COMPARATIVE PLANT VIROLOGY

SECOND EDITION

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CHAPTER

13

Plant Viruses in the Field: Diagnosis, Epidemiology, and Ecology

Plant viruses are a significant agricultural problem and cause major losses. In any disease situation it is important to know what virus is causing the problem, where it comes from, and how it spreads before developing control measures.

OUTLINE

I. DIAGNOSIS

A. Introduction

There are three basic situations in which the techniques for recognising and identifying a virus are needed: diagnosis of a viral infection in the field to determine whether it is caused by a known or unknown virus; detection of a known virus, usually in an epidemiological or quarantine situation; and assay for a known virus—for instance, when purifying or manipulating it.

When appraising the relative merits of different methods, the following important factors must be considered:

- What question is being addressed? Is one just determining if the plant is virus infected, what virus is infecting the plant, or what strain of the virus is infecting the plant?

- Sensitivity: How small an amount of virus can be measured or detected?
- Accuracy and reproducibility
- Numbers of samples that can be processed in a given time by one operator
- Cost and sophistication of the apparatus and materials needed
- The degree of operator training required
- Adaptability to field conditions

It must always be remembered that diseased plants in the field may be infected by more than one virus. Thus, an early step in diagnosis for an unknown disease must be to determine whether more than one virus is involved. The methods involved in assay, detection, and diagnosis can be placed in four groups according to the properties of the virus on which they depend: biological activities, physical properties of the virus particle, properties of viral proteins, and properties of the viral nucleic acid.

B. Methods Involving Biology of the Virus

Biological methods for the assay, detection, and diagnosis of viruses are much more time-consuming than most other methods now available. Nevertheless, they remain very important. For diagnosis, in most circumstances only inoculation to an appropriate host species can determine whether a particular virus isolate causes severe or mild disease. However, this group of methods does have a major drawback, especially when used on a new virus or in quarantine situations. It raises the possibility that the production of infected plants might be a source of infection for local crops even in the face of the strictest containment conditions.

1. *Indicator Hosts*

Disease symptoms on plants in the field are frequently inadequate on their own to give a positive identification. This is particularly so when several viruses cause similar symptoms, as do yellowing diseases in beet; when a single virus, such as *Cucumber mosaic virus*, is very variable in the symptoms it causes; or when both of these factors are relevant in a single host. Thus, since the early days of plant virology, searches have been made for suitable species or varieties of host plant that will give clear, characteristic, and consistent symptoms for the virus or viruses being studied, usually under glasshouse conditions. Many good indicator species have been found in the genera *Nicotiana, Solanum, Chenopodium, Cucumis, Phaseolus, Vicia*, and *Brassica*. Certain plant species such as *Chenopodium amaranticolor, C. quinoa,* and *Nicotiana benthamiana* react to a wide range of viruses.

Testing large numbers of samples using indicator hosts requires glasshouse facilities that may be occupied for weeks or longer. However, the actual manipulations involved in mechanical inoculation may take less time per sample than many other methods of testing, and many attempts have been made to streamline the procedures for sap extraction and inoculation. As just noted, care must be taken to keep inoculated test plants under suitable containment to minimise escape of the virus(es) to the outside environment.

2. *Host Range*

Host range is an important criterion in diagnosis. However, there are various factors that must be considered:

- In many of the reported host range studies, only positive results have been recorded.
- Absence of symptoms following inoculation of a test plant has not always been followed by back inoculation to an indicator species to test for masked infection.
- The manner of inoculation may well affect the results. Mechanical inoculation has almost always been used in extensive host range studies because of its convenience, but many plants contain inhibitors of infection

that prevent mechanical inoculation to the species, or from it, or both.
- In studying large numbers of species, it is usually practicable to make tests only under one set of conditions, but it is known that a given species may vary widely in susceptibility to a virus, depending on the conditions of growth.
- Quite closely related strains of a virus may differ in the range of plants they will infect. Host range data may apply only to the virus strain studied.

3. *Methods of Transmission*

The different methods of virus transmission, discussed in Chapter 12, may be useful diagnostic criteria. Their usefulness may depend on the particular circumstances. For example, a virus with an icosahedral particle that is transmitted through the seed and by nematodes is very probably a nepovirus. On the other hand, such a virus transmitted mechanically and by the aphid *Myzus persicae* might belong to any one of several groups.

4. *Cytological Effects*

Cytological effects (see Chapter 2) detectable by light microscopy can sometimes be used effectively to supplement macroscopic symptoms in diagnosis. The light microscope has a number of advantages when the inclusions are large enough to be easily observed:

- It is a readily available instrument.
- Specimen preparation techniques can be simple.
- There is a wide field of view, thus allowing many cells to be readily examined.
- A variety of cytochemical procedures are available.

5. *Mixed Infections*

Simultaneous infection with two or even more viruses is not uncommon. Such mixed infections may complicate a diagnosis based on biological properties alone, especially if the host response is variable. However, several possible differences in biological properties may be used to separate the viruses:

- If one virus is confined to the inoculated leaf in a particular host, while the other moves systemically
- If a host can be found that only one of the viruses infects
- If the two viruses cause distinctive local lesions in a single host
- If the two viruses have different methods of transmission—for example, by different species of invertebrate vectors

On the other hand, difficulties may arise in sorting out certain diseases in the field that result from a more or less stable association between two or more viruses. For instance, groundnut rosette disease is caused by two viruses and a satellite (see Box 2.1).

C. Methods That Depend on Physical Properties of the Virus Particle

1. *Stability and Physicochemical Properties*

A virus has certain independently measurable properties of the particle that depend on its detailed composition and architecture. These properties can be useful for identification and as criteria for establishing relationships. The most commonly measured properties are density, sedimentation coefficient and diffusion coefficient, and ultraviolet absorption spectrum (Box 13.1).

2. *Electron Microscopy*

Knowledge of the size, shape, and any surface features of the virus particle is a basic requirement for virus identification. Electron microscopy can provide this information quickly and, in general, reliably. For the examination of virus particles in crude extracts or purified preparations, a negative-staining procedure is now used

BOX 13.1

ULTRAVIOLET SPECTRA OF VIRUSES

The ultraviolet absorption spectrum of a virus is a combination of the absorption spectra of the nucleic acid and the coat protein (Figure A). Nucleic acids have maximum absorption at about 260 nm and a trough at about 230 nm, whereas the absorption spectrum for proteins peaks at about 280 nm and troughs at about 250 nm. The specific absorption for nucleic acids (20–25 OD units/mg/ml/ at 260 nm) is much greater than that for proteins (about 1 OD unit/mg/ml/ at 280 nm). The combination gives an absorption spectrum for a virus that peaks at about 260 nm and troughs at about 230–240 nm (Figures A, B).

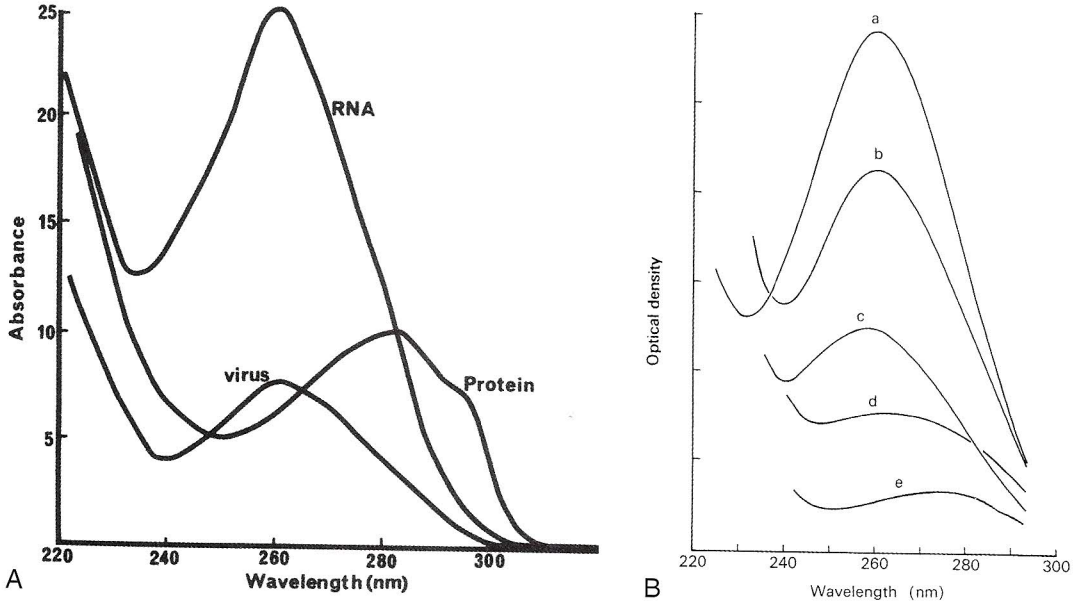


Fig. Ultraviolet absorption spectra. A. Comparative absorption spectra of 1 mg/ml RNA, a virus containing 20% RNA (1 mg/ml) and a typical protein (10 mg/ml); note the shoulder on the protein spectrum at 290 nm due to absorbance by tryptophan. [From Hull (1985; in *Virology, a practical approach,* B.W.J. Mahy, Ed., pp. 1–14, IRL Press, Oxford).] B. Absorption spectra of a suspension of (a) *Turnip yellow mosaic virus* (TYMV) RNA; (b) TYMV nucleoprotein particles (33% RNA); (c) *Cucumber mosaic virus* particles (19% RNA); (d) *Tobacco mosaic virus* particles (5% RNA); and (e) TYMV protein particles (top component). All suspensions contain the same weight of material except (a), which contained half as much as the others. [From Gibbs and Harrison (1976; *Plant virology, the principles,* Edward Arnold, London).]

Thus, this property depends mainly on the ratio of nucleic acid to protein in the virus; the amino acid composition of the protein can also influence the spectrum. Many unrelated viruses with similar compositions have similar absorption spectra. Ultraviolet absorption provides a useful assay for purified virus preparations, provided other criteria have eliminated the possibility of contamination with nonviral nucleic acids or proteins, especially ribosomes. Measurements of A_{260} may be unreliable for rod-shaped viruses that vary substantially in their degree of aggregation from one preparation to another, thus leading to changes in the amount of light scattering for a given virus concentration.

almost universally. Of particular use for rapid results is the epidermal strip technique, in which the broken surface of a strip of epidermis is removed from the leaf is wiped through negative stain on the electron microscope grid. Commonly used negative stains are sodium phosphotungstate, ammonium molybdate, or uranyl acetate, depending on the stability of the virus to these stains.

Approximate particle dimensions can be determined. This is particularly useful for rod-shaped viruses for which particle length distributions can be obtained. Surface features may be seen best in isolated particles on the grid. Depending on size and morphology, a virus may be tentatively assigned to a particular taxonomic group. However, some small icosahedral viruses cannot be distinguished from members of unrelated groups on morphology alone.

For many viruses, examination of thin sections by electron microscopy is a valuable procedure for detecting virus within cells and tissues, but this has its limitations such as differentiating virus particles from normal cell constituents. The large, enveloped viruses, the plant reoviruses, and the rod-shaped viruses can usually be readily distinguished because their appearance in thin sections generally differs from that of any normal structures.

D. Methods That Depend on Properties of Viral Proteins

Some of the most important and widely used methods for assay, detection, and diagnosis depend on the surface properties of viral proteins. For most plant viruses, this means the protein or proteins that make up the viral coat. Different procedures may use the protein in the intact virus, the protein subunits from disrupted virus, or proteins expressed from cloned cDNA or DNA in a system such as *E. coli* or insect cells. More recently nonstructural proteins coded for by a virus have been used in diagnosis.

1. Serology

Serological procedures are based on the interaction between a protein or proteins (termed the antigen) in the pathogen with antibodies raised against them in a vertebrate. The components of a serological reaction are shown in Box 13.2.

2. Types of Antisera

There are two basic types of antisera: polyclonal, which contain antibodies to all the available epitopes on the antigen, and monoclonal (Mab), which contain antibodies to one epitope. There is much discussion as to which is the best for diagnosis, but this will depend on what question the diagnostician is addressing. Monoclonal antisera are much more specific than polyclonal antisera and can be used to differentiate strains of many pathogens. On the other hand, specificity can be a disadvantage, and a variant of the pathogen may not be detected.

The bringing together of two technologies, the production of single-chain antibodies, and the display of recombinant proteins on the surface of bacteriophage (phage display) have resulted in the ability to produce a large range of molecules with the properties of single-type antibodies similar to Mabs without involving injection of animals.

3. Methods for Detecting Antibody-Virus Combination

A wide variety of methods has been developed for demonstrating and estimating combination between antibodies and antigens. The most widely used are the enzyme-linked immunoabsorbent assay (ELISA), immunoabsorbent electron microscopy (ISEM), and "dot blots" that employ either polyclonal or monoclonal antibodies.

a. ELISA Procedures. Many variations of the basic procedure have been described (Box 13.3),

BOX 13.2

COMPONENTS OF A SEROLOGICAL REACTION

1. Antibodies: An antibody is a molecule that binds to a known antigen. Antibodies are secreted by B lymphocytes. Structurally they are composed of one or more copies of a characteristic unit that can be visualised as forming a Y shape (Fig.).

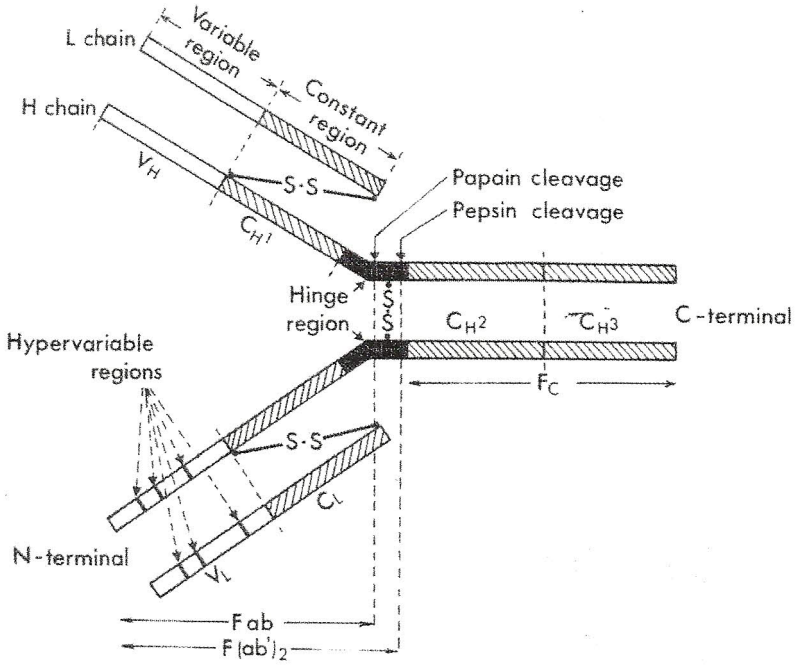


Fig. IgG antibody molecule. Arrows indicate specific sites where the molecule is cleaved by the enzymes papain and pepsin to give the Fab, Fc, and $(FabN)_2$ fragments. L, light chains; H, heavy chains. The four polypeptide molecules are joined by disulfide bridges. The two antigen-combining sites are made up from the variable regions of the L and H chains. [This article was published in *Serology and immunochemistry of plant viruses*, M.H.V. Van Regenmortel, Copyright Elsevier (1982), Academic Press, New York.]

Each Y contains four polypeptides: two identical copies of the heavy chain and two identical copies of the light chain joined by disulfide bonds. Antibodies are divided into five classes—IgG, IgM, IgA, IgE, and IgD—based on the number of Y-like units and the type of heavy-chain polypeptide they contain.

IgG molecules have three protein domains. Two of the domains, forming the arms of the Y, are identical and are termed the Fab domain. They each contain an antigen-binding site at the end, making the IgG molecule bivalent. The third domain, the Fc domain, forms the stem of the Y. The three domains may be separated from each other by cleavage with the protease papain. The Fc region binds protein A, a protein obtained from the cell wall of *Staphylococcus aureus*, with very high affinity. This property is used in several serological procedures.

(continued)

BOX 13.2 *(continued)*

The N-terminal regions of both the light and heavy chains in the arms of the Y-shaped IgG molecule comprise very heterogeneous sequences. This is known as the variable (V) region. The C-terminal region of the light chains and the rest of the heavy chains form the constant (C) region. The V regions of one heavy and one light chain combine to form one antigen binding site. The heterogeneity of the V regions provides the structural basis for the large repertoire of binding sites used in an effective immune response.

2. Antigens: Antigens are usually fairly large molecules or particles consisting of, or containing, protein or polysaccharides that are foreign to the vertebrate species into which they are introduced. Most have a molecular weight greater than 10 kDa, although smaller peptides can elicit antibody production. There are two aspects to the activity of an antigen. First, the antigen can stimulate the animal to produce antibody proteins that will react specifically with the antigen. This aspect is known as the immunogenicity of the antigen. Second, the antigen must be able to combine with the specific antibody produced. This is generally referred to as the antigenicity of the molecule.

Large molecules are usually more effective immunogens than small ones. Thus, plant viruses containing protein macromolecules are often very effective in stimulating specific antibody production; the subunits of a viral protein coat are much less efficient.

It is specific regions on antigens, termed epitopes, which induce and interact with specific antibodies. Epitopes can be composed of amino acids, carbohydrates, lipids, nucleic acids, and a wide range of synthetic organic chemicals. About 7–15 amino acids at the surface of a protein may be involved in an antigenic site.

There are several different grouping of epitopes:

The *continuous epitope* is a linear stretch of amino acids.

The *discontinuous epitope* is formed from a group of spatially adjacent surface residues brought together by the folding of the polypeptide chain or from the juxtaposition of residues from two or more separate peptide chains.

Cryptotopes are hidden epitopes revealed only on dissociation or denaturation of the antigen.

Neotopes are formed by the juxtaposition of adjacent polypeptides (e.g., adjacent viral coat protein subunits).

Metatopes are epitopes present on both the dissociated and polymerised forms of the antigen.

Neutralisation epitopes are specifically recognised by antibody molecules able to neutralise the infectivity of a virus.

The epitope type giving rise to a monoclonal antibody (MAb) and the relative proportions of different epitopes recognised by a polyclonal antiserum can affect the outcome of certain serological tests. For instance, an antibody to a cryptotope is unlikely to recognise an antigen in DAS-ELISA but is likely to in a Western blot.

3. Interaction Between Antibodies and Antigens. The interaction between an epitope and antibody is affected by both affinity and avidity. Affinity is a measure of the strength of the binding of an epitope to an antibody. As this binding is a reversible bimolecular interaction, affinity describes the amount of antibody-antigen complex that will be found at equilibrium. High-affinity antibodies perform better in all immunochemical techniques, not only because of their higher capacity but also because of thestability of the complex. Avidity is a measure of the overall stability of the complex between antibodies and

(continued)

BOX 13.2 *(continued)*

antigens and is governed by three factors: the intrinsic affinity of the antibody for the epitope, the valency of the antibody and antigen, and the geometric arrangement of the interacting components.

Titre is a relative measure of the concentration of a specific antibody in an antiserum. It is often used to define the dilution end point of the antiserum for detection of an antigen. Thus, as the sensitivities of various serological tests differ, the apparent titre is applicable only to the test under discussion.

The serological differentiation index (SDI) is a measure of the serological cross-reactivity of two antigens. It is the number of two-fold dilution steps separating the homologous and heterologous titres. The homologous titre is that of the antiserum with respect to the antigen used for immunising the animal, while the heterologous titre is that with respect to another related antigen.

BOX 13.3

ELISA PROCEDURES

Direct Double-Antibody Sandwich Method

The principle of the direct double-antibody sandwich procedure is summarised in the Figure (A). The technique is widely used but suffers two limitations:

1. It may be very strain specific. For discrimination between virus strains, this can be a useful feature, but for routine diagnostic tests, it means that different viral serotypes may escape detection. This high specificity is almost certainly due to the fact that the coupling of the enzyme to the antibody interferes with weaker combining reactions with strains that are not closely related.
2. It requires a different antivirus enzyme-antibody complex to be prepared for each virus to be tested.

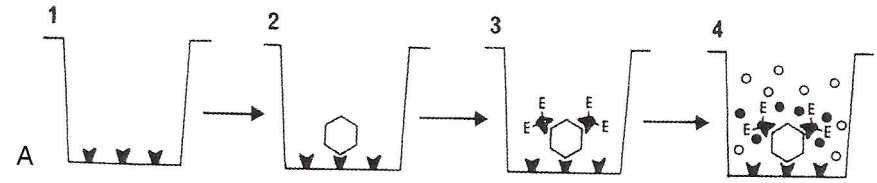


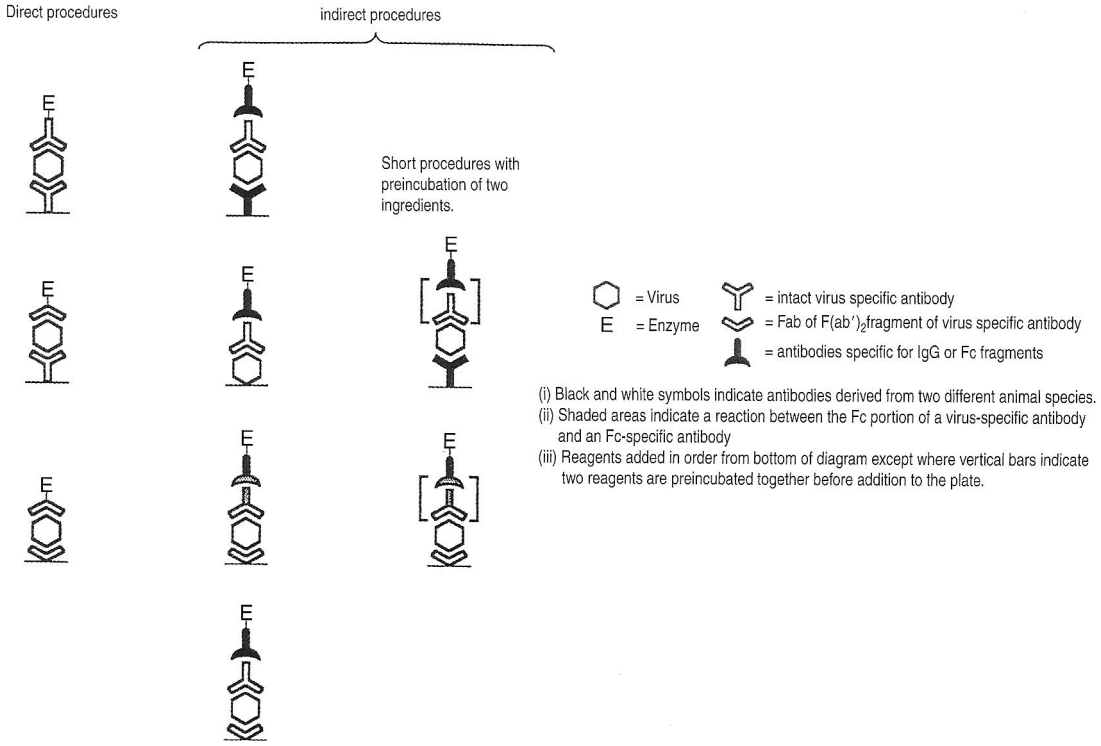
Fig. Immune detection of viruses. A. Principle of the ELISA technique for plant viruses (direct double-antibody sandwich method). (1) The gamma globulin fraction from an antiserum is allowed to coat the surface of wells in a polystyrene microtitre plate. The plates are then washed. (2) The test sample containing virus is added and combination with the fixed antibody is allowed to occur. (3) After washing again, enzyme-labeled specific antibody is allowed to combine with any virus attached to the fixed antibody. For instance, alkaline phosphatase is linked to the antibody with glutaraldehyde.) (4) The plate is again washed and enzyme substrate is added. The colourless substrate *p*-nitrophenyl phosphate (open circle) gives rise to a yellow product with alkaline phosphatase (filled circle), which can be observed visually in field applications or measured at 405 nm using an automated spectrophotometer. [Modified from Clark and Adams (1977; *J. Gen. Virol.* **34**, 475–483).]

(continued)

Indirect Double-Antibody Sandwich Methods

In the indirect procedure, the enzyme used in the final detection and assay step is conjugated to an anti-globulin antibody. For example, if the virus antibodies were raised in a rabbit, a chicken antirabbit globulin might be used. Thus, one conjugated globulin preparation can be used to assay bound rabbit antibody for a range of viruses.

Many variations of these procedures are possible (Figure (B)).



B

Fig.B. Diagrammatic representation of some variants of ELISA. [Modified from Koenig and Paul (1982; *J. Virol. Methods* **5**, 113–125).]

The direct double-antibody sandwich method is the most convenient for the routine detection of plant viruses in situations where strain specificity and very low virus concentrations cause no problems.

Dot ELISA

Several procedures have been developed that use nitrocellulose or nylon membranes as the solid substrate for ELISA tests. For the final colour development, a substrate is added that the enzyme linked to the IgG converts to an insoluble coloured material. An example of a dot blot assay is given in Figure (C).

(continued)

BOX 13.3 *(continued)*

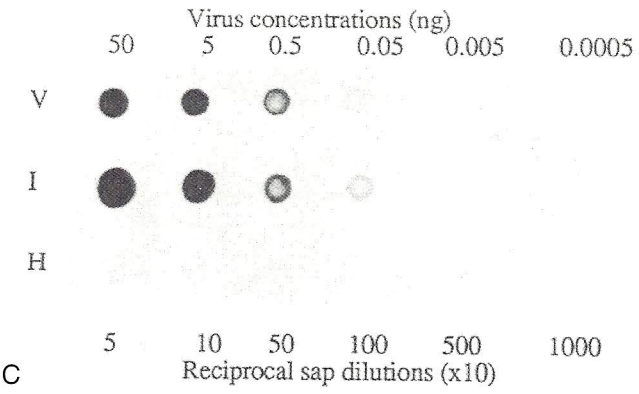


Fig.C. The sensitivity of a dot immunobinding assay. V = purified *Strawberry pseudo mild yellow edge virus;* I = crude sap from infected leaves; and H = crude sap from healthy leaves. The conjugated enzyme was alkaline phosphatase and the substrate for colour development was Fast Red TR salt. [From Yoshikawa *et al.* (1986; *Ann. Phytopathol. Soc. Jpn.* **52**, 728–731).]

The main advantages of the method are speed (three hours to complete a test), low cost, and the small amount of reagents required. Dot blot immunoassays may be particularly useful for routine detection of virus in seeds or seed samples, especially for laboratories where an inexpensive and simple test is needed. However, endogenous insect enzymes may interfere with tests using insect extracts.

Immuno-Tissue Printing

As an extension to dot ELISA, immuno-tissue printing involves applying the cut surface of a leaf or stem (or any other plant organ) to a nitrocellulose membrane and revealing the presence of an antigen (say viral coat protein) by immunoprobing.

Tissue printing has several advantages:

1. It gives detailed information on the tissue distribution of a virus.
2. Extraction of sap from leaves in which a virus has limited tissue distribution leads to the dilution of the virus with sap from uninfected cells. Since this technique samples the contents of each cell on the cut surface individually there is increased sensitivity.
3. The technique is easily applicable to field sampling; tissue blots can be made in the field, and there is no need to collect leaf samples for sap extraction in the laboratory.

with the objective of optimising the tests for particular purposes. The method is very economical in the use of reactants and readily adapted to quantitative measurement. It can be applied to viruses of various morphological types in both purified preparations and crude extracts. It is particularly convenient when large numbers of tests are needed. It is very sensitive, detecting concentrations as low as 1–10 ng/ml.

As with other diagnostic tests, it is necessary to define and optimise the time of sampling and the tissue to be sampled to achieve a reliable routine detection procedure. The main factor limiting the number of tests that can be done using ELISA procedures is the preparation of tissue extracts.

When many hundreds of field samples have to be processed, it is often necessary to store them for a time before ELISA tests are carried out.

Storage conditions may be critical for reliable results. For this reason, conditions need to be optimised for each virus and host.

b. Serologically Specific Electron Microscopy. A combination of electron microscopy and serology can provide rapid diagnosis and reveal features of a virus. The method offers a diagnostic procedure based on two properties of the virus: serological reactivity with the antiserum used and particle morphology. Various terms have been used to describe the process: serologically specific electron microscopy (SSEM), immunosorbent electron microscopy (ISEM), solid-phase immune electron microscopy, and electron microscope serology. Basically there are two approaches. Virus particles can be trapped on an electron microscope grid previously coated with an antiserum. The particles can be then negatively stained and reacted with another antiserum that decorates particles of the virus against which the antiserum is directed (Figure 13.1A). The other method is, after being adsorbed onto the EM grid, the virus particles are coated with a virus-specific antibody to which gold particles have been attached (Figure 13.1B).

This method has many advantages:

1. The result is usually clear in the form of virus particles of a particular morphology, and thus false positive results are rare.
2. Sensitivity may be of the same order as with ELISA procedures and may be 1,000 times more sensitive for the detection of some viruses than conventional EM.
3. When the support film is coated with an antibody, much less host background material is bound to the grid.
4. Antisera can be used without fractionation or conjugation, low-titre sera can be satisfactory, and only small volumes are required.
5. Very small volumes (0.1 μl) of virus extract may be sufficient.
6. Antibodies against host components are not a problem inasmuch as they do not bind to virus.
7. One antiserum may detect a range of serological variants (on the other hand, the use of monoclonal antibodies may greatly increase the specificity of the test).
8. Results may be obtained within 30 minutes.

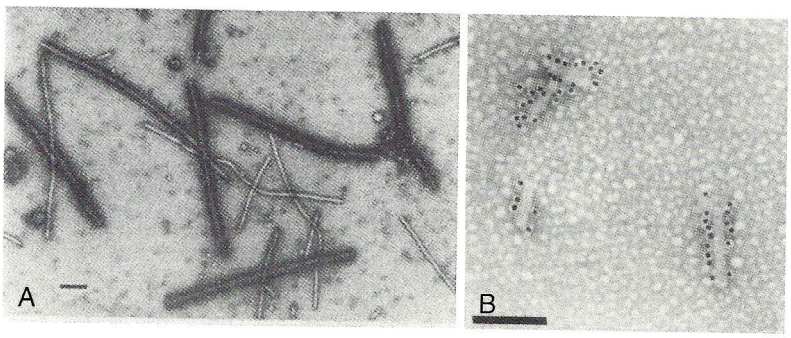


FIGURE 13.1 Immune specific electron microscopy (ISEM). A. A natural mixture of two potyviruses from a perennial cucurbit, *Bryonia cretica*. The antiserum used has decorated particles of only one of the viruses in the mixture. One particle near the centre is longer than normal and decorated for only part of its length. This particle probably arose by end-to-end aggregation between a particle of each of the two viruses. Bar = 100 nm. [From Milne (1991; in *Electron microscopy of plant pathogens*, K. Mendgen and D.E. Lesemann, Eds., pp. 87–102, Springer-Verlag, New York).] B. Gold particle labeling of virus particles. *Rice tungro bacilliform virus* (RTBV) particles were treated first with anti-RTBV protease rabbit antiserum and then with gold-labeled goat antirabbit serum. Bar = 200 nm. [This article was published in *Virology*, **205**, J.M. Hay, F. Grieco, A. Druka, M. Pinner, S.-C. Lee, and R. Hull, Detection of *rice tungro bacilliform virus* gene products *in vivo*, pp. 430–437, Copyright Elsevier (1994).]

9. When decoration is used, unrelated undecorated virus particles on the grid are readily detected (Figure 13.1A).
10. Different proteins on a particle can be decorated (e.g., the two coat proteins on closteroviruses; see Figure 5.1).
11. Prepared grids may be sent to a distant laboratory for application of virus extracts and returned to a base laboratory for further steps and EM examination.

The following are some disadvantages of the procedure:

1. It will not detect virus structures too small to be resolved in the EM (e.g., coat protein monomers).
2. Sometimes the method works inconsistently or not at all for reasons that are not well understood.
3. It involves the use of expensive EM equipment, which requires skilled technical work and is labour-intensive. For these reasons it cannot compete with, say, ELISA for large-scale routine testing.
4. When quantitative results are required, particle counting is laborious, variability of particle numbers per grid square may be high, and control grids are required.

c. Electrophoretic Procedures. Electrophoresis in a suitable substrate separates proteins according to size and net electric charge at the pH used. Polyacrylamide gel electrophoresis in a medium containing sodium dodecyl sulfate (SDS-PAGE) is commonly used. The position and amount of the proteins can then be visualised by a nonspecific procedure such as staining or by a specific procedure such as immunoassay (termed *Western blotting*).

d. Dot Blots. Protein dot blots resemble Western blotting, except that the constituent proteins in the sample are not separated. The samples are spotted onto a solid matrix such as nylon or nitrocellulose and probed with an antiserum to which a reporter group has been attached (see Figure C in Box 13.3). Reporter groups can be either enzymes that give a colour reaction with a specific chemical or a fluorescent compound. Unlike ELISA, the colour product must be insoluble.

Similar to dot blots, there are commercially available kits that use the same technique as pregnancy testing kits, in which the antibody is immobilised on a strip and the sample is diffused along the strip.

E. Methods That Involve Properties of the Viral Nucleic Acid

General properties of a viral nucleic acid, such as whether it is DNA or RNA, double- (ds) or single-stranded (ss), or if it consists of one or more pieces, are fundamental for allocating an unknown virus to a particular family or group. However, with the exception of dsRNA, these properties are usually of little use for routine diagnosis, detection, or assay. The ability to make DNA copies (cDNA) of parts or all of a plant viral RNA genome has opened up many new possibilities. The nucleotide sequence of the DNA copy can be determined, but this is far too time-consuming to be considered as a diagnostic procedure, except in special circumstances.

Basically, the four approaches to using nucleic acids for detection and diagnosis of viruses are the type and molecular sizes of the virion-associated nucleic acids; the cleavage pattern of viral DNA or cDNA; hybridisation between nucleic acids; and the polymerase chain reaction.

1. Type and Size of Nucleic Acid

Double-stranded RNAs are associated with plant RNA viruses in two ways: The plant reoviruses, and cryptoviruses have genomes that consist of dsRNA pieces, and in tissues infected with ssRNA viruses, a ds form of the genome RNA accumulates that is twice the size of the

genomic RNA. This is known as the replicative form (Figure 8.1). These dsRNAs have been used for diagnosis following characterisation of the ds forms by PAGE.

2. *Cleavage Patterns of DNA*

Cleaving cDNAs of RNA genomes and the genomes of DNA viruses at specific sites with restriction enzymes and determining the sizes of the fragments by PAGE are possible procedures for distinguishing viruses in a particular group. For instance, isolates of *Cauliflower mosaic virus* could be distinguished on the restriction endonuclease patterns of the virion DNA.

3. *Hybridisation Procedures*

These procedures depend on the fact that ss nucleic acid molecules of opposite polarity and with sufficient similarity in their nucleotide sequence will hybridise to form a ds molecule. The theory concerning nucleic acid hybridisation is complex. The Watson and Crick model for the structure of dsDNA showed that the two strands were held together by hydrogen bonds between specific (complementary) bases—namely, adenine and thymidine, cytosine and guanine. This interaction of base-pairing is the basis of all molecular hybridisation. Essentially, there are two stages, disruption of the base-pairing (termed melting or denaturing the nucleic acid) and reinstatement of base-pairing (termed reassociation, renaturation, or hybridisation). Among the factors that affect denaturation are temperature, nucleic acid composition, type and concentration of salt in the buffer, the pH of the buffer, presence of organic solvents, and base-pair mismatch. Factors that affect reassociation include temperature, salt concentration, base-pair mismatch, the length of the nucleic acid fragments, the type of nucleic acid (DNA and/or RNA), the concentration of nucleic acid, and the presence of various anionic polymers.

For diagnosis of virus by hybridisation, the unknown nucleic acid is termed the target and the known nucleic acid is termed the probe. In many of the systems the target is immobilised on a solid matrix and the probe applied in liquid. The probe comprises sequences complementary to the target sequences to which a reporter system, either radioactive or nonradioactive, is attached to reveal when hybridisation has taken place. There are three basic types of nonradioactive reporter: those that directly modify bases in the probe DNA, those that attach precursors (e.g., horseradish peroxidase or alkaline phosphatase) to the probe DNA or RNA, and those that incorporate labeled precursors (e.g., biotinylated nucleotides) into the probe. The detection of the reporter group is usually by an enzyme that gives a coloured product or a luminescent compound on reaction with a substrate. The coloured product has to be insoluble (unlike the coloured product of the ELISA reaction).

Excess probe hybridised to the target immobilized on a solid matrix has to be removed before detection. This is done by washing at selected temperatures and salt concentrations. At this stage relatedness of the probe to the target can be ascertained by using different "stringencies" that relate to the effect of hybridisation and/or wash conditions on the interaction between complementary nucleic acids that may be incompletely matched. The use of different stringencies is one of the more powerful tools of the hybridisation technology. There are various hybridisation formats (Box 13.4).

4. *Dot Blots*

Dot blots, as just described for proteins, are widely used for detecting nucleic acids using labeled probes (see Box 13.4).

5. *Polymerase Chain Reaction*

DNA fragments of interest can be enzymatically amplified *in vitro* by the polymerase chain reaction (PCR). The technique involves the hybridisation of synthetic complementary oligonucleotide primers to the target sequence

BOX 13.4

HYBRIDISATION FORMATS

Southern Blotting

See standard molecular biology text books.

Dot Blot Hybridisation

Dot blot hybridisation is now the most commonly used procedure for testing of large numbers of samples. These are the main steps in dot blot hybridisation:

1. A small amount of sap is extracted from the plant under test.
2. The viral nucleic acid is denatured by heating or, if it is DNA, by alkali treatment.
3. A spot of the extract is applied to a membrane.
4. The membrane is baked or exposed to ultraviolet light to bind the nucleic acid firmly to it.
5. Nonspecific binding sites on the membrane are blocked by incubation in a prehybridisation solution containing a protein, usually bovine serum albumin or non-fat dried milk, and small ss fragments of an unrelated DNA, together with salt and other ingredients.
6. Hybridisation of a labeled probe nucleic acid to the test nucleic acid bound to the substrate.
7. Washing off excess (unhybridised) probe and estimation of the amount of probe bound by a method appropriate to the kind of label used for the probe. The prehybridisation step (about two hours) and the hybridisation step (overnight) are carried out in heat-sealable plastic bags in a water bath at about 65°C.

The technique is now widely used in plant virology. For instance, a dot blot technique has been successfully applied to screening large numbers of potato plants in a programme of breeding potatoes for resistance to several viruses. A nonradioactive dot blot system using minimal equipment was developed for the routine diagnosis of a range of insect-transmitted viruses. The method has been used to assess relationships between tombusviruses, but some unexpected cross-hybridisations were observed. A sensitive nonradioactive procedure has been developed for detecting *Bamboo mosaic virus* and its associated satellite RNA in meristem-tip cultured plants.

Tissue Print Hybridisation

This is similar to immuno-tissue printing as described in Box 13.3 but using labeled nucleic acid probes. Probes can be specifically designed for detecting (+) or (–) strands or specific parts of the genome. Tissue print hybridisation is of especial use for viroids that do not have proteins that can be detected immunologically. Viroids have been detected by molecular hybridisation of imprinted membranes. An adaptation of the procedure, termed *squash-blot* (or *swat blot*), has been designed to assay *Maize streak virus* in single leafhopper vectors squashed directly onto the nitrocellulose filter.

In Situ Hybridisation

In situ hybridisation can give information of the distribution of the target nucleic acid within a cell. The loci at which *Banana streak virus* (BSV) is integrated into the banana chromosomes were identified by *in situ* hybridisation. As an extension of this procedure, fibre stretch hybridisation, in which denatured chromosomal DNA is stretched out on slides before hybridisation, gives information on the detailed structure of the integrated locus; this is described for BSV in Box 8.9.

and synthesis of multiple copies of complementary DNA of the sequence between the primers using heat-stable DNA polymerase. The process goes through a series of amplification cycles, each consisting of melting the ds template DNA molecules in the presence of the oligonucleotide primers and the four deoxyribonucleotide triphosphates at high temperature (melting), hybridisation of the primers with the complementary sequences in the template DNAs at lower temperature (annealing), and extension of the primers with DNA polymerase (DNA synthesis). During each cycle, the sequence between the primers is doubled so that after n cycles, a 2^n amplification should be obtained. Usually the reaction is of 30–50 cycles.

As PCR is based on DNA, it is not directly applicable to most plant viruses that have RNA genomes. However, a cDNA can be made to the desired region of the RNA genome using a primer and reverse transcriptase and this used as the initial template. This procedure, now widely used, is termed *RT-PCR*. A further refinement is to couple PCR with the capture of the virus particles by immobilised antibodies, termed immune capture PCR (*IC-PCR* or *IC-RT-PCR*).

PCR (and RT-PCR) has proved to be a very powerful tool for virus detection and diagnosis. It can be used to directly produce a DNA product of predicted size that can be confirmed by gel electrophoresis. The choice of primers can be used to distinguish between strains of a virus or, with primers containing a variety of nucleotides at specific positions (degenerate primers), be used for more generic determinations. Strains can also be distinguished by amplifying a region that has differences in restriction endonuclease sites. PCR is widely used in producing probes for hybridisation by incorporating reporter nucleotides in the reaction. To illustrate the widespread applicability to the detection and diagnosis of plant viruses, we will look at some examples; these are by no means a comprehensive collection of the uses to which this technique can be applied.

As described in Box 8.9, *Banana streak virus* (BSV) sequences are frequently found integrated in the host genome. This precludes the use of straightforward PCR for diagnosis of episomal infections of this virus, but IC-PCR can be used in which the episomal virus particles are captured and the host chromosomal DNA (containing the integrant) is removed before PCR. Using an IC-RT-PCR method in a single closed tube, *Tomato spotted wilt virus* can be detected in leaf and root tissue, and *Citrus tristeza virus* and *Plum pox virus* can be detected in plant tissue and single aphid samples.

In an RFLP analysis of 10 *Potato virus Y* isolates representative of four symptomatic groups, the whole genomes were each amplified in two fragments by RT-PCR. Using seven restriction enzymes, three RFLP groups were determined in the 5′ fragment and two in the 3′ fragment that correlated with the biological characters. One group of isolates appeared to have resulted from a recombination event.

Degenerate primers to highly conserved regions have been used to detect whitefly-transmitted geminiviruses. Strains of the leafhopper-transmitted geminivirus *Wheat dwarf virus* can be differentiated by using universal and strain-specific promoters. A highly sensitive procedure for the early detection of *Beet necrotic yellow vein virus* (BNYVV) in plant, soil, and vector samples involves PCR and digoxigenin labeling. PCR is being widely used in the detection and identification of phytoplasmas in both plant and insect samples.

6. DNA Microarray

The principle of microarrays or DNA chips is the hybridisation of fluorescently labeled target sequences to probe sequences spotted onto a solid surface, usually a glass microscope slide. Total RNA from the infected plant is converted to DNA by RT-PCR, and the cDNA is labeled by reaction with a fluorescent dye. Probes to many

different viruses or variants can be spotted onto the glass slide, and hybridisation can reveal joint infections with more than one virus.

F. Decision Making on Diagnosis

The application of biological, physical, and molecular techniques has given a large "toolbag" for detection and diagnosis of plant viruses. This emphasizes the point made in the introduction to this chapter that it is important to identify the question to be addressed. If one wishes to determine if a plant is virus infected—say, for quarantine purposes—one does not necessarily need a sophisticated technique that identifies a virus strain. On the other hand, if one is studying the durability of a potential resistance gene (or transgene), it is very useful to have an understanding of the range of variation of the virus. Thus, one has to select the best technique for what is wanted. In making this decision various points have to be taken into account, including the following.

The sensitivity required. There is much discussion about the relative sensitivity of detection procedures. However, the sensitivity of many of the serological and nucleic acid–based tests is adequate for most purposes, so one should use the system that is most convenient. For ease and speed of operation, and low cost, dot blots based on either an immunological test or nucleic acid hybridisation have a lot to offer. There are field kits available for testing for, say, potato viruses that are based on the same technology as home pregnancy kits. However, for each virus situation it is advisable to compare tests to see which is the most appropriate.

The number of samples. Where large numbers of samples have to be handled, the following factors will be important in choosing a test procedure: specificity; sensitivity; ease and speed of operation; and cost of equipment, consumable supplies, and labour.

The material being sampled. In many cases, and especially in trees, the distribution of virus is not uniform. Thus, one has to be careful with the taking of samples, and it is advisable to take several samples from each tree.

The reliability of the technique. A technique can be unreliable in two ways. False negatives can result from sampling part of the plant not containing virus, inhibition of the reaction by a plant constituent, or limitation in the materials being used (e.g., mismatch in a primer for PCR). False positives can be due to plant constituents, especially when testing new plant species.

The equipment and expertise available. The routine reliable detection of most viruses usually does not require expensive equipment. There are some exceptions, such as the detection of BSV described earlier in this chapter, which requires either ISEM or IC-PCR. However, it is important to have a good, reliable supply of consumables and means for storing them without deterioration. Similarly, most of the basic techniques are relatively easy to learn, but it is important that they are learned properly so potential sources of error can be recognised.

There are international guidelines (FAO/IPGRI *Technical Guidelines for the Safe Movement of Germplasm*) that have been drawn up by expert panels to assist with international germplasm movement. These detail the current ideas on the safest and simplest tests for ensuring that plant propagules do not contain the viruses that are known to infect that species. It should always be remembered that one cannot, of course, test for unknown viruses.

II. EPIDEMIOLOGY AND ECOLOGY

To survive, a plant virus must have (1) one or more host plant species in which it can multiply, (2) an effective means of spreading to and infecting fresh individual host plants, and (3) a supply of suitable healthy host plants to which it can spread. The actual situation that exists for any given virus in a particular locality, or on the global scale, will be the result of complex