 83% depending on the variety and location (Hillocks et al. 2001). The peak period of spread was from crops planted at the beginning of the year in April and May, just prior to the main dry season (Hillocks unpublished data). This spread peak corresponds with the period of peak whitefly (principally *B. tabaci*) numbers which provides further circumstantial evidence suggesting that *B. tabaci* is the vector of CBSV. Variable symptom expression between varieties and seasons complicates epidemiological study, but it is hoped that the improved virus diagnostics that are now available and the future definitive identification of the vector will aid the further and more detailed study of this important aspect of CBSD ecology.

Economic impact of CBSD

Distribution and incidence

Little information was produced during the early years of CBSD research on the geographical significance of CBSD other than descriptive notes on its general distribution within East Africa (Nichols 1950), as described earlier. The first comprehensive survey

of CBSD was conducted in Tanzania in 1993/1994 (Legg and Raya 1998), although this was restricted to an assessment of leaf symptoms and did not take account of stems or roots. Apart from one observation in the western midaltitude (*c.* 1 200 m.a.s.l.) region of Tabora, CBSD was confined to the lowland coastal plain. Average incidence for the country was 8.6%, although this rose to 36% for Mtwara region in the southeast, and three other coastal regions had incidences exceeding 19%. There was considerable variation between varieties in terms of CBSD incidence. In the most affected areas in which some varieties had high incidences of infection, others were entirely free of leaf symptoms. A more intensive survey in southern Tanzania broadly confirmed the results of the 1993/1994 survey, and highlighted the decrease in incidence from the low altitude coastal zone (29%) to the higher altitude (500–700 m.a.s.l.) hinterland (7%) (Hillocks et al. 1999).

New observations of CBSD were made from a single location in southeastern Zambia in 1996 (Muimba-Kankalongo, personal communication), but much more significant was the confirmation of the occurrence of CBSD as a major constraint to cassava production in northern Mozambique in 1999 (Hillocks et al. 2002). Extensive surveys in Mozambique confirmed that CBSD was not present south of the Limpopo River, but that in coastal areas of northern Mozambique, incidences were 90–100% in some fields (Hillocks et al. 2002; Thresh and Hillocks 2003). Associated with these levels of aboveground symptoms, root necrosis symptoms were commonly associated with leaf symptoms and some farmers harvesting roots during the time of the survey found them almost totally affected by root necrosis. CBSD incidence has also been assessed in Zanzibar (Thresh and Mbwana 1998) and coastal Kenya (Munga and Thresh 2002). These results are summarized with incidence data from Mozambique and Tanzania in a distribution map (Fig. 4).

Effects of CBSD on yield and root quality

It has been recognized from the earliest research on CBSD that the main economically significant effect of the disease was through the root necrosis symptoms (Nichols 1950), but it has also been observed that the relationship between leaf and root symptoms is ill-defined, as some varieties or plants with clear leaf symptoms may fail to show root symptoms, while others not expressing leaf symptoms may produce root symptoms. Studies in Tanzania, however, have demonstrated that most (> 90%) plants of sensitive varieties sprouting from cuttings taken from diseased stems express leaf symptoms, and that many of the same plants (12–59%, depending on variety) show root symptoms at harvest (Hillocks et al. 2001). It was also shown that sensitive varieties may lose up to 70% of fresh root yield, due principally to the effects of die-back. These losses are then compounded by the effects of necrosis on root quality, which prevent harvested

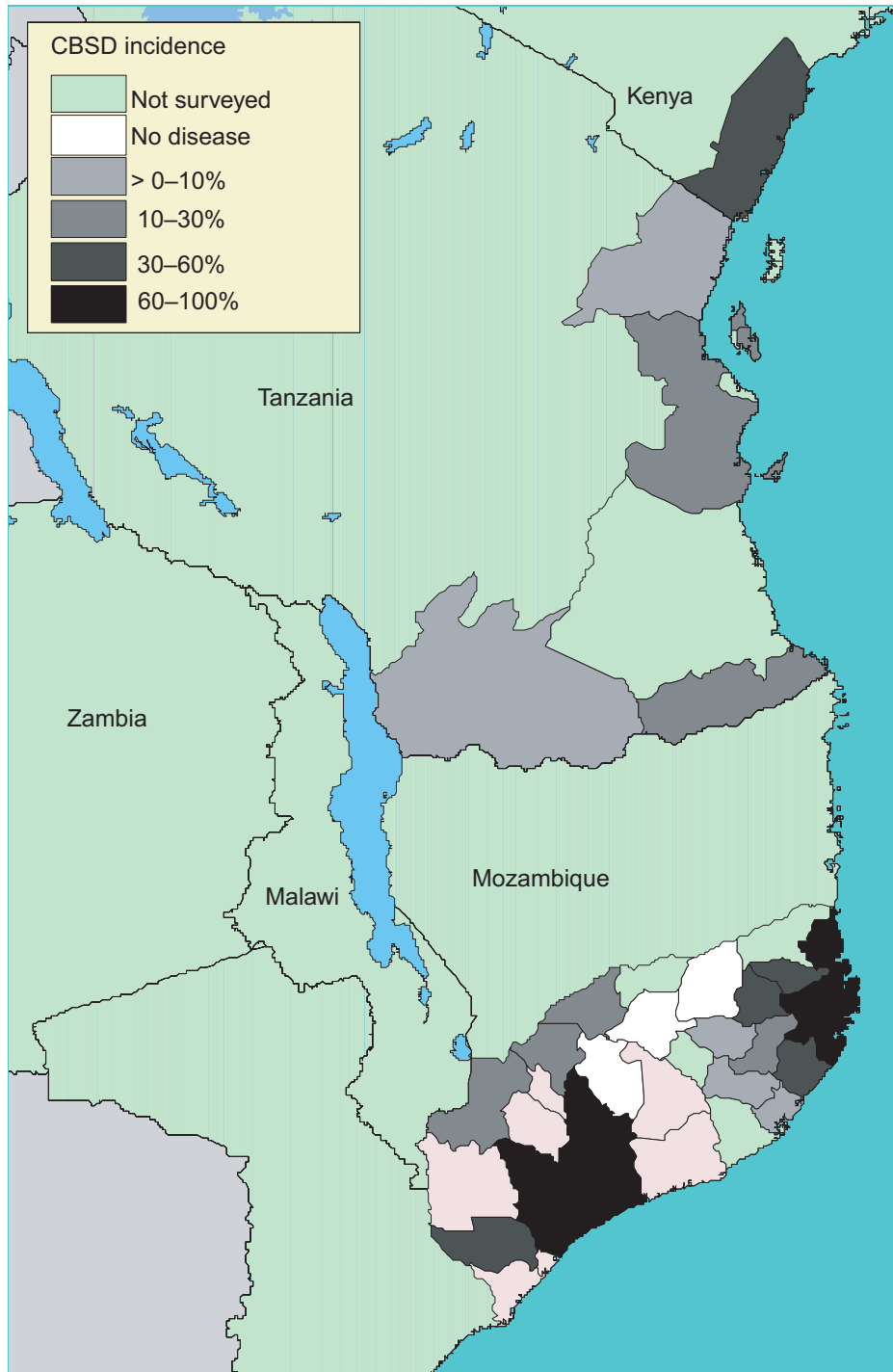


Figure 4. Incidence of cassava brown streak disease (CBSD) in East Africa. Tanzania (Legg and Raya 1998); Zanzibar (Thresh and Mbwana 1998); Kenya (Munga and Thresh 2002); Mozambique (Hillocks et al. 2002; Thresh and Hillocks 2003).

roots from being marketed or encourage premature harvesting to avoid the most severe damage. For susceptible varieties, these additional losses ranged from 2 to 29%. Symptoms of root necrosis and yield loss increase as the age of the crop increases (Nichols 1950). The losses are particularly acute for local varieties in which root necrosis begins to increase from six months after planting, encouraging farmers to harvest prematurely (Hillocks et al. 2001). An important consequence is that such varieties cannot be relied on as a food reserve for use in times of drought. By contrast, other apparently tolerant varieties, such as Nanchinyaya in southern Tanzania, begin to show mild root necrosis only beyond 12 months after planting, and as such, incur virtually no yield loss and can be harvested at the optimal and most convenient time.

Overall impact on production of CBSD

Substantial gaps in knowledge mean that it is difficult to make an overall estimate of the current impact of CBSD on cassava production in East and Central Africa. Good quality shoot *and* root survey data are still lacking for much of the known affected area. More data are needed on the relationship between shoot and root symptoms and on yield and quality losses, and there are new reports of CBSD from DRC (Mahungu personal communication) and ROC which need to be investigated. What is clear, however, is that a grossly inadequate amount of research attention has been directed towards what is evidently a major threat to the livelihoods of millions of smallholder farmers in sub-Saharan Africa, and this is a situation that urgently needs to be addressed.

Management

Research on breeding resistance initiated at the Amani Station in the 1930s considered both CMD and CBSD. Initially, a large collection of cassava varieties was made from many international and local sources, but none appeared to have adequate levels of resistance to either CMD or CBSD. Two Brazil-derived varieties, Aipin Valenca and Macaxeira, did have limited levels of resistance to CBSD. Aipin Valenca continues to be grown widely in Tanzania, but neither of these two varieties was considered sufficiently resistant to CBSD for large-scale promotion. Following the success of the interspecific crossing technique using *M. glaziovii* to introduce CMD resistance to cultivated cassava, a similar approach was explored for CBSD, although in this case, using the other wild relative *Manihot melanobasis* Muell. Arg. The first crosses were made in 1950, and over the subsequent decade, substantial progress was made in developing cassava that combined good levels of resistance to both CMD and CBSD. Materials developed through the Amani program over this period were maintained in a

germplasm collection at research stations in coastal Kenya, and subsequent evaluation trials conducted between 1984 and 1987 still included two CBSD-resistant Amani selections: 5543/156 and 46106/27 (Kaleso). Visits to the Msabaha Research Station, coastal Kenya, in 2001, (Thresh, personal observation) revealed a substantial number of Amani-derived clones still being maintained and some were being grown by farmers nearby. Efforts are underway to make use of this material in a reestablished regional breeding program for CBSD resistance.

In southern Tanzania and Mozambique, management efforts have focused on the identification of local tolerant varieties, such as “Nanchinyaya” in Tanzania (Hillocks et al. 2001) and “Muendowaloya, Mulaleia”, and “Nikwaha” in Mozambique (Mangana unpublished data). Introductions of the tolerant varieties “Nanchinyaya” and “Chigoma Mafia” from Cabo Delgado to Nampula and Zambézia in northern Mozambique, have also been successful as both have performed well in evaluation trials. Much of the management work in Mozambique is being run by a consortium of NGOs, including World Vision, Save the Children, and CARE. Each has developed a significant cassava program primarily to address the CBSD problem in view of the impact of the disease on the livelihoods of farming communities in northern Mozambique. The programs remain limited, however, through lack of highly resistant material, emphasizing again the importance of the reestablishment of a breeding program specifically targeting CBSD resistance.

The benefits of selecting of CBSD-free stems when replanting have been clearly demonstrated (Hillocks et al. 2001), although advocating a phytosanitation program to farmers has two major drawbacks. The first is the major educational and training input required and the second is the difficulty that farmers or even researchers can face in correctly identifying CBSD-free material. Farmers have been introduced to the control of virus diseases in cassava through phytosanitation in southern Tanzania on a small scale (Katanila, unpublished data). However, the relative merits of such an approach compared to the promotion of tolerant varieties are yet to be evaluated. It seems that resistant and tolerant varieties and phytosanitation may all have an important role to play in managing CBSD, but considerable research remains to be done on the conditions under which each is most appropriate and on how best to combine them into an integrated strategy. In future, there might be opportunities for the development of improved resistance through genetic transformation techniques. Viruses of the family *Potyviridae* are particularly amenable to coat protein-mediated resistance approaches, and CBSV might therefore be an appropriate target for such a strategy.

Another key facet of CBSD management, given its apparently restricted distribution, will be the prevention of movement between countries through the implementation

of strict quarantine procedures. Some of Africa's major cassava producers, including Nigeria (no. 1), Ghana (no. 2), Bénin, and Côte d'Ivoire appear to have favorable environments for CBSD. It is therefore critical that movements of germplasm in vegetative form should to be strictly controlled (through tissue culture) and that virus indexing laboratories that test tissue culture material prior to export are fully equipped to test for CBSV.

Summary

Research into CMD and CBSD over more than 70 years has been characterized by sporadic progress in particular locations, followed by long periods of relative inactivity. Encouragingly, the beginning of the 21st Century has seen a previously unprecedented level of interest in both diseases, with activities spanning a broad range of countries, disciplines, and institutions. While this is a positive development, CMD in particular seems to be providing a continually increasing threat, as the African pandemic continues to spread, and with new provisional reports of CBSD from DRC and ROC, both diseases still pose a massive threat to African agriculture. However, the gravity of the situation, together with renewed research interest and awareness among African governments, may provide a unique opportunity. Enormous benefits in terms of food security, poverty reduction, and improvements in social welfare, could be gained from effective management of cassava virus diseases. It is hoped that researchers, governments, and the donor community will seize the opportunity to transform the lives of millions in the continent through the achievement of this goal.

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The epidemiology of African plant viruses: basic principles and concepts

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Abstract

In recent years there have been important advances in epidemiology, as in other aspects of plant virology. Those concerned with the collection and mathematical analysis of data, modeling, and the impact of molecular studies of viruses, hosts, and vectors are particularly notable. Consequently, the terms quantitative ecology and molecular epidemiology are now widely accepted. Recent achievements using these approaches in plant virus epidemiology in sub-Saharan Africa are considered here.

Compared to biological assays and serological techniques, molecular typing of the pathogens generally facilitates a more detailed and comprehensive analysis of the structure of pathogen populations. A more precise identification of virus strains and a more reliable assessment of their prevalence and geographical distribution within cultivated and/or alternative hosts became possible. Comparisons of genome sequences through phylogenetic studies and systematic searches for recombination events make it possible to assess the relationships between strains, and to formulate hypotheses on their origin and evolution. This has been achieved recently with several major African viruses including banana streak, cassava mosaic, maize streak, rice yellow mottle, sugarcane mosaic, tomato leaf curl, and yam mosaic.

Such information is critical in order to understand, assess, and predict the spatial and temporal spread of the diseases they cause. It is useful also in implementing and enforcing phytosanitation measures introduced to avoid disseminating potentially dangerous strains within and between continents.

A comprehensive knowledge of virus genetic diversity is necessary also to elucidate plant–pathogen interactions. In practice, it is useful to evaluate sources of resistance derived originally against one or a few local strains against a more comprehensive range of isolates, bearing in mind that many of the major African viruses occur widely in the continent and there is a strong geographical basis to their diversity. Molecular

studies also help to identify the pathogenic determinants of the viruses being studied. Complementarily, there have been recent advances in the characterization of host resistance genes. Such information on plant–pathogen interactions will also be useful in breeding programs, especially when gene-for-gene interactions occur or are suspected, and to assess the possible risks of resistance breakdown when plants are challenged by virulent strains. It is realistic to predict that over the next few years molecular data will continue to accumulate at an ever increasing rate—providing epidemiologically relevant information on each member of the “ecological trinity”.

Mathematical analysis of field data and modelling studies have already been applied successfully with several tropical viruses to test epidemiological scenarios and control strategies. For example, the impact of different eradication schemes to control *Cacao swollen shoot virus* has been simulated, and the long-term impact of resistance components in strategies for controlling cassava mosaic viruses has been tested. However, mathematical analysis of data is still grossly underutilized in tropical virus epidemiology in Africa compared to other continents. It is likely that several pertinent and long-standing epidemiological questions could be resolved by utilizing such approaches (e.g., the “equilibrium concept”, cycles of infection–reinfection, mutual virus/host adaptation). Modelling studies will also be necessary in adopting the holistic, ecological approach to elucidating the complex interactions involving “the epidemiological triangle” of host, pathogen, and environment.

A possible objective of the PVSSA 2001 conference will be to identify such unresolved epidemiological problems, and to investigate, in the light of the ecological information available, the directions to take in molecular and quantitative ecological programs, to provide solutions, and to determine which information can reasonably be expected in the immediate future.

Résumé

Ces dernières années, d'énormes progrès ont été réalisés dans le domaine épidémiologique et dans d'autres domaines de la virologie végétale, les plus remarquables étant ceux relatifs à la collecte et l'analyse mathématique des données, la modélisation et l'impact des études moléculaires des virus, de leurs hôtes et vecteurs. Aussi, les termes « écologie quantitative » et « épidémiologie moléculaire » sont-ils devenus d'usage très courant. Les réalisations enregistrées récemment en matière d'épidémiologie des virus végétaux, en Afrique subsaharienne, à partir de ces approches sont ici abordées.

Comparé aux essais biologiques et aux techniques sérologiques, le typage moléculaire des pathogènes facilite généralement une analyse plus complète de la structure des populations de pathogènes. Aussi, est-il désormais possible d'obtenir une identification

plus précise des souches virales et une évaluation plus fiable de leur prévalence et de leur répartition géographique sur les plantes cultivées ou les hôtes de remplacement. Des comparaisons de séquences génomiques au moyen d'études phylogénétiques et de quêtes méthodiques de recombinaisons permettent d'apprécier les relations entre les souches, et de mettre des hypothèses quant à leur origine et évolution. Cette voie fut récemment adoptée pour l'étude de plusieurs principaux virus africains dont la striure du bananier, la mosaïque du manioc, la striure du maïs, la marbrure jaune du riz, la mosaïque de la canne à sucre, l'enroulement de la feuille de tomate et la mosaïque de l'igname. Cette information est essentielle pour comprendre, évaluer et prédire la répartition spatio-temporelle des maladies dont ils sont les agents causaux. Dans la mise en application des mesures phytosanitaires introduites, il convient également d'éviter la diffusion de souches potentiellement dangereuses aussi bien à l'intérieur des continents qu'entre eux.

Une connaissance approfondie de la diversité génétique des virus est requise afin d'élucider les relations plante-pathogène. En pratique, il est utile d'évaluer les sources de résistance originaires obtenues pour une ou quelques souches locales, par rapport à un éventail plus complet d'isolats, tout en sachant que bon nombre des principaux virus africains se sont répandus sur le continent et que leur diversité géographique est bien établie. En outre, les études moléculaires aident également à reconnaître les déterminants pathogéniques des virus étudiés. Des avancées complémentaires ont été récemment enregistrées quant à la caractérisation des gènes qui régissent la résistance chez l'hôte. Ces renseignements sur les interactions plante-pathogène seront aussi utiles pour les programmes de sélection, surtout en présence réelle ou soupçonnée d'interactions gène-pour-gène, et pour évaluer les risques possibles de rupture de résistance lorsque les plantes sont soumises à des souches virulentes. On peut à juste titre prédire que les données moléculaires continueront de s'accumuler à un rythme de plus en plus élevé au cours des prochaines années. Ainsi, de pertinentes informations à caractère épidémiologique seront obtenues sur chaque membre de la 'trinité écologique'.

L'analyse mathématique des données collectées au champ et les études de modélisation impliquant plusieurs virus tropicaux ont été déjà appliquées avec succès dans des tests d'options épidémiologiques et des stratégies antivirales. Par exemple, l'impact de divers plans conçus pour éradiquer le CSSV a été simulé et celui à long terme des composantes de la résistance dans les méthodes de lutte contre les virus de la mosaïque a été testé. Toutefois, en Afrique, l'analyse mathématique des données demeure un outil très peu utilisé dans l'épidémiologie des virus tropicaux, par rapport aux autres continents. Il est probable que bon nombre de questions épidémiologiques pertinentes trouvent enfin des solutions grâce à ces approches (ex : "concept d'équilibre",

cycles infection-réinfection, adaptation mutuelle virus/hôte). Des études de modélisation seront également requises si l'on adopte une démarche holistique et écologique dans le but d'élucider les interactions complexes inhérentes au 'triangle épidémiologique' hôte-pathogène-environnement.

Un objectif possible de la conférence PVSSA-2001 consisterait à identifier ces problèmes épidémiologiques non encore résolus, et à déterminer, à la lumière des informations écologiques disponibles, les orientations à prendre dans le cadre des programmes moléculaires et d'écologie quantitative à mettre en place pour trouver des solutions et se fixer sur les informations qu'on est à même d'espérer dans un proche avenir.

Introduction

Viruses were first distinguished as a separate group of plant pathogens in 1898 in early studies on the aetiology of tobacco mosaic disease in the Netherlands (Bos 2000). Much of the subsequent research in the early decades of the 20th century in Africa and elsewhere was by plant pathologists or entomologists involved in transmission studies with aphids, leafhoppers, whiteflies, or thrips as virus vectors. Virus research has since become more specialized and wide-ranging. Some researchers have put the main emphasis on aetiology and virus characterization. Others are primarily concerned with resistance breeding, vector ecology, or mechanisms of transmission. Moreover, epidemiology has become a separate subdiscipline which requires inputs from several diverse specialists. It is defined as the study of disease in host populations and is the subject of this review. Basic epidemiological concepts and some of the most important areas of study are discussed, with particular reference to virus diseases of African crops. One of the main aims is to show the value of a holistic ecological approach and the role of epidemiology in developing and evaluating control measures.

Modes of spread

Viruses must have one or more effective means of spread from infected to uninfected plants if they are to persist in host populations and not be at risk of extinction (Thresh 1985). This is an important aspect of the "epidemiological competence" of all pathogens (Crosse 1967) and one which has received considerable attention in studying plant viruses in the tropics as elsewhere.

The principal means of dissemination are shown in Figure 1 in which a distinction is made between virus spread above-(top) and belowground (bottom) and also between autonomous spread (left) and by means of man or vectors (right). However, it is important to appreciate that the diagram illustrates all the known means of dispersal and no single

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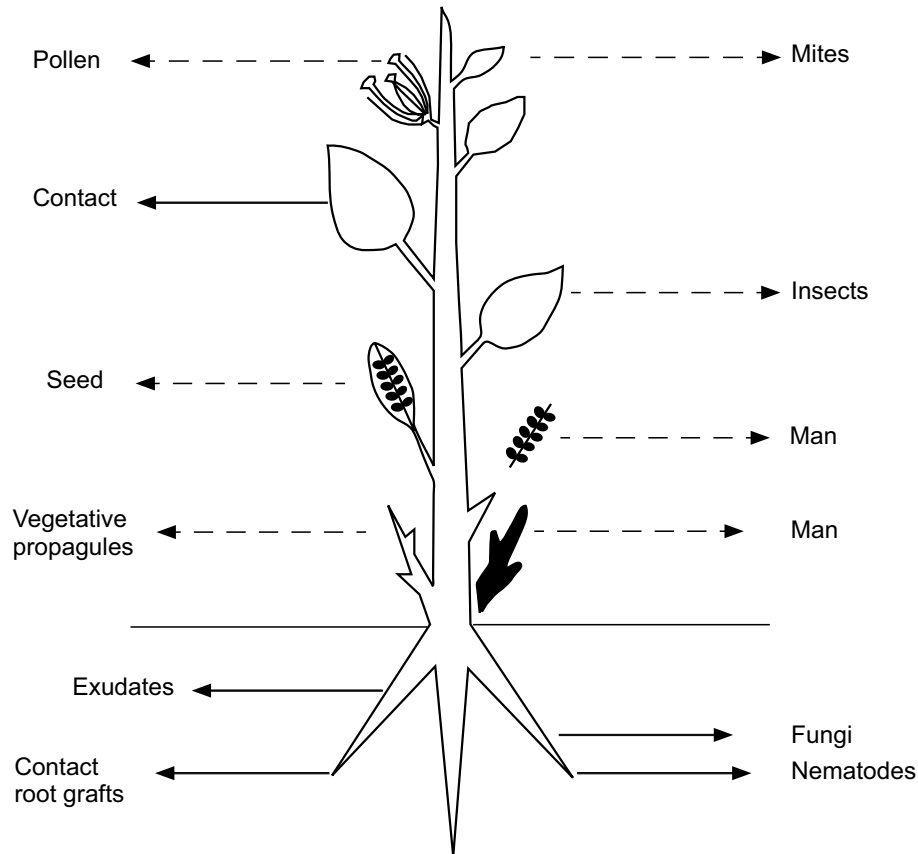


Figure 1. Diagrammatic representation of the principal means of plant virus spread above-(top) and belowground (bottom) and autonomously (left) or by means of vectors (right).
 ————— = Local spread; - - - - - = Local and distant spread.

Table 1. Representative viruses of African crops and their means of spread.

Contact-borne	<i>Tobacco mosaic virus</i>
Fungus-borne	<i>Peanut clump virus</i> (S)
Aphid-borne (N-P)	<i>Sugarcane mosaic virus</i> (V)
	<i>Bean common mosaic virus</i>
Aphid-borne (P)	Groundnut rosette viruses
Leafhopper-borne	<i>Maize streak virus</i>
Whitefly-borne	Cassava mosaic viruses (V)
	<i>Okra leaf curl virus</i>
Thrips-borne	<i>Groundnut bud necrosis virus</i>
	<i>Tomato spotted wilt virus</i>
Mealybug-borne	<i>Cacao swollen shoot virus</i>
	<i>Banana streak virus</i>
Beetle-borne	<i>Rice yellow mottle virus</i>
	<i>Cowpea mottle virus</i> (S)

Also disseminated in seed (S) or vegetative propagules (V)
 N-P = Nonpersistent. P = Persistent.

virus exploits each of the different routes. This is apparent from Table 1, which lists the means of spread of some of the most important viruses of African crops. It will be seen that only some of these viruses are seedborne (S), or disseminated in vegetative propagules (V). Others have fungal or insect vectors and there is marked specificity in that each virus is transmitted by only one or more species of a single taxonomic group of vectors. For example, aphid-borne viruses are not transmitted by other types of insect or by mites, nematodes, or fungi.

Notable omissions from the viruses listed in Table 1 are any that are transmitted by free-living, ectoparasitic nematodes or by eriophyid mites. Longidorid or Trichodorid nematodes are known to transmit many viruses of temperate crops, and closely related nematode species occur widely in the tropics. Moreover, mites are known to transmit at least one virus in tropical regions of Asia (*Pigeon pea sterility mosaic virus*). This suggests that nematode-borne and mite-borne viruses may also occur in Africa and await discovery. There are certainly many tropical viruses that spread naturally into or within crops, but for which no vector has yet been determined (Brunt et al. 1990).

In considering the effectiveness of the different means of dissemination, it is important to distinguish between virus spread over short distances from foci of infection within crops and that occurring over greater distances into or between crops. In ecological terms, local spread within crops is effective in enabling viruses to exploit habitats already colonized, whereas distant spread is more hazardous and difficult to achieve, but of crucial importance in leading to the colonization of entirely new habitats (Vanderplank 1963; Thresh 1974b). It is notable that each of the different means of dissemination can lead to local spread, but only some can achieve effective colonization over longer distances (Table 2). This emphasizes the importance of active winged vectors that can fly or be blown far and also of virus dissemination in infected seed or vegetative propagules that can be transported over long distances between regions, or even continents, by natural means or by human activity.

Insect vectors have very diverse life cycles and characteristics, although a crucial

Table 2. The effectiveness of the different means of dispersal locally and over greater distances.

Method	Local	Distant
Contact	+	–
Seed transmission	+	+
Pollen transmission*	+	+
Active vectors	+	+
Less active vectors	+	–
Vegetative propagation	+	+

* Transmission by pollen to the plant pollinated has been demonstrated with several viruses of temperate crops but not with any of tropical crops.

feature of all species is that they are active at one or more stages of their life cycle (Thresh 1974b; 1985). For example, the first-instar nymphal stages of mealybug vectors are small and well-adapted to dispersal by wind, whereas the later stages and wingless female adults are relatively immobile and make little contribution to distant spread. Although adult male mealybugs have wings, they do not have functional mouthparts and so cannot transmit viruses. With other types of insect vector including leafhoppers, planthoppers, thrips, whiteflies, beetles, and aphids, at least some of the adults are winged and they can travel far to reach new habitats, especially when blown by wind currents. This emphasizes the important role played by insect polymorphism and the ability of many species to produce particularly active stages that are well adapted to dispersal and to colonizing new habitats (Thresh 1974b; Table 3).

From this brief discussion, it is apparent that a knowledge of the means of spread is of crucial importance in studying any virus and in attempts to develop effective methods of control. For example, there are obvious benefits to be gained from selecting and using only virus-free propagules to minimize the losses caused by seedborne viruses and those of vegetatively propagated crops. It is also important to know which viruses are transmitted by soil-inhabiting fungi so that infested sites can be avoided and appropriate crop rotations or virus-resistant varieties can be adopted to decrease the risk of infection. Moreover, identification of an arthropod vector and of the mechanism of transmission can lead to effective control by pesticides, or by more benign means such as mineral oils, intercropping, and the use of natural enemies, barrier crops, or other cultural practices.

Table 3. Arthropod vectors having forms of contrasting mobility.

Group	“Colonizers”		“Exploiters”	
Mites and mealybugs	Wind-borne	(-)	Sedentary	(-)
Whiteflies and beetles	Active forms	(W)	Less active	(W)
Leafhoppers	Long-fliers	(W)	Short-fliers	(W)
Planthoppers	Macropterae	(W)	Brachypterae	(±W)
Aphids	Alatae	(W)	Apterae	(-W)

W = Winged forms ±W = Rudimentary wings.

Epidemiological cycles and cycles of infection

When healthy plants of a susceptible variety are exposed to virus inoculum, the virus may or may not become established in the plants and replicate. If infection does occur, and the virus multiplies and becomes systemic, the host plant becomes infectious and can act as a source of inoculum from which further spread may occur. Moreover, symptoms may or may not develop at the original site of inoculation and/or in subsequent plant growth. The time between inoculation and first symptom expression is usually termed

the latent period and that between inoculation and the plant becoming infectious is the incubation period.

The duration of the incubation period (IP) is important epidemiologically because it influences the potential number of infection cycles (N) that can occur during the life span of the host (Time: T). The maximum hypothetical number is T/IP, but in reality N is likely to be considerably less because inoculum may not be present from the outset and plants usually become resistant to infection as they age and approach maturity. This so-called “mature plant resistance” is known to be an important feature of many crops including *Solanum* potato (Beemster 1957) and cassava (Fargette et al. 1994). Moreover, vectors may not occur or they are not abundant throughout the duration of the crop and their activity may be curtailed during periods of unfavorable weather. Thus, spread may be restricted to brief “windows of opportunity” when vectors are present and active while plants are at a susceptible stage of growth (Thresh 1983).

The concept of infection cycles is simplistic, as both IP and T are influenced by temperature and other factors that influence crop growth and virus multiplication. Nevertheless, it is useful in indicating the potential for rapid spread, as reported in the literature on viruses of cowpea, groundnut, maize, rice, and many other herbaceous annual crops that are grown from seed. Incubation periods are short (days), especially during the early, most active phase of crop growth, and repeated cycles of infection are possible. Cassava is propagated vegetatively and with cassava mosaic geminiviruses the incubation period is longer (weeks) and the number of infection cycles is restricted by the onset of mature plant resistance. Viruses of tree crops such as cocoa and citrus have even longer incubation periods (months), vegetative growth is sporadic, and the virus is slow to become systemic throughout the canopy of branches. Consequently, repeated cycles of infection are possible only because of the longevity of the crop.

Such differences in epidemiological behavior can be considered in ecological and evolutionary terms and related to the type, continuity, and stability of the habitats that crops provide for viruses (Vanderplank 1949a; Thresh 1980a). Those infecting short-lived herbaceous annuals are unlikely to become prevalent and are prone to extinction unless they have the ecological competence to invade and multiply rapidly whenever and wherever suitable habitats occur. They must also have effective means of survival between growing seasons, especially in environments where crop growth is restricted by long periods of drought, cold, or other constraints. For example, *Bean common mosaic virus* is seedborne, *Peanut clump virus* persists in the long-lived resting spores of a soil-inhabiting fungus vector (*Polymyxa graminis*), and *Maize dwarf mosaic virus* infects *Sorghum halepense* and other perennial weeds that occur commonly in or near maize fields. Viruses of long-lived perennials are not subject to such limitations. Indeed,

extreme host vulnerability that permits repeated, short cycles of infection would be a grave evolutionary disadvantage and jeopardize host survival, unless the plants sustain limited damage when infected (Vanderplank 1949a).

Spatial patterns of spread into and within crops

Valuable information can be gained from observations on the distribution of diseased plants within crop stands. For example, it may facilitate identification of the initial source(s) of inoculum from which spread occurs. It may also be possible to infer the means of spread and the most effective control strategy to adopt. This has been apparent from observations made over many years on a wide range of virus diseases of temperate and tropical crops.

Some of the main findings are illustrated diagrammatically in the accompanying figures in which a distinction is made between local spread from infected sources within crops and spread from sources that are nearby or remote. Figure 2 considers spread from weeds or wild plant species and Figure 3 that from crop species. The distinctions between local and distant and between crop and noncrop hosts can be somewhat arbitrary, as spread may occur from multiple and diverse sources within and/or outside the crop(s) being considered. Nevertheless, the distinctions are helpful in elucidating the role of the different sources, the magnitude of the threat they pose, and the likely effectiveness of control measures. Clearly, initial foci that occur within crops from the outset present the greatest risk and can lead to rapid infection, even if spread occurs only over short distances by contact, or by vectors of limited mobility. Spread into crops from outside sources is less likely to occur, especially if the sources are remote and spread is entirely dependent on vectors that are active and able to fly, or can be blown far by wind currents.

Such considerations explain why there are such big differences between diseases in their patterns and rates of spread. Patches of disease often occur within crops as a consequence of local spread around initial foci of infection due to the debris of previous crops, or to the use of infected seed or vegetative propagules, or to the occurrence of weed or wild hosts within the stand. Patches can also occur as a consequence of soilborne inoculum, or spread around primary foci of infection that are initiated by incoming arthropod vectors, such as the aphid vector (*Aphis craccivora*) of groundnut rosette disease (Evans 1954; Naidu et al. 1998).

Figure 4 illustrates the temporal sequence of spread of *Cacao swollen shoot virus*, which is transmitted by several species of mealybug (Thresh 1958). New outbreaks are initiated by “jump spread” of viruliferous, windborne, first-instar nymphs (Cornwell 1960). Subsequent “radial spread” is by mealybugs walking mainly short distances

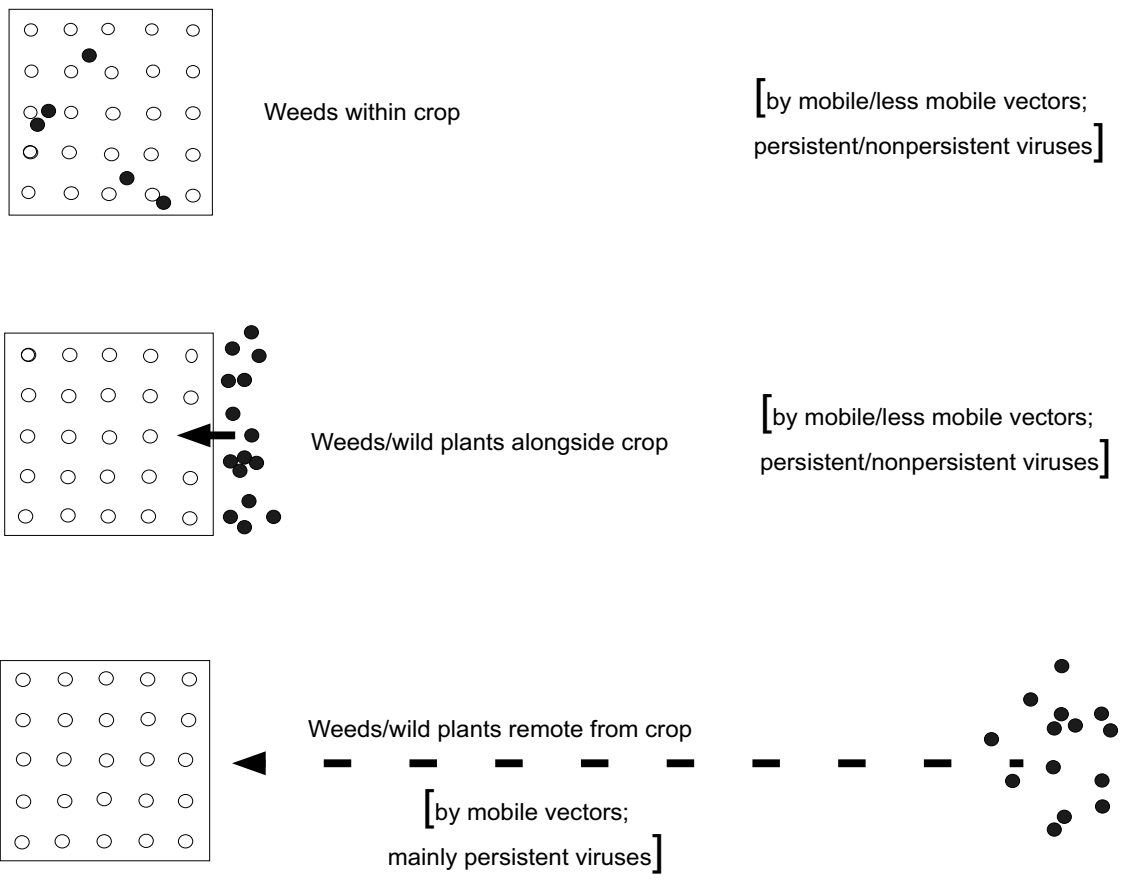


Figure 2. Diagrammatic representation of virus spread into or within crops from weeds or wild plants.

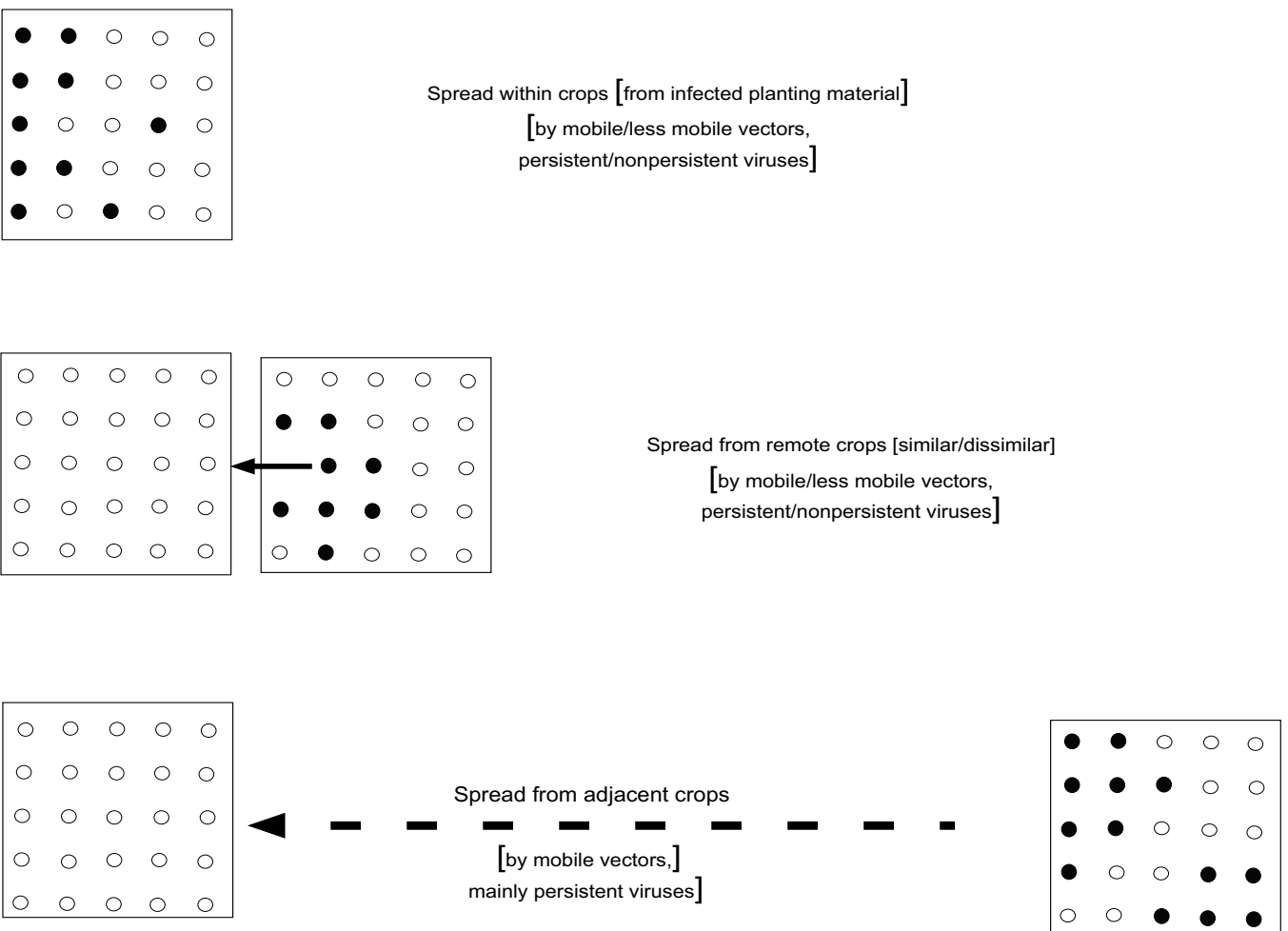


Figure 3. Diagrammatic representation of virus spread into or within crops from crop hosts.

between the canopy branches of adjacent trees to form obvious patches of disease within plantations (Cornwell 1958). The patches continue to expand and give rise to additional “satellite” outbreaks that ultimately coalesce and form continuous “areas of mass infection”. Such patterns of spread have been reported with many other pathogens and Figure 4 has been generalized so as to apply to a wide range of virus diseases (Vanderplank 1963). However, there can be big differences between diseases in their rate of progress and in the relative importance of “jump” and “radial” spread.

When spread is caused mainly by incoming viruliferous arthropod vectors from outside sources there may be “edge effects” around the margins of crop stands, especially those facing the direction of the prevailing wind or alongside nearby sources of infection, as discussed in the following section. Such information can be helpful in devising management practices to decrease the incidence of infection by planting away from and upwind of known sources of inoculum (Fig. 5) and in large compact blocks to decrease the proportion of plants in the vulnerable peripheral areas (Fig. 6). There may also be advantages in planting a barrier crop or windbreak around the field margin, or border rows of a resistant variety. However, there is limited scope for such approaches in the many parts of Africa where land is scarce, fields are typically small, and farmers have little latitude in choice of site.

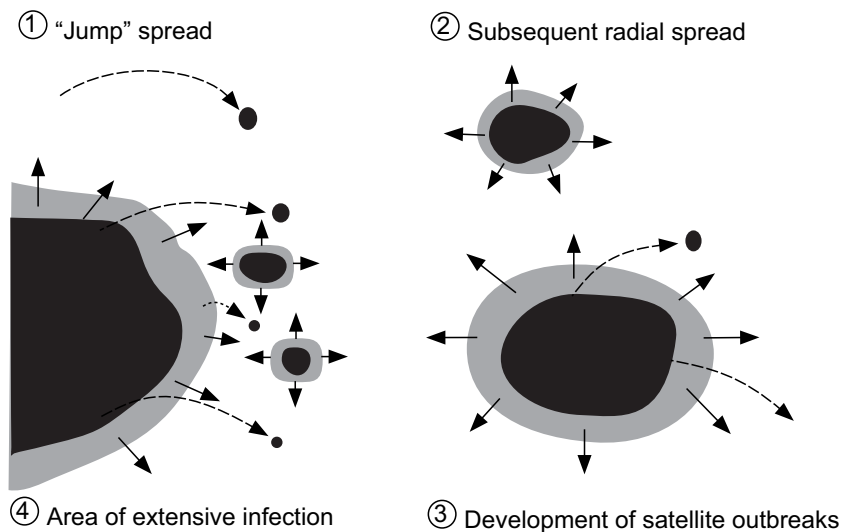


Figure 4. Successive stages in the spread of cocoa swollen shoot disease by wind-borne mealybug (“jump-spread”) and mealybugs walking between the canopy branches of adjacent trees (radial spread).

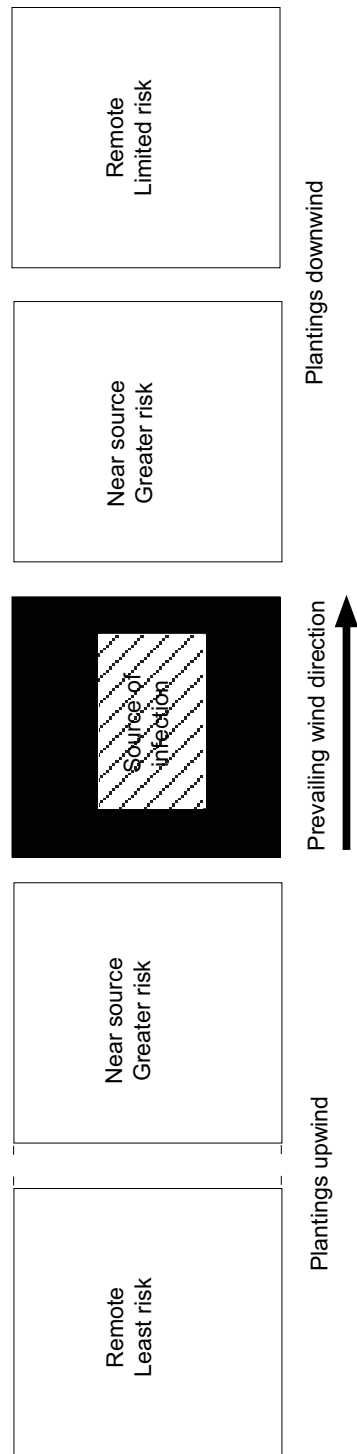


Figure 5. The disposition of plots/fields/plantings in relation to the direction of the prevailing wind and the risk of spread from a major source of infection (solid block).

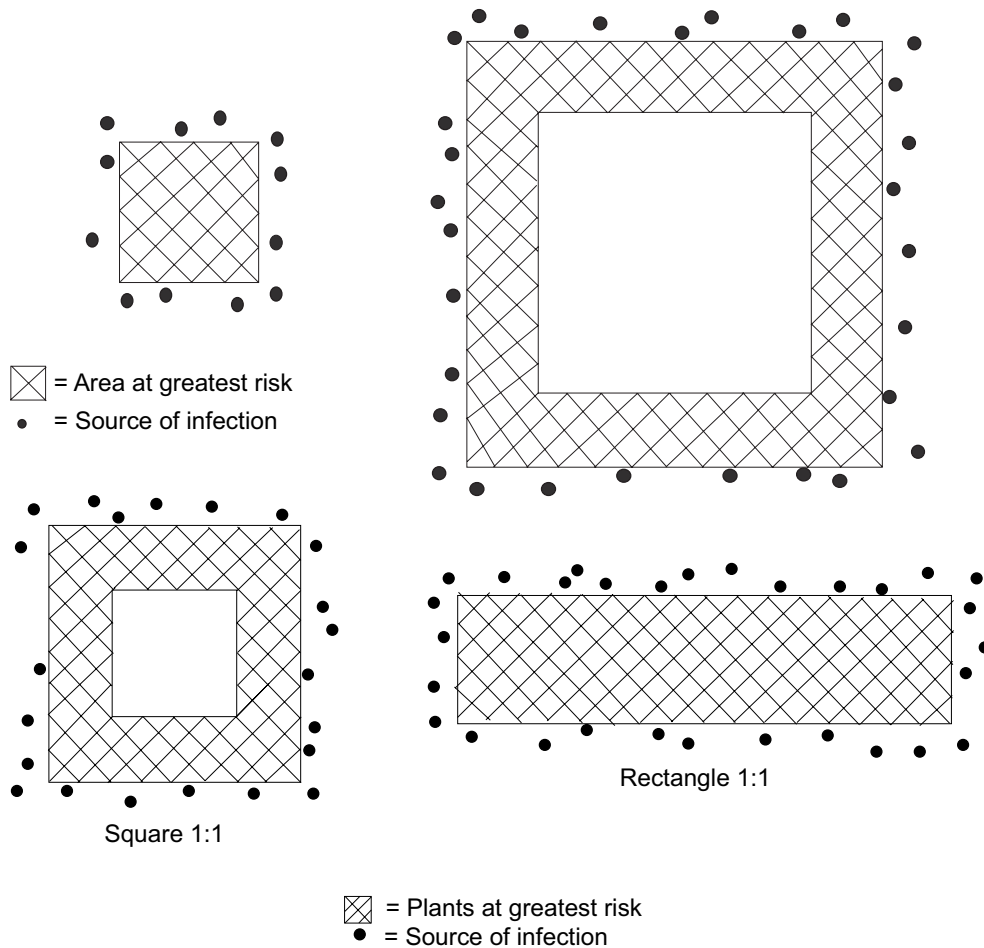


Figure 6. The influence of plot/field size (top) and shape (bottom) on virus spread from surrounding sources of infection.

Disease gradients

A feature of many pathosystems is that there are clear trends in the incidence of disease with increasing distance along one or more transects across the planting being assessed. Such changes are referred to as “disease gradients” and they are of two main types (Gregory 1968). “Environmental gradients” are associated with changes in soil fertility or microclimate and also with features such as windbreaks, buildings, or other physical obstacles that influence the vulnerability of host plants or the distribution and deposition of vectors and inoculum. Tropical examples include the gradients in the incidence of cassava mosaic disease, which tend to be greatest at the margins of plantings, especially those facing the direction of the prevailing wind. This is associated with the tendency of incoming whitefly vectors to alight preferentially and become most numerous on plants in the outer rows, as observed in field studies in Côte d’Ivoire in

which there were pronounced edge effects on the distribution of disease (Fargette et al. 1985; Colvin et al. 1998).

“Infection gradients” differ from environmental gradients in that they are associated with spread from initial foci of infection and incidence tends to decrease with increasing distance from the source of inoculum. Such gradients have been reported with many virus diseases including several of African crops (Rose 1973; Thresh and Lister 1960; Thresh 1976; Atiri and Varma 1991; Fargette et al. 1993). Infection gradients are obtained by plotting disease incidence on the vertical axis against distance on the horizontal axis using appropriate scales. The gradients tend to be curvilinear and concave as disease incidence decreases most rapidly near the source and then declines to zero, or reaches a low “horizon of infection” (Fig. 7). This is the distance beyond which spread is so infrequent that infection becomes insignificant, or so unimportant that it can be disregarded (Vanderplank 1949b).

Many factors influence the shape of infection gradients, the amount of spread and the distances over which it occurs. For example, for any particular disease, gradients tend to be shallower and over longer distances when conditions favor spread, compared with situations or seasons when conditions are less favorable. Moreover, for viruses with windborne vectors, the disease gradients tend to be steeper and more circumscribed up-wind than downwind from the source. An important consequence is that it is seldom possible to give a simple, unqualified recommendation in response to requests for advice on the most appropriate isolation distance to adopt to avoid serious risk of infection. The “safe” distance depends not only on site and season but also on the size and potency of the source of inoculum and the degree of risk and the extent of the losses that are considered acceptable. Clearly, the smaller the source and the greater the isolation, the less the risk, but it may be necessary to compromise because extreme isolation may be difficult to achieve and may also be very inconvenient. This explains why substantial isolation may be justified and appropriate for producing “elite”, specially selected, virus-free propagules for further propagation or distribution to farmers, but not for routine crop production.

The mode of spread and type of vector are other important considerations. Shallow gradients of spread over great distances are due solely to the most mobile vectors and their occurrence may be restricted to certain clearly defined migration periods. Spread at other times and by less active individuals or development stages of the vector species results in relatively steep gradients over mainly short distances around the source. Thus, the initial patterns of infection by migrants moving considerable distances into or between plantings may be quite different from those due to subsequent local movement by the incoming vectors, or their less mobile progeny

Plant virology in sub-Saharan Africa

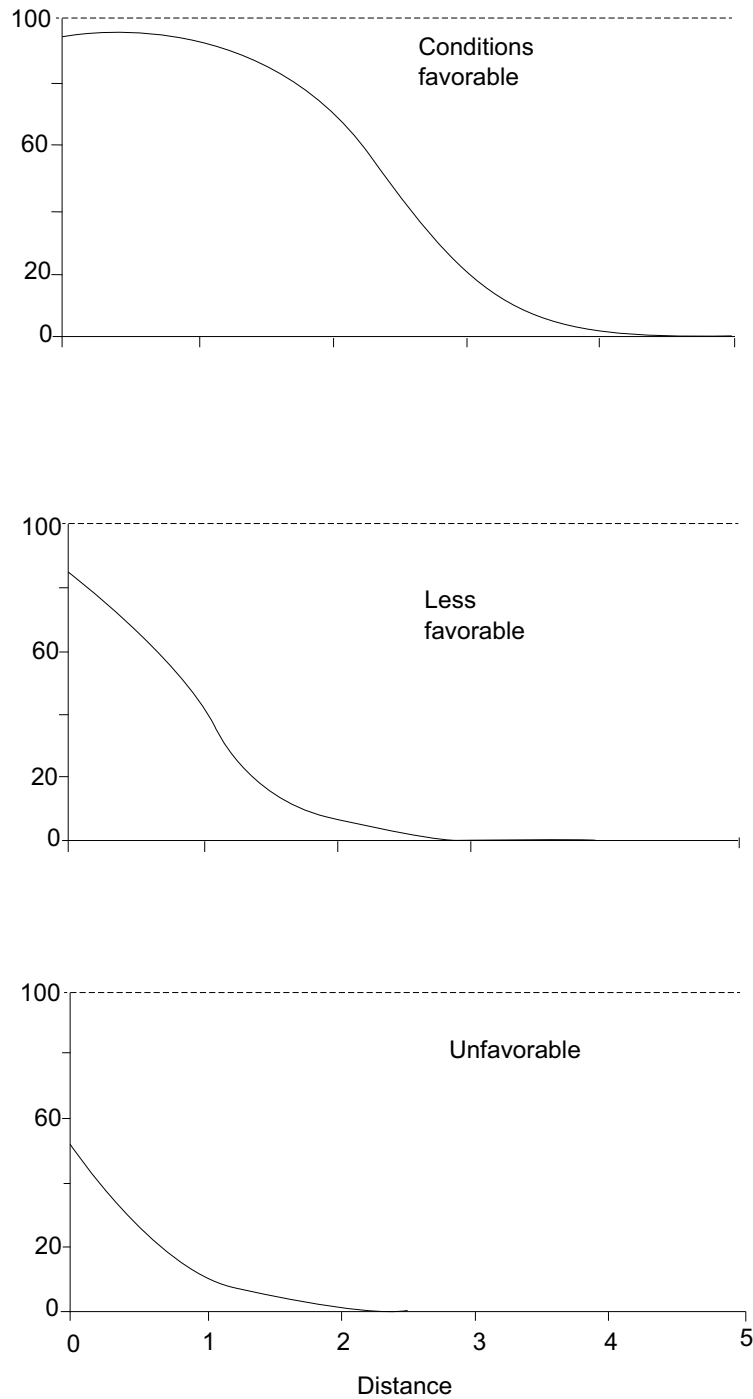


Figure 7. Generalized gradients of disease obtained by plotting incidence (%) on the vertical scale against distance on the horizontal scale (arbitrary units) for spread under favorable (top), less favorable (middle), and unfavorable conditions (bottom).

(Fig. 4). Where different forms of the vector coexist and each contributes to spread, the observed gradients of infection represent the superimposition of two or more separate distributions. However, there is seldom any discontinuity or inflexion in the disease gradients observed to make it possible to distinguish distinct zones or means of spread. The zones tend to merge imperceptibly and there is considerable uncertainty as to the relative importance in local spread of windborne and crawling mealybugs and of the short- and long-flying forms of certain leafhopper vectors, including the *Cicadulina* spp. that transmit *Maize streak virus* (Rose 1972b). Conflicting claims have also been made on the performance of wingless (apterous) and winged (alate) aphids as vectors and their relative importance seems to differ between viruses and between regions (Broadbent 1959; Hodgson 1991). Nevertheless, there is general agreement that in some crops, apterous aphids or the relatively immobile forms of other types of vector, often reach plants that have been infected already by the more active members of the vector population and so make little direct contribution to the local spread of disease.

For all diseases the amount and extent of spread tends to increase as outbreaks increase in size or potency and several factors contribute to the resulting tendency for gradients to flatten with time:

- For any particular disease the rate of dilution of inoculum with increasing distance is greatest around small discrete “point” sources, less around several infected source plants in a line or small group, and least around large groups of infected plants.
- As outbreaks enlarge, the number and/or activity of the vectors contributing to virus spread tend to increase and there is an increased probability of some migrating far. This can occur in unusual circumstances, or by the atypical behavior of particularly active individuals or migrant forms comprising only a small proportion of the total population.
- Gradients become increasingly distorted due to a progressive increase in the extent of “multiple infection”, which occurs as increasing amounts of inoculum reach plants that are already infected (Gregory 1948). The effect is to underestimate the number of inoculations taking place and the extent to which gradients are being distorted.
- There is an increased probability that plants infected by primary spread from the original source will themselves become infectious and act as secondary foci for further spread.

A major outcome of these trends is that there is a general “blurring” of disease gradients with time and it becomes increasingly difficult to assess whether new infections are due to local spread from secondary foci, or to continuing spread from the original ones. The spread of some diseases appears to increase dramatically once a critical

level of infection has been exceeded and control then becomes increasingly difficult to achieve. This is apparent from experience with cocoa swollen shoot disease in West Africa, where the effectiveness of eradication measures decreases as outbreaks increase in size and much larger outbreaks require more drastic treatment than small ones (Thresh and Lister 1960; Thresh and Owusu 1986).

Dispersal curves are difficult to handle mathematically in seeking to compare gradients between sites, seasons, and diseases. Consequently, transformations have been used widely to convert the usual curvilinear relationships between disease incidence and distance from the source to straight lines and so enable the use of conventional regression techniques. One approach is to transform disease incidence by using logarithms of the percentage values, or after transforming these to allow for the inevitable multiple infection (Gregory 1948). With data for many virus diseases this give a straight line relationship between the logarithm of disease incidence ($\log_{10} Y$) and some power (p) of distance (x) from the source:

$$\log_{10} Y = a + bx^p$$

The constants a and b vary independently according to the height and slope of the regression line, respectively, and so indicate the amount of spread and the rate of decrease with distance. This is a robust and simple approach of wide applicability (Gregory and Read 1949; Gregory 1968) that has been used with cocoa swollen shoot (Thresh and Lister 1960) and other diseases. However, use has also been made of double logarithmic transformations of both incidence and distance. Other more complex mathematical approaches are also possible, as discussed by Minogue (1986).

Temporal patterns of spread

Temporal patterns of disease spread are concerned with changes in disease incidence with time. They receive much attention from epidemiologists and for obvious reasons. The amount of disease and the rapidity with which spread occurs within the life span of a crop are important in determining the losses caused. Moreover, such data are required in evaluating the effectiveness of host plant resistance and other approaches to control.

Temporal patterns of spread are apparent from the disease progress curves produced by plotting cumulative incidence against time (Fig. 8). The incidence of virus diseases is usually recorded as the percentage or proportion (P) of diseased plants within the stand being assessed on scales of 0 to 100 (%) or 0 to 1 (P). The most appropriate time scale depends on the nature and longevity of the crop being studied and ranges from days, weeks, or months for short-lived annuals and vegetatively propagated herbaceous crops, to years for shrubs and trees. In collecting data it is necessary to compromise between

the need for frequent observations to follow the details of disease progress and the time and expense incurred in carrying out numerous observations (Nutter 1997).

Disease progress curves are often sigmoid in shape as the incidence of many diseases tends to increase slowly at the outset and also in the later stages of crop growth as crops mature, or when almost all plants become diseased and “saturation” occurs (Fig. 8). Spread is usually most rapid at the intermediate stages of crop growth, as indicated by plotting successive increments of disease against time, which tend to increase to a maximum and then decline.

Disease progress curves merit detailed scrutiny to obtain the maximum possible information from the observations made. The various possible approaches and the mathematical procedures used are discussed in the general epidemiology texts of Vanderplank (1963), Zadoks and Schein (1979), and Campbell and Madden (1990) and in reviews dealing specifically with plant virus diseases (Thresh 1974a, 1983; Nutter 1997). These publications discuss the derivation and utility of the various means of quantifying spread including:

- time of onset (first appearance of disease)
- duration of spread (time: t)
- rate of spread (r)

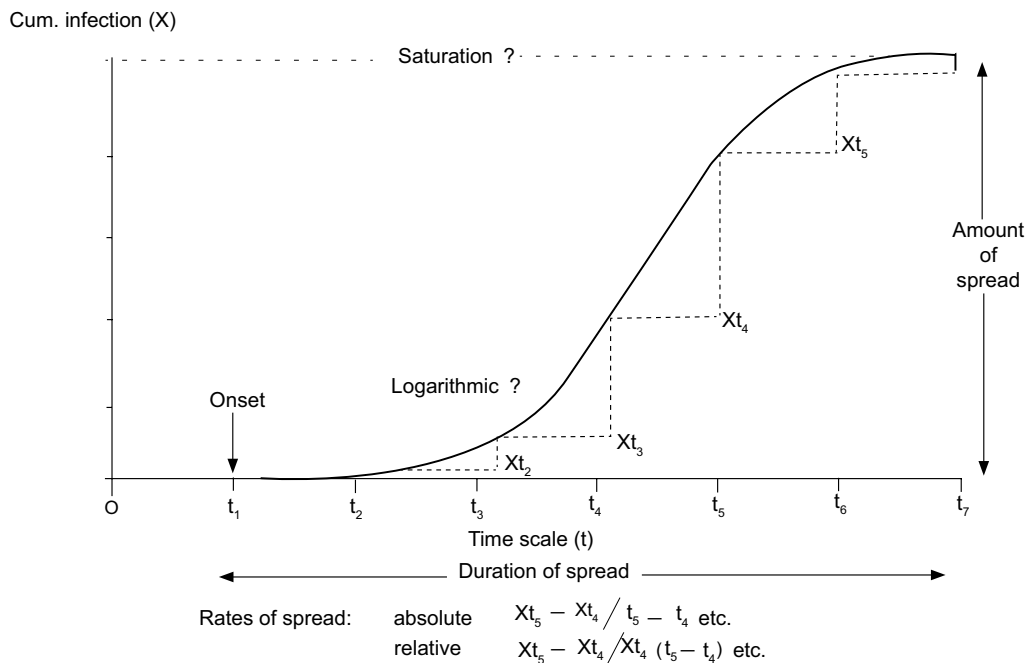


Figure 8. Generalized curve of disease progress obtained by plotting cumulative incidence (x) against time (t) and the derivation of absolute and relative spread.

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- final or maximum disease incidence (x_{max})
- area under the disease progress curve (AUDPC)
- the increment in disease between successive observations
- time to reach 50% disease incidence (t_{50})
- initial disease incidence at the outset of crop growth (x_o)

These criteria have been used widely in studying the temporal dynamics and control of many virus diseases of African crops, ranging from short-lived annuals to long-lived perennials, including several important vegetatively propagated crops.

In the monetary analogy used by Vanderplank (1963), the initial incidence of disease at the outset (x_o) is regarded as the original capital sum invested and r is the rate of interest. The two variables are independent, but interrelated in their effects and both must be considered in evaluating the increase in capital (= incidence) that occurs over the entire investment period (= crop duration: t). Clearly, the greatest disease problems occur when there is rapid spread over a prolonged period and from many initial foci. However, numerous initial foci, or a prolonged period of spread, can lead to high incidences of disease despite low rates of disease progress. All three variables (x_o , r , t) can be manipulated to influence the final outcome and in evaluating control measures it is helpful to distinguish phytosanitation or other means of decreasing x_o and “rate reducing” measures such as host plant resistance, or the use of pesticides to control vectors and so decrease r (Zadoks and Schein 1979; Thresh 1983). It is also important to appreciate that the effectiveness of phytosanitation in decreasing x_o is inversely related to values of r that determine the “explosiveness” of an epidemic (Putter 1980). This is consistent with the finding that the removal of infected plants (roguing) and other approaches to phytosanitation are effective means of controlling slow-spreading diseases such as cocoa swollen shoot, but not rapidly spreading diseases of annual crops (Thresh 1988).

The monetary analogy has been used to clarify the difference between “simple interest” (monocyclic) and “compound interest” (polycyclic) diseases (Vanderplank 1963) (Fig. 9). Monocyclic diseases spread mainly or exclusively from initial sources of inoculum (= starting capital) and the newly infected plants (= interest) do not contribute to further spread (= monetary growth). In contrast, newly affected plants contribute to the spread of polycyclic diseases and there are repeated cycles of infection depending on the duration of the crop and the length of the incubation period, as discussed earlier.

Plant virus diseases are usually polycyclic and cocoa swollen shoot disease provides a well-known African example (Thresh 1958; Vanderplank 1965), but monocyclic diseases have also been reported in sub-Saharan Africa. For example, “kromnek” disease of tobacco is spread into crops by thrips vectors dispersing from infected weed hosts and there is no subsequent spread within crops. This led to the realization that the incidence of kromnek disease is decreased if thinning the stand is delayed until the

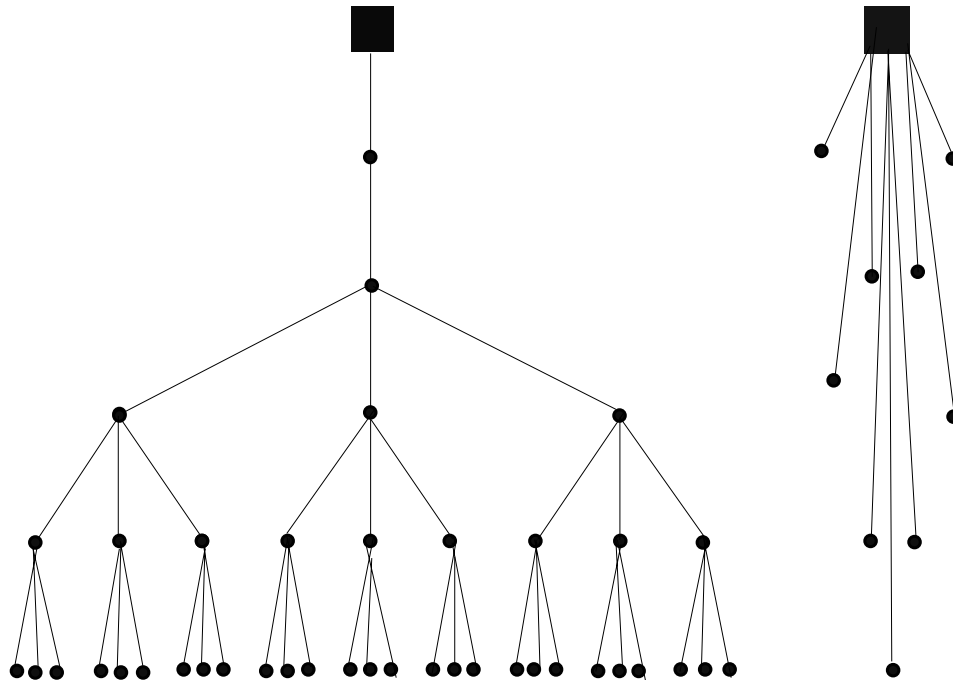


Figure 9. Diagrammatic representation polycyclic (“compound interest”) disease (left) and monocyclic (simple interest) disease (right).

main influx of infective vectors has occurred. Some of the diseased or latently infected plants are then removed as stands are thinned to provide what was referred to as “the mathematical solution to the problem of disease” (Vanderplank and Anderssen 1944; 1945). A ringspot disease of sunflower in eastern and southern Africa also behaves monocyclically as inoculum is carried into crops by aphids from a composite weed host (*Tridax procumbens*) and there is no spread between sunflower plants (Theuri et al. 1987). Maize streak differs in that it seems to behave as a monocyclic disease in some situations and as a polycyclic disease in others (Rose 1974; 1978). The behavior of *Bean common mosaic virus* is also complex as it usually causes a polycyclic disease, but not in stands of bean varieties that develop a severe hypersensitive necrosis when infected and become poor sources of inoculum from which little or no further spread can occur (D. Roose, personal communication). Consequently, the incidence of infection in a hypersensitive variety increases monocyclically at a rate dependent on the extent of infection in nonhypersensitive varieties nearby that act as sources of inoculum to the aphid vectors.

Disease incidence and crop loss

Information on disease incidence and crop loss is required by researchers, policymakers, and donors in order to determine priorities for research and extension projects and to

make effective use of the personnel and other resources available (Barnett 1986). Such information is also required to evaluate the need for and the cost-effectiveness of control measures. However, reliable data that are truly representative are not readily obtained because of the huge scale of crop production in a large continent such as Africa, the great diversity of crops and varieties grown, the wide range of agroecologies utilized, and the big differences in disease incidence that can occur between sites and seasons. This explains the paucity of relevant information on the incidence and effects of many important African diseases, including maize streak, bean common mosaic, groundnut rosette, and rice yellow mottle.

Crop loss information is obtained by comparing the growth and yield of healthy and diseased plants. This can be done in specially planted trials involving controlled inoculations, or the use of infected and uninfected propagules, or by careful sampling of healthy and naturally infected plants within farmers' fields. These approaches have been used widely and with diverse crops. Important results have been obtained as with maize streak and cassava mosaic diseases in many African countries (Van Rensberg 1981; Fargette et al. 1988; Fauquet and Fargette 1990; Thresh et al. 1994; Bosque-Perez et al. 1998). However, it is difficult to assess the overall significance of such findings because the losses are greatly influenced by the virulence of the virus strain(s) present in the infected plants, the sensitivity of the crop variety or varieties grown, and by the soil fertility, cropping practices, and environmental conditions encountered.

Another complication is that the healthy plants within a stand may at least partially compensate for the impaired growth of their diseased neighbors (Otim-Nape et al. 1997). The magnitude of the effect is likely to be influenced by agronomic factors including the spacing adopted, the growth habit of the plants, and the overall fertility of the site. The extent to which compensation occurs will also depend on the distribution of diseased plants and will be less when they occur in patches than when scattered throughout the stand (Hughes 1988). There may also be a critical incidence of disease in a crop stand below which there is little or no effect on overall productivity (Reestman 1970). Consequently, the results of yield trials cannot readily be extrapolated to estimate the losses occurring in a country or region as a whole, although they can be useful in providing a broad indication of the losses sustained, especially if the results of field surveys are also available on the incidence and severity of disease in the principal varieties grown in the main areas of production. For example, the results of crop loss studies and surveys of cassava mosaic disease in several countries of Africa have been used to estimate the annual losses caused by the disease in the continent as 12–23 million tonnes, compared with actual production at the time of 73 million tonnes (Thresh et al. 1997).

Surveys of disease incidence and severity are expensive to carry out and very demanding of time, resources, and expertise. Ideally they should be done throughout

the region being considered and over a sequence of several years to take account of seasonal differences in disease incidence and growing conditions (Barnett 1986). These are exacting requirements that are seldom met and this largely explains the paucity of the information available. However, very detailed and repeated surveys of the distribution of cocoa swollen shoot disease were made throughout the cocoa growing areas of Ghana and elsewhere in West Africa as an essential part of the eradication campaigns mounted after the Second World War (1939–1945) in attempts to eliminate or at least contain the disease (Thresh and Owusu 1986). More recently there have been surveys of the incidence and severity of cassava mosaic disease in Uganda (Otim-Nape et al. 1998; Otim-Nape et al. 2001) and in several of the other most important cassava growing countries of Africa (Thresh et al. 1997; 1998c). These have been undertaken as part of more comprehensive assessments of the status of cassava (Nweke 1994), or its pests and diseases (Wydra and Msikita 1998). Cassava is appropriate for surveys of this type because it is propagated vegetatively and stem cuttings for use as planting material are usually obtained by farmers from the stands being harvested. Consequently, the observed incidence of cassava mosaic disease in new plantings provides a reliable indication of the overall status of the disease in the area, except under epidemic conditions when rapid spread is occurring (Otim-Nape et al. 1998; Otim-Nape et al. 2000).

Similar considerations apply to yam, potato, sugarcane, and sweetpotato and to woody perennials such as cocoa and citrus in which spread is usually slow and the disease situation does not change greatly from year to year. However, annual crops grown from seed present difficulties as the results of surveys will be greatly influenced by the stage of growth and the season when observations are made. Moreover, there can be big differences in disease incidence between years and between seasons and it is difficult to draw meaningful conclusions from the results of a single survey, as evident from experience with groundnut rosette (Naidu et al. 1998) and maize streak diseases (Rose 1978). Both diseases occur sporadically and they can be prevalent in some seasons and almost absent in others. Such issues must be addressed if definitive results are to be obtained on the magnitude of the losses sustained and on possible long-term trends in the prevalence and importance of virus diseases (see later section). Meanwhile, caution is required when interpreting some of the data and statements that appear in the literature in which it is not always stated whether the estimates of crop loss are based on the results of specific field trials or inferred from comprehensive regional surveys.

In evaluating the losses caused by virus disease it is important to consider their effects in restricting the areas that are considered suitable for crop production, or the range of varieties that can be grown reliably and successfully. Such indirect effects can be very

important, even though they are not obvious and may be overlooked or ignored. This is apparent from experience with banana and cassava. With these crops the movement of genotypes and breeding lines has been prevented or severely curtailed by the need for stringent quarantine regulations to restrict virus spread between and within continents. There have also been difficulties in developing diagnostic techniques that are suitably sensitive and reliable for the different cassava mosaic geminiviruses, *Banana streak virus* (BSV) and *Banana bunchy top virus* (BBTV). Special quarantine procedures were developed to overcome the problem posed by BBTV and so facilitate the movement of banana germplasm (Diekmann and Putter 1996). BSV then became a problem and it was found to be very variable and difficult to detect (Lockhart and Olszewski 1993). Moreover, it can be integrated in the host genome and then cause symptoms only when activated by stress factors as yet poorly defined (Ndowora et al. 1999). This behavior has led to further restrictions on the movement of banana material which has impeded germplasm exchange and breeding programs. Quarantine problems have arisen with cassava and partly explain why so little use has been made of African genotypes by breeding programs in South America and India. Moreover, South American genotypes tend to be so severely affected by cassava mosaic disease if grown in Africa that they can only be used after intercrossing with CMD-resistant material. Difficulties have also been encountered in utilizing exotic high yielding varieties of sweetpotato in Africa where they soon succumb to the viruses present there.

Cropping practices and virus spread

There is abundant evidence from different continents of the importance of cropping practices in influencing the incidence and effects of virus diseases and the prospects for control (Thresh 1982). The situation is particularly complex in Africa because of the very diverse crops grown and the wide range of agroecologies utilized. Moreover, rain-fed agriculture as practised over large areas of the continent is increasingly being supplemented by irrigation, using traditional or modern techniques. A further complication is that cropping practices that have been little changed for centuries and depend on human labor or animal traction are still being used widely, together with modern, industrial techniques involving improved varieties, mechanization, pesticides, herbicides, and other innovations (Thresh 1991). This creates difficulties by complicating virus epidemiology and in achieving effective control because measures shown to be suitable for some groups of farmers and their cropping systems may be inappropriate for others.

In assessing the impact of cropping practices on virus spread it is convenient to distinguish between those adopted at or before planting and those deployed later. Some

of the main features to be considered are listed in Table 4, and their importance has long been recognized. This is apparent from experience with groundnut rosette disease in Gambia where the incidence of infection was increased by weeding early (Hayes 1932). Sowing late and at wide spacing were shown to have similar effects in enhancing spread in later trials on groundnut rosette in Malawi, Nigeria, and Uganda and led to the recommendation that farmers should sow early and at close spacing to decrease the risk of infection (A'Brook 1964; Davies 1976, Farrell 1976a). Planting date is also an important factor influencing the spread of cassava mosaic disease as demonstrated in Côte d'Ivoire (Fargette et al. 1994) and Uganda (Adipala et al. 1998).

A detailed consideration of the extensive literature on such effects is beyond the scope of this review. Nevertheless, it is appropriate to consider some of the implications of current trends in crop production. One of the most important of these is the increased use of irrigation to permit crops to be grown at times or in places when or where it would otherwise be difficult or impossible. This has the effect of shortening or eliminating any

Table 4. Cropping practices that influence virus spread.

Preplanting	Site selection: cropping history/isolation field size/shape/orientation/aspect
	Crop/cultivar selection: single/multiple crops single/multiple cultivars seed/vegetative propagules source of propagules
Planting	Sowing/planting: direct planted/transplanted planting/transplanting dates
	Crop spacing/arrangement: plant population in-row/between row spacing
	Pesticide/fertilizer application: at or before planting amount/type
Postplanting	Weed control/tillage:- method/frequency/effectiveness
	Fertilizer: amount/type/timing/method of application
	Thinning/pruning: crop growth stage/extent/method
	Roguing: intensity/timing/frequency/extent
	Irrigation: amount/mode/frequency

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natural break between growing seasons and enables crops to be grown in continuous overlapping sequence through the year. There is enhanced survival of viruses, vectors, weed hosts, crops, and crop debris and virus spread is greatly facilitated, as reported with *Rice yellow mottle virus* in Kenya (Bakker 1974) and more recently in many other parts of Africa. Similar consequences have been reported with maize streak disease in several African countries (Rose 1973; 1978; Fajemisin et al. 1976) and with groundnut rosette in Nigeria (Yaycock et al. 1976). The increased use of irrigation and intensive cropping practices are also likely to accentuate the problems caused by virus diseases in the vegetable crops now being grown increasingly in many parts of sub-Saharan Africa and especially in nurseries established around large urban centers.

Jones (1981) discussed some of the contrasting features of traditional and modern agriculture based on experience with viruses of *Solanum* potato in South America and Europe/North America. Some of the main differences are summarized in Table 5 and similar considerations apply to potato, cereals, cotton, banana, tobacco, and many other crops that are cultivated in different ways in Africa. There is little information on the extent to which the different practices influence the prevalence of virus diseases and the effectiveness of control measures. However, there has been considerable debate on the implications of the trend towards decreased crop diversity as multiple cropping systems are replaced by single crops that may be grown over large contiguous areas and as the numerous landraces of crops such as rice, bean, maize, cowpea, and cassava are displaced by relatively small numbers of specially bred cultivars.

Diversity in the crops and varieties grown is considered to be an important and robust feature of traditional agriculture that provides a substantial degree of resilience and

Table 5. Contrasting features of traditional and modern methods of crop production adapted from Jones (1981) and Thresh (1991).

Feature	Traditional	Modern
Fields	Small, irregular	Large, regular
Crop species	Often intermixed	Usually single
Cultivars	Often intermixed Usually landraces	Usually grown singly Usually specially bred
Propagules	Own-grown or produced locally	Usually specially bred Usually purchased Seldom produced locally
Inorganic fertilizers	Seldom used	Used routinely
Herbicides/pesticides	Seldom used	Often used
Rotations	Much use of bush fallow	Limited use of fallow
Traction	Mainly human/animal	Mechanical

enables farmers to sustain production despite the damage caused by pests and diseases and the vagaries of the weather (Francis 1986; Smithson and Lenné 1996; Wolfe 1985). This is explained by the ability of particular crop species or varieties to withstand pests and diseases or adverse weather conditions and to yield satisfactorily, even though others succumb. Moreover, diverse cropping systems can make the most effective use of the land and other environmental resources available (Fordham 1983).

There is little evidence to support or deny the merits of crop diversity as a means of avoiding virus disease problems because epidemiology trials are usually done with single crops and varieties. Nevertheless, some of the few experiments on intercropping in Africa have provided evidence of beneficial effects in decreasing virus spread. This occurred in Kenya with *Bean common mosaic virus* in bean grown together with maize (van Rheenan et al. 1981) and with cassava mosaic viruses in Bénin, Cameroon, and Côte d'Ivoire when cassava was intercropped with maize, groundnut, or cowpea (Fargette and Fauquet 1998; Ahohuendo and Sarkar 1995; Fondong et al. 1997; 2002). However, the consequences of intercropping are not always consistent or substantial, as noted with *Maize streak virus* in Uganda when maize was interplanted with sorghum or cowpea (Page et al. 1999). It was also apparent from trials in Malawi that field beans suppressed the growth of groundnut intercrops, even though there was a lower incidence of rosette disease compared with groundnut grown alone (Farrell 1976b). These results emphasize the complex effects of intercropping and the need for additional multidisciplinary research on the epidemiological, agronomic, and socioeconomic issues involved if clear and acceptable recommendations are to be made for use by farmers.

There is even less information on the implications of the trend away from the traditional use of varietal mixtures and the adoption of a relatively small number of improved genotypes. However, it was observed recently in Uganda that cassava mosaic disease was more damaging in areas where only a single variety predominated than where many varieties were being grown (Otim-Nape et al. 2001). It was also demonstrated in field trials in Uganda that cassava mosaic disease spread less rapidly to a susceptible variety grown with resistant ones as a mixture than when the susceptible variety was grown alone (Sserubombwe et al. 2001). These results indicate the potential benefits of varietal mixtures in decreasing virus spread, but further research is required on this and the whole range of agricultural practices. Such studies merit high priority and require close collaboration with agronomists, socioeconomists, and plant breeders if modern cropping systems are to be developed that possess the apparent stability and resilience of traditional systems.

Forecasting

The possibility of forecasting the prevalence of virus diseases and the losses that will occur at harvest or at crop maturity, has received considerable attention from epidemiologists (see review by Thresh 1986). Much of the relevant research has been on temperate crops, but early studies by Storey and Bottomley (1928) established a positive relationship between the final incidence of groundnut rosette disease in Natal, South Africa, and the total rainfall during the preceding dry season. This influenced the survival of “volunteer”, self-grown groundnut seedlings and crop debris and hence the abundance of sources of inoculum from which the aphid vector (*Aphis craccivora*) could transmit rosette viruses. Such information is important in indicating the need to adopt virus-resistant varieties or other control measures. An ability to forecast disease incidence is also an indication that the main features of the pathosystem have been elucidated and that the most important details of the epidemiological cycle have been determined.

Other positive relationships between disease incidence and pre-season rainfall that influence the prevalence of vectors and inoculum sources have been established with cotton leafcurl disease in Sudan (Boughey 1947) and maize streak disease in Zimbabwe (Rose 1972a). Data on the probability of cocoa swollen shoot disease spreading to neighboring trees has also been used to evaluate and modify the eradication measures being used to treat outbreaks in Nigeria (Thresh and Lister 1960) and later in Ghana (Thresh and Owusu 1986). The strategy developed, recommended, and eventually adopted was based on the size of the outbreak being treated and the estimated probability of latent infection in the adjoining symptomless trees. More recently, observations on the progress of the 1990s epidemic of a particularly severe form of cassava mosaic disease in Uganda have been used to anticipate the threat to neighboring countries and the need to build up stocks of virus-resistant varieties for release to farmers (Legg 1999). These examples indicate the potential value of being able to forecast disease spread and emphasize the need for additional studies on the diseases of a wider range of crops and in different environments.

The ecological approach

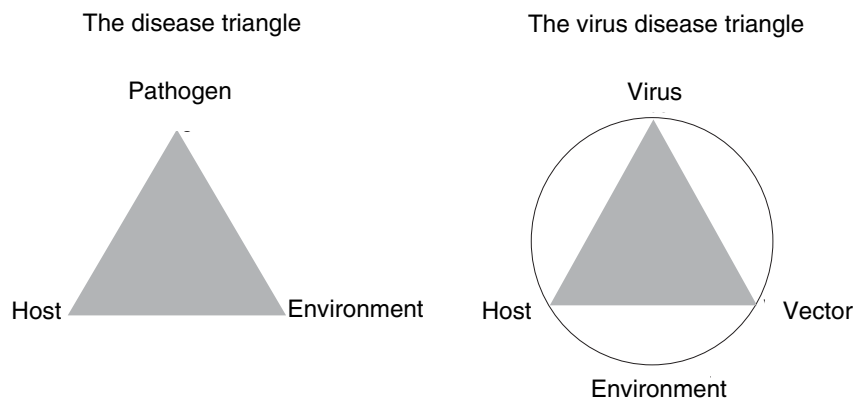
Plant pathologists have made considerable use of the so-called “epidemiological triangle” to facilitate an understanding of the complex interrelationships between pathogens, hosts, and their environment (Fig. 10a). With many plant virus diseases, this approach must be modified because of the involvement of animal or fungal vectors (Fig. 10b). This led to the concept of the “ecological trinity” of viruses, hosts, and vectors within the overall context of the environment. The concept was developed by the American vector entomologist Walter Carter from his experience with *Yellow leaf*

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spot virus disease of pineapple and its thrips vector in Hawaii (Carter 1939) and from his previous studies on *Sugarbeet curly top virus* disease and the sugarbeet leafhopper vector in the southwest states of USA (Carter 1930). With both these diseases, it was necessary to consider the interactions of viruses and vectors with crops, weeds, and wild hosts and the overall influence of cropping practices and other environmental factors in creating the conditions for epidemics to occur. This necessitated a holistic ecological approach, as adopted in subsequent studies on cocoa swollen shoot disease in Ghana and elsewhere in West Africa.

The need for such an approach with cocoa swollen shoot disease became apparent following the detection of wild indigenous tree hosts of the causal virus and its mealybug vectors and by the finding that there are several vector species that occur in association with more than 120 other insect species. They include 75 ant species, 16 Hymenoptera species, and three species of predatory beetle (Strickland 1951; Tinsley 1964). The role of ants was shown to be particularly complex and important in influencing vector populations and virus spread because some ant species are antagonistic to mealybugs, whereas others protect and tend them. Moreover, the competing groups of ants occupy distinct territories that form a continually changing mosaic (Leston 1971).

An ecological approach can provide valuable insights into the role and status of viruses, hosts, and vectors. Crop hosts can be viewed in ecological terms as apparent (easily found) or nonapparent (cryptic) species, depending on their longevity, size, and other growth characteristics. These features influence the ease with which plants are located and colonized by arthropod vectors and the need for chemical or other host defence mechanisms. Apparent species include trees and shrubs, especially if these occur



Figures 10a and b. The “epidemiological triangle” of host, virus, and environment (left), and when modified to take account of vectors (right).

widely and in large continuous stands. Nonapparent species are typified by herbaceous hosts and these are often ephemeral and restricted to particular sites or seasons.

Similar considerations explain why crops can be regarded as providing either transient or stable “island” habitats that are invaded readily or with difficulty, depending on their size, stability, and degree of isolation and on the mobility of the vector. Concepts derived from biogeography and from practical and theoretical studies on the colonization of islands by plant and animal species are relevant in plant virus epidemiology (Thresh 1980a). It is also advantageous to consider the evolutionary history and origin of viruses, vectors, and hosts and whether they are indigenous or exotic species. Important crops that have been introduced to Africa include maize, groundnut, cocoa, sweetpotato, cassava, and Asian rice (*Oryza sativa*), whereas indigenous crops include yam, sorghum, millet, cowpea, and African rice (*O. glaberrima*). Such considerations determine whether crops have had long or short periods of coexistence and coevolution (Buddenhagen 1977). The merits of adopting this approach and the insights provided are considered further in the following section.

The equilibrium concept

The plant pathology literature contains numerous references to damaging outbreaks, severe epidemics, and serious losses (Thurston 1973; Klinkowski 1974; Thresh 1980b, 1991; Bos 1992; Rybicki and Pietersen 2000; Morales and Anderson 2001). Such reports are notable and understandable, especially as researchers are now enjoined to provide strong justification for their applications for funds and are expected to demonstrate the practical relevance and benefits to farmers of the studies proposed. However, the inevitable “professional pessimism” of the plant pathologist can give a somewhat exaggerated, biased, and misleading impression of the magnitude of the problems caused by viruses and other pathogens. Severe epidemics undoubtedly occur and cause serious losses, but usually they can be regarded as infrequent or even rare events that are often restricted to certain crops, areas, and seasons. Otherwise crops generally are seldom severely affected, production is not seriously impaired, and virus diseases are but one of many constraints that must be addressed if productivity and overall yields are to be increased.

As discussed earlier, there is so little information on the prevalence of African diseases and the losses they cause that the validity of this relatively optimistic proposition can be regarded as contentious, unproven, and unsustainable. Nevertheless, it merits detailed debate and scrutiny, not least because of the insights it can provide into the role of cropping systems and breeding strategies in contributing to disease problems and also to their solution. If damaging outbreaks are indeed exceptional or even rare events, then

this implies that there are usually effective constraints that impede their occurrence and restrict further spread. Clearly, it is important to identify any such constraints if they are to be sustained, manipulated, exploited, and even enhanced to improve crop health and so increase productivity. Moreover, it is also important to identify the underlying causes of epidemics in attempts to facilitate their control and to prevent any recurrence by changing the cropping systems being used.

In considering these issues it is appropriate to adopt an ecological, evolutionary approach, as discussed previously. The main features of crops and other plants that limit their vulnerability to viruses are illustrated in Figure 11. This emphasizes the role of three attributes or mechanisms that operate singly or in combination: (i) evasion, (ii) resistance to virus infection or vectors, and (iii) tolerance of infection. Evasion can be in time or in space and operates if plants escape infection because they grow at times or in places when/where viruses or their vectors do not occur, or when/where they are seldom a serious problem. Clearly this is more likely to occur with transient, sparsely distributed species growing seasonally and in mixed stands with nonhost plants and weeds than it is with long-lived species that are grown widely, throughout the year and in stands of a single species or variety subject to rigorous weed control. Moreover, the situation in nature is dynamic in that there are usually big seasonal differences in inoculum pressure and a period of increased disease pressure can be expected to lead to a decrease in host prevalence, or in the proportion of susceptible genotypes and so to an eventual relaxation of disease pressure. Host prevalence or the proportion of susceptibles is then likely to increase, facilitating a resurgence of disease and leading

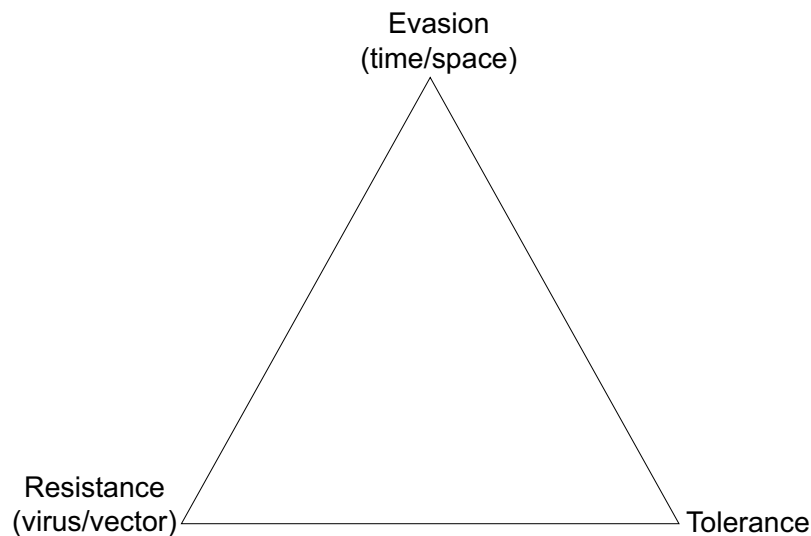


Figure 11. Diagrammatic representation of the ways in which plants avoid the harmful effects of plant viruses by some combination of resistance, tolerance, and evasion in time or in space.

to continuing cycles of increasing and decreasing prevalence (Buddenhagen 1977). This type of cyclical behavior is largely inimical to modern methods of crop production. However, as discussed earlier, the full implications of modern trends in cropping practices are difficult to evaluate because of the limited information on their effects on virus spread and on the losses caused.

Resistance to or tolerance of infection is an alternative supplement or complement to evasion as an effective means of avoiding the detrimental effects of diseases. Many types of resistance have been described and they can be effective against viruses (Fraser 1987) or their vectors (Jones 1987). Mature plant resistance, as discussed earlier, and the apparent immunity of any one plant species to all but a small number of viruses are distinct and epidemiologically important characteristics that have not been fully explained. Other types of resistance include those associated with gene-for-gene relationships between host and pathogen, as reported with *Bean common mosaic virus* (Drijfhout 1978). There is also hypersensitivity, other types of resistance due to major genes, and the quantitative “rate reducing” resistance, as described with several viruses including *Cacao swollen shoot virus* (Thresh et al. 1988) and cassava mosaic geminiviruses (Thresh et al. 1998a). Tolerance of infection is quite different and, in the strict sense of the term, is the ability of plants to grow and yield satisfactorily despite sustaining a virus content that causes serious damage in sensitive varieties (Clarke 1986). However, the term is also used more widely for the ability of plants to withstand infection irrespective of virus content (Posnette 1969).

A limitation of both resistance and tolerance to viruses and other pathogens is that they may be overcome by new or more aggressive pathogenic strains; “breakdown” of resistance to virus vectors has also been reported. Moreover, varieties that are resistant in one region or country may succumb to the same disease elsewhere, as reported with sweetpotato. Thus, host plant resistance is not necessarily durable or universal and there are examples of large decreases in virus incidence due to the introduction of resistant varieties that have been followed some years later by a resurgence as new virus strains or vector biotypes have appeared and become prevalent. Examples include *Sugarcane mosaic virus* and *Tomato mosaic virus* and the breakdown of resistance to the main leafhopper vector of rice tungro viruses (Thresh 1989). This emphasizes the labile nature of the situation illustrated in Figure 12, which portrays a dynamic equilibrium in which neither host nor pathogen gains permanent ascendancy.

The concept of an equilibrium between hosts and their pathogens provides a convenient and appropriate basis from which to evaluate the impact of agriculture and the adoption of intensified cropping practices. Clearly, the ability of plants to avoid or withstand diseases when growing in natural habitats is undermined when they are

cultivated as crops, especially when grown in dense stands, in extensive monocultures, at the same sites in successive years, and with effective weed control and much use of inorganic fertilizers. The situation is further exacerbated by the use of irrigation or other means of extending the natural growing season to facilitate crop production throughout the year (Thresh 1982). There has also been a tendency to decrease the genetic diversity of crops by selecting a small number of high yielding genotypes for use on a large scale (Thresh 1980b, 1982; Bos 1992). Moreover, crops and varieties have been transferred to entirely new areas where they have been affected by novel pathogens or more virulent strains of viruses than those encountered previously. The increased traffic in plant material has also facilitated the dissemination of viruses and their vectors and imposed exacting demands on quarantine controls that have been difficult to implement effectively.

These developments have led some to the view that disease epidemics and problems due to pests seldom occur in undisturbed natural ecosystems; that they are largely the outcome of agricultural practices; and that they are increasing as traditional methods are abandoned, cropping systems become increasingly specialized, and there is increased traffic in plant material (Thresh 1980b; Bos 1992). There is insufficient evidence to confirm or deny these suppositions. Nevertheless, many of the epidemics reported in recent decades can be interpreted as major perturbations of previously stable equilibria as a consequence of changes in the crops grown and the method of cultivation adopted. Cocoa swollen shoot, cassava mosaic, groundnut rosette, and maize streak are all prime examples of “new encounter” diseases in the sense of Buddenhagen (1977). They have occurred following the transfer of their hosts from the New World to Africa, where they have been severely affected by viruses that were already present in indigenous

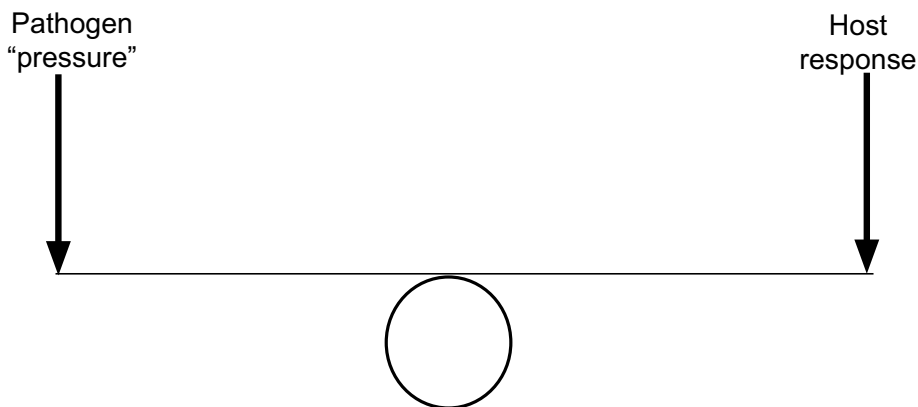


Figure 12. Diagrammatic representation of the dynamic equilibrium between pathogen “pressure” and host response.

hosts. Moreover, the problems caused by some of these and other diseases have been accentuated by the cropping practices adopted and the extensive use of particularly vulnerable genotypes. For example, when cocoa was first established in Ghana and elsewhere in West Africa, it was grown in many small farms that were usually heavily shaded by forest trees. There was seldom any separation between farms and they were often established alongside or even beneath indigenous tree hosts (Thresh et al. 1988). The spread of cocoa swollen shoot disease was also facilitated by the almost exclusive use of the South American Amelonado variety of cocoa. Initially, this grew well and seemed ideally suited to conditions in West Africa. The situation changed in the 1930s as swollen shoot disease became a problem and the extreme vulnerability of Amelonado became apparent. It was necessary to adopt more resistant varieties (Thresh et al. 1988).

More recently the widespread adoption of the Ebwanateraka variety of cassava in Uganda has contributed to the 1990s pandemic of cassava mosaic disease. This is associated with a particularly virulent strain of the causal virus that is considered to be a novel recombinant of two different cassava mosaic geminiviruses (Deng et al. 1997; Harrison et al. 1997; Pita et al. 2001; Zhou et al. 1997). Other perturbations of this type are likely to occur and it seems inevitable that there will be problems with other whitefly-borne viruses as a consequence of the recent introduction to Africa of the B biotype of *Bemisia tabaci* (Bedford et al. 1993). This has a wider host range than the biotypes already present and is associated with recent virus epidemics in tomato, beans, and other crops in the Americas (Brown 1994, 2000; Morales and Anderson 2001) and India (Banks et al. 2001). Other viruses and vectors that already occur elsewhere could also be introduced to Africa. This emphasizes the importance of quarantine measures and the need for epidemiological studies to monitor the continually changing situation.

Viruses, virus strains, and molecular epidemiology

Epidemiological data are usually obtained by assessing the incidence of diseased plants as apparent from visual assessments at one or more stages of crop growth. It is seldom feasible to test for the occurrence of the virus or viruses responsible, except in small samples of representative plants to check the validity of the diagnoses being made on the basis of symptoms. This is because suitable methods of virus detection that are robust, reliable, and able to achieve the large throughput required are seldom available and would be prohibitively expensive and time-consuming to adopt. Similar considerations explain why it is seldom possible to determine the strains of virus that are encountered in epidemiology trials and field surveys, except where special attention is given to these issues, as with *Bean common mosaic virus* (Spence and Walkey 1994).

These are important constraints and limitations, especially in sub-Saharan Africa where there is a general lack of laboratory facilities and trained personnel.

The epidemiological importance of virus strains has long been apparent, as in the early studies on groundnut rosette, cassava mosaic, and cocoa swollen shoot diseases (Storey and Bottomley 1928; Hayes 1932; Storey and Nichols 1938; Crowdy and Posnette 1947). In these and other studies, distinct virus strains were recognized from the type and severity of the symptoms expressed by the crop host and some virus strains were shown to be more damaging than others. Moreover, the virulent 1A (New Juaben) strain of *Cacao swollen shoot virus* (CSSV) spread more rapidly than two other less virulent strains of the virus when compared in a replicated field trial in Ghana (Crowdy and Posnette 1947).

There have since been many other reports of differences between virus strains, on the interactions between them, and on their epidemiological importance. For example, avirulent forms of the 1A strain of CSSV that occur naturally in Ghana will protect against many of the most damaging virulent strains and provide a possible means of control (Posnette and Todd 1955; Hughes and Ollennu 1994). There have been similar findings with *Citrus tristeza virus* (van Vuuren et al. 1993). Moreover, there are strains of *Sugarcane mosaic virus* (SCMV) and *Bean common mosaic virus* (BCMV) that can infect and damage varieties selected as being resistant to other virus strains (Spence and Walkey 1994). Such results emphasize the importance of strain variation as a crucial feature of the epidemiological competence of viruses that enables them to respond, adapt, and persist, despite the sometimes big changes in physical and biotic environment and in the crops and varieties being grown.

Initially, virus strains were distinguished by the symptoms they cause in crop plants or when inoculated to specially selected indicator hosts. This led to the selection of a range of differential varieties to distinguish strains, as with SCMV, BCMV, *Tomato mosaic virus*, *Soybean mosaic virus*, and many others. Much use has also been made of other approaches to strain identification including serological techniques using polyclonal antisera or, more recently, monoclonal antibodies (MABs). Serology provides a convenient means of testing many samples and has provided important information on the occurrence and variability of many African viruses, including those causing rice yellow mottle and cassava mosaic diseases (N'Guessan et al. 2000; Swanson and Harrison 1994). Originally, cassava mosaic disease was assumed to be caused by a single whitefly-borne geminivirus, although different strains were distinguished using polyclonal antisera (Bock and Harrison 1985). Three distinct cassava mosaic geminiviruses were recognized in later studies with MABs and shown to have distinct, largely nonoverlapping distributions in West/Central Africa, East Africa, and the Indian

subcontinent (Swanson and Harrison 1994). The validity and status of the three viruses was confirmed by PCR and DNA sequencing (Hong et al. 1993), but they are now known to be more widespread than suggested by the initial findings. Both *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) have been detected in West Africa and also in East Africa and they sometimes occur as mixed infections. Moreover, other cassava mosaic viruses have been distinguished recently in South Africa (Berrie et al. 1998) and Zanzibar (Maruthi et al. 2001) and recombinant strains having properties of both ACMV and EACMV have been distinguished in Uganda and adjoining countries and associated with the pandemic now affecting the Great Lakes region of East Africa and the Democratic Republic of Congo (Deng et al. 1997; Harrison et al. 1997; Zhou et al. 1997; Legg 1999; Pita et al. 2001; Neuenschwander et al. 2002).

The experience with cassava mosaic disease has shown the value of biochemical techniques that enable the detailed characterization of the viruses involved. Other examples of this are groundnut rosette and banana streak diseases. The latter was first reported in Côte d'Ivoire and Morocco and it is now known to occur in many countries of sub-Saharan Africa and elsewhere in the tropics. The disease is caused by DNA containing viruses that are so variable that it has been difficult to develop reliable methods of detection based on serology or PCR (Lockhart and Olszewski 1993). Moreover, it has been shown recently that sequences of viral DNA can be incorporated within the host genome and that these can be activated under certain conditions to produce episomal virus and cause typical streak symptoms (Ndowora et al. 1999). This creates problems in epidemiology and in developing appropriate control measures because it is necessary to distinguish between an integrated virus that has been activated by stress factors from that already present in vegetative propagules or introduced by the mealybug vector. Studies are now in progress to resolve these issues, to determine the factors responsible for activation, and to devise appropriate control measures and quarantine procedures to enable the safe movement of *Musa* germplasm for breeding and crop improvement programs.

Complexity of a different type has become apparent in molecular studies on groundnut rosette disease. It has long been known that the aetiology of rosette is complex and that a distinct assistor virus is required for the virus that causes rosette symptoms to be transmitted by the aphid vectors (Hull and Adams 1964). More recent studies have established the role of a satellite RNA in causing rosette symptoms (Murant et al. 1988) and in facilitating the transmission of *Groundnut rosette virus* (GRV) by vectors (Murant 1990). Moreover, techniques have been developed to assay each of the three

components of the pathosystem and they are now being used in epidemiology and to assess the behavior of rosette-resistant varieties of groundnut (Naidu et al. 1998, 1999a, 1999b). It is already apparent that the *Groundnut rosette assistor virus* (GRAV) can occur alone in rosette-resistant and other varieties and that such plants remain symptomless. Furthermore, GRAV is not always present in diseased plants containing both GRV and satellite RNA. Such plants develop rosette symptoms, but GRV is not transmissible by aphids in the absence of GRAV and so behaves monocyclically.

These examples indicate how the molecular analysis of virus isolates can yield information that facilitates an understanding of epidemiology and have led to the term “molecular epidemiology” (Garcia-Arenal et al. 2000). It is now apparent that the spatial and temporal structure of virus populations is critical in epidemiology and that different strains of a given virus species may induce epidemics having very different characteristics. Accordingly, molecular techniques are being used increasingly in tropical plant virus epidemiology, as elsewhere in virology, to facilitate the characterization of virus populations and strains, and to elucidate virus/vector relationships. Such techniques are also being applied to study the interactions between viruses and strains to determine their origins and epidemiological effects. Molecular epidemiology complements, supplements, and refines earlier information obtained through serological and biological tests. This leads to a better characterization of epidemics, of the spatial and temporal patterns of spread, and of the mode of spread; all topics considered earlier and which require a precise characterization of the virus populations involved.

Mutations accumulate with time in the genomes of pathogens and particularly with viruses, because many lack fidelity during replication. Some mutations confer phenotypic differences, such as enabling the virus to infect different host species, or to be transmitted by different routes, while others can be used to make inferences on the history of epidemics. The rapid progress in techniques of gene sequencing that has occurred recently makes it possible to produce numerous sequences quickly and conveniently. One of the aims of molecular epidemiology is to collate and analyze this information in order to reconstruct the history of a pathogen’s spread through host populations and to predict future developments. Phylogenetic studies elucidate the relationships between strains, whereas such information was not accessible, or could only be interpreted broadly and indirectly, using earlier serological and biological data.

The information now being obtained broadens the scope of earlier “classical” epidemiology as evolutionary features which are highly relevant to crucial unresolved aspects of the ecology of plant viruses can now be considered and elucidated. This approach reveals geographic, climatic, and biological correlates in the structure of phylogenetic trees which provide insights into epidemiological processes and

the history of epidemics (Harvey et al. 1996; Page and Holmes 1998). Accordingly, the epidemiological and aetiological characteristics of several virus groups have been mapped onto their phylogeny to reveal striking correlations between the topological arrangements of the viruses and their epidemiological characteristics. Much progress has been made already in medical and veterinary studies (Harvey et al. 1996), and it is inevitable that plant virus epidemiology will follow a similar trend (Harrison and Robinson 1988; Gibbs et al. 1995, 1999). For instance, the quasi-species structure of a virus population, its spatial and temporal variation, recombination phenomenon, and virus interactions are critical for an understanding of several of the key epidemiological aspects considered in this text. They include the emergence of new epidemics, virus evolution, plant–virus coevolution, and host switching. Ultimately, the correlation between epidemiology, disease spread, and biogeography will begin to define the complex evolutionary relationships between viruses, vectors, and plant hosts and the ecological niches they exploit (Gaunt et al. 2001).

Discussion

The epidemiology of plant viruses in the tropics, and in sub-Saharan Africa in particular, has a long history and features prominently in the plant pathology literature (Thresh 1991). Indeed, experience gained in South Africa was used by Vanderplank (1946) to develop his “doublet” test of contagion to analyze patterns of disease spread and also to provide a “mathematical solution to a problem of disease”. The concept of “crowd” diseases was also developed from experience with those such as cocoa swollen shoot disease that do not spread quickly or far in any considerable amount (Vanderplank 1948; Thresh et al. 1988). Moreover, this and several other African viruses feature in the seminal and highly influential text *Plant Disease Epidemiology* (Vanderplank 1963). This prominence is entirely appropriate because of the importance of agriculture in Africa and the severity of the damage caused by virus diseases in many subsistence and export crops. However, it should be appreciated that there is totally inadequate information on the prevalence and distribution of many of the plant viruses known to occur in Africa, on the losses they cause, and on their means of spread. Indeed, there are likely to be many viruses in Africa as yet undiscovered and there is detailed epidemiological information on few of the viruses known to occur. One reason for this is that many of the studies that have been undertaken have been of limited duration and restricted to specific agroecologies that are not always truly representative.

These are important limitations and the information available is seldom adequate to mount and sustain effective control measures. The problems that arise are apparent from experience with the two most important virus diseases of cassava in Africa.

Cassava mosaic disease (CMD) has been known since 1894 and arguably it has received more attention than any other virus disease of an African food crop (Thresh 1991). Nevertheless, the information available in the early 1990s was not sufficient to explain the very damaging regional pandemic that was first reported in Uganda in 1988, or to provide an effective means of control (Thresh et al. 1994; Otim-Nape et al. 2000). This has necessitated much additional research and led to important new findings on the whitefly vector and on the nature and distribution of the geminiviruses responsible and on the interactions between them. Nevertheless, many uncertainties remain on the causes of the current pandemic in East Africa, on the distribution and implications of the different cassava mosaic geminiviruses now known to occur, and on the epidemiological significance of the recent observations that the whitefly vector (*Bemisia tabaci*) breeds more rapidly on CMD-affected plants than on healthy ones (Colvin et al. 1999) and that an apparently distinct race of *B. tabaci* was associated with the onset of the epidemic in Uganda (Legg et al. 2002). There is also a need to resolve the long-standing uncertainty concerning the need for phytosanitation if CMD-resistant varieties are adopted (Thresh et al. 1998b). One view is that phytosanitation is unnecessary if the varieties used are sufficiently resistant, whereas the counter argument is that the two approaches are complementary and should be deployed together.

Cassava brown streak disease has been relatively neglected compared with cassava mosaic and there is inadequate information on its distribution, effects, and mode of spread. Moreover, brown streak disease has been recognized only recently in Mozambique, even though it is prevalent in large populous areas of the country and undermines food security (Hillocks et al. 2002). There is no explanation as to why the disease seems to be confined to southern and eastern Africa, or why it seldom occurs at altitudes exceeding 700 m above sea level (Hillocks et al. 1999). The original, indigenous host from which brown streak is assumed to have spread to cassava after the crop was introduced to Africa has not been determined and the putative insect vector is not known.

There is similar uncertainty over the origins and indigenous hosts of the viruses responsible for groundnut rosette disease and no information on the source(s) of inoculum from which spread occurs to groundnut crops in areas where there is such a prolonged dry season that volunteers and other sources of inoculum do not persist between growing seasons (Naidu et al. 1998). Moreover, there is a need to assess the epidemiological significance of the early observation that infection of groundnut with rosette disease enhances the production of winged (alate) forms of the aphid vector (Rèal 1955), as this can be expected to enhance spread. Additional studies are also required on *Rice yellow mottle virus* because it is still unclear whether the main means of spread is mechanical or by beetle vectors and there are conflicting claims on the relative importance of the two processes (Abo et al. 2000).

These and the many other similar deficiencies of current knowledge will not easily be overcome because epidemiology is an exacting science and there are few, fully trained

practitioners, especially in Africa where virologists seldom have the opportunity to specialize in particular crops or diseases. Furthermore, experience has shown the merits of a multidisciplinary approach involving not only virologists but also those concerned with vectors who require accurate diagnostics and access to modellers, biometricians, plant breeders, and other specialists. Such teams have operated at various times and places but for restricted periods in studies on only a few particularly important African diseases, including cocoa swollen shoot, maize streak, groundnut rosette, and cassava mosaic. This explains why these few diseases feature so prominently in this review. However, multidisciplinary teams are not easily assembled or sustained, because they require a substantial commitment of funds over a prolonged period in order to overcome the inevitable difficulties and vagaries of field experimentation and the need for studies in different agroecologies and in contrasting seasons to take account of climatic and other variables.

These factors explain why recent efforts in Africa have been limited, sporadic, and largely funded by outside donors. National programs seldom have the resources required to undertake such studies, and at international agricultural research centers, the main emphasis in plant virology has been on diagnosis and to support resistance breeding projects, rather than on epidemiology. Moreover, with few exceptions, the emphasis of collaborators in advanced laboratories has been on virus characterization and aetiology and more recently on biochemical and biotechnological aspects. Collaboration between African scientists and those in Europe or North America is easier on these topics than on field-based studies and this explains why so little of the training provided to the African PhD students who study outside the continent is concerned with epidemiology. A major change of attitude will be required if progress is to be made in solving the many intractable problems that remain and in enabling the introduction, evaluation, and considered use of transgenic sources of resistance in a sustainable and environmentally sound manner. Only then will it be possible to develop for use in Africa control measures that are comparable in effectiveness to those that have made such a big contribution to food production in temperate regions and in Asia and South/Central America. The challenge is to obtain a basic understanding of virus epidemiology so that effective and sustainable control measures can be developed that enable agriculturalists and horticulturalists to exploit fully the advances being made in other aspects of crop technology. Moreover, they should be able to do so despite decreasing soil fertility and a decline in the rural workforce and without damaging the environment. It is also important to avoid undue reliance on the use or misuse of pesticides or other practices that have caused such concerns elsewhere. These are exacting requirements, but it is important that they are fulfilled if agricultural production in Africa is to meet the continually increasing needs

of a burgeoning human population despite the expected reduction in the rural workforce due to AIDS and movement to urban centers.

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