Methods for the detection of plant virus diseases

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Abstract

Plant viruses cause major losses to several agricultural and horticultural crops around the world. Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the viral diseases. Hence, methods for detection and identification of viruses, both in plants and vectors, play a critical role in virus disease management. Diagnostic techniques for viruses fall into two broad categories: biological properties related to the interaction of the virus with its host and/or vector (e.g., symptomatology and transmission tests) and intrinsic properties of the virus itself (coat protein and nucleic acid). Detection methods based on coat protein include precipitation/agglutination tests, enzyme-linked immunosorbent assays, and immunoblotting. Viral nucleic acid-based techniques like dot-blot hybridization assays and polymerase chain reaction are more sensitive than other methods. Availability of these diagnostic methods provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of virus diseases in disease surveys, epidemiological studies, plant quarantine and seed certification, and breeding programs. Nevertheless, deployment and effective utilization of these techniques and diagnostic reagents (viz. antibodies and kits) to address plant virus disease problems in sub-Saharan Africa depends on having minimum research facilities and critical scientific expertise in the national agricultural research systems. Thus, programs to improve knowledge in plant virology and strengthen skills for virus diagnosis are vital for crop improvement and agricultural sustainability in the region.

Résumé

Les virus des plantes causent d'importantes pertes aux cultures agricoles et horticoles dans le monde. Contrairement aux autres agents pathogènes, il n'existe aucune méthode directe de lutte contre ces virus. Les seules méthodes actuelles consistent en des techniques indirectes de gestion. Ainsi, la mise au point de méthodes de détection

et d'identification des virus chez les plantes comme chez les vecteurs constituent un aspect essentiel dans la gestion des ces maladies. Les techniques de diagnostic des virus entrent dans deux grandes catégories : les propriétés biologiques liées à l'interaction du virus avec son hôte et/ou son vecteur (symptomatologie et test de transmission) et les propriétés intrinsèques du virus (protéine de capside et acide nucléique). Les méthodes de détection basées sur la protéine de capside portent sur des test de précipitation/agglutination, des tests immuno-enzymatiques et de buvardage. Les techniques basées sur l'acide nucléique viral telles que les méthodes d'hybridation par « dot blot » et la réaction en chaîne de la polymérase sont plus efficaces que les autres méthodes. Ces techniques de diagnostic permettent une plus grande flexibilité, une efficacité et une spécificité accrues pour un diagnostic rapide dans le cadre de l'évaluation des maladies, des études épidémiologiques de la quarantaine des plantes, de la certification des semences et des programmes de sélection. Toutefois, la diffusion et l'utilization efficaces de ces techniques et réactifs (anticorps et kits) pour résoudre les problèmes de maladies virales en Afrique subsaharienne requièrent l'existence d'infrastructures minimum de recherches de même qu'une expertise scientifique critique au sein des systèmes nationaux de recherche agricole. Il convient dont de mettre en place des programmes destinés à améliorer les niveaux de connaissance en matière de virologie végétale et à renforcer les compétences en matière de diagnostic pour une amélioration culturale et une agriculture durable dans la région.

Introduction

Viruses infect many different plant species. Unfortunately, there are also no economically feasible chemical agents similar to fungicides and bactericides that are effective against plant viruses. Strategies aimed at plant virus disease management are largely directed at preventing virus infection by: (i) eradicating the source of infection to prevent the virus from reaching the crop, (ii) minimizing the spread of the disease by controlling its vector, (iii) utilizing virus-free planting material, and (iv) incorporating host-plant resistance to the virus. An essential precursor of the implementation of control measures, however, is an accurate diagnosis of a virus disease and mapping of its geographical and temporal distribution in an area or crop. Because of the increased worldwide movement of germplasm through seed and other propagative material in global trade and agriculture, diagnosis of viruses in these materials assumes greater importance for national quarantine services to ensure the safe movement of germplasm across the borders.

Many methods have been developed for the detection and identification of plant viruses. A single diagnostic test or assay may provide adequate information on the identity of

a virus, but a combination of methods is generally needed for unequivocal diagnosis. Optimally, methods for detection of plant viruses are sensitive, specific, and can be completed within a relatively short period of time and are inexpensive. Recent advances in techniques for the detection of proteins and nucleic acids have provided an opportunity to develop methods with these qualities for the diagnosis of plant virus diseases.

The type of diagnostic test used ultimately depends on resources, facilities, availability of reagents, level of specificity and sensitivity required, expertise and skills available to carry out these assays, type and number of samples to be tested, and the amount of information available on the virus to be detected. An overview of various methods available for the detection of plant virus diseases is provided in the following sections with emphasis on how they can be used by scientists in developing countries, especially in sub-Saharan Africa.

Methods of detection based on biological properties

Symptomatology

Symptoms on plants commonly are used to characterize a disease having viral aetiology and for roguing of diseased plants in an attempt to control the disease. Visual inspection is relatively easy when symptoms clearly are characteristic of a specific disease. However, many factors such as virus strain, host plant cultivar/variety, time of infection, and the environment can influence the symptoms exhibited (Matthews 1980). Plants can also exhibit virus-like symptoms as a response to unfavorable weather conditions, soil mineral/nutrient imbalances, infection by nonviral pathogens, damage caused by insect/mite/nematode pests, air pollution, and pesticides (particularly herbicides). Some viruses may induce no apparent symptoms or cause symptomless infection. In addition, different viruses can produce similar symptoms or different strains of a virus cause distinct symptoms in the same host. While symptoms provide vital information on virus diseases, adequate field experience is required when making a decision on symptomatology alone. Usually, it is necessary that visual inspection for symptoms in the field is done in conjunction with other confirmatory tests to ensure accurate diagnosis of virus infection (Bock 1982).

Transmission tests

Virus detection and identification techniques originated with mechanical, graft, and vector transmission of the viruses to susceptible indicator plants (Jones 1993). Mechanical transmission by sap inoculation to herbaceous indicator plants can be done with minimal facilities and characteristic symptoms produced by these plants allow

both the detection and identification of many viruses (Horvath 1983, 1993). Although host-range may not be a precise guide for virus identification (Hamilton et al. 1981), it is still used in many laboratories as an important assay in virus diagnosis. The reliability of host-range tests for diagnosis can be increased with hands-on experience and by using a suitable range of plant species.

Viruses that are not mechanically transmissible and viruses of tree fruit and small fruit can be diagnosed by vector transmission or grafting onto suitable indicator hosts (Fridlund 1980; Nemeth 1986; Martelli 1993). While these assays are used in many laboratories both for diagnosis and maintaining virus cultures, they are time and resource consuming, and beset with the same difficulties in discerning viruses based on symptoms expressed in the field.

Physical properties

Physical properties of a virus (e.g., thermal inactivation point, dilution end point, and longevity in vitro), taken to be a measure of infectivity of the virus in sap extracts, were previously used to identify plant viruses. However, these properties are unreliable and no longer recommended for virus diagnosis (Francki 1980).

Microscopy

Electron microscopy (EM) provides very useful information on the morphology of the virus particles and is commonly used for virus detection when EM facilities are readily available (Baker et al. 1985; Milne 1993). Filamentous and rod-shaped viruses such as potyviruses, potexviruses, and tobamoviruses can more readily be differentiated in negatively stained leaf-dip preparations than isometric viruses and other viruses. Viruses that occur in low concentrations in plant sap are not easily seen unless the virus in the test material is concentrated before visualization. The efficiency of virus visualization can be improved in combination with serology (see section: Immunosorbent electron microscopy). As EM is labor intensive and expensive, it cannot often be used for the rapid processing of multiple samples. Many agricultural research institutions cannot afford to have an electron microscope facility due to the prohibitively high costs involved in installation and maintenance of the facility.

Many plant viruses induce distinctive intracellular inclusions, or develop large crystalline accumulations of virus particles, and their detection by light microscopy or EM can provide a simple, rapid, and relatively inexpensive method to confirm viral infection (Edwardson et al. 1993). Because of the uniqueness of inclusions produced as a result of infection by some viruses, unknown viruses can sometimes be identified to the genus level based on inclusion bodies observed using selective stains. Plant virus

inclusion technology, however, requires extensive hands-on experience and is seldom used by the novice for routine viral disease identification.

Detection methods based on viral coat protein

Serological or immunological assays have been developed and used successfully for a number of years for the detection of plant viruses. These tests are broadly subdivided into liquid and solid phase tests. In the former, both antigen and antibody react in solution to form a visible precipitate (precipitin or microprecipitin tests, gel diffusion assays) or agglutination of cells (agglutination methods). In the latter, assays are conducted on a solid surface such as on a microtitre plate or nitrocellulose membrane and the antigen–antibody reaction is visualized by means of a suitable detection system such as an enzyme-labelled antibody. These methods are reviewed briefly here and details can be found in Hampton et al. (1990), Van Regenmortel (1982), and Van Regenmortel and Dubs (1993).

Precipitation and agglutination tests

Precipitin tests (either in liquid medium or in agar/agarose) rely on the formation of a visible precipitate when adequate quantities of virus and specific antibodies are in contact with each other (Ball 1974; Van Regenmortel 1982). Precipitin and microprecipitin tests are routinely used by some investigators, but agglutination and double diffusion tests are more commonly used. In double diffusion tests, the antibodies and antigen (either purified virus or virus-infected plant sap) diffuse through a gel matrix and a visible precipitin line is formed where the two diffusing reactants meet in the gel (Ouchterlony 1962). The Ouchterlony double diffusion method can be used to distinguish related, but distinct, strains of a virus or even different but serologically related viruses. However, disadvantages of this method include a lack of sensitivity in detecting viruses that occur in low concentration (e.g., luteoviruses and most viruses of woody hosts), the need to dissociate filamentous or rod-shaped viruses to allow them to diffuse through the gel matrix, and the need for large quantities of antibodies.

In an agglutination test, the antibody is coated on the surface of an inert carrier particle (e.g., red blood cells, latex, or *Staphylococcus aureus* cells), and a positive antigen–antibody reaction results in clumping/agglutination of the carrier particles which can be visualized by the naked eye or under a microscope. Agglutination tests are more sensitive than other precipitin tests and can be carried out with lower concentrations of reactants than are necessary for precipitation tests (Koenig et al. 1979; Walkey et al. 1992; Hughes and Ollennu 1993).

Although the precipitation and agglutination tests lack the sensitivity of other serological assays, they are excellent methods for detecting viruses that occur in a

reasonable concentration in plants. Tests can be conducted simply by squeezing out a drop of plant sap and testing it with the appropriate antisera. These techniques can be performed with minimum facilities and expertise and, therefore, are suitable for many laboratories with limited facilities but which have an adequate supply of antiserum.

Immunosorbent electron microscopy

This technique was introduced by Derrick (1973) as "serologically specific electron microscopy" (SSEM) and has become widely used in plant virology (Milne 1991). Because of its similarity with solid phase immunoassays, the method has become known as "immunosorbent electron microscopy" (ISEM, Roberts and Harrison 1979). ISEM combines the specificity of serological assays with the visualization capabilities of the EM. Virus particles are selectively "trapped" on antibody-coated grids with little contaminating host-plant material. Hence, the technique is more sensitive for detecting viruses than the leaf dip method. In addition to diagnosis, ISEM can also be used to estimate the degree of serological relationship between viruses. Although ISEM is a sensitive technique, it has the same drawbacks as EM. Nonetheless, it is ideal for confirmatory tests using small numbers of samples, if the EM facility and specific antisera are available.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) has been very popular for detection of viruses in plant material, insect vectors, seeds, and vegetative propagules since it was introduced to plant virology by Clark and Adams (1977). Unlike precipitin and agglutination tests, ELISA is a solid phase heterogeneous immunoassay and usually done in microtitre plates made up of either polystyrene (inflexible "rigid" plates) or polyvinyl chloride (PVC, flexible plates). Due to its adaptability, sensitivity, and economy in use of reagents, ELISA is used in a wide range of situations, especially to test a large number of samples in a relatively short period of time. Many variations of ELISA have been developed (Clark and Bar-Joseph 1984; Cooper and Edwards 1986; Van Regenmortel and Dubs 1993) and fall into two broad categories: "direct" and "indirect" ELISA procedures. They differ in the way the antigen–antibody complex is detected, but the underlying theory and the final results are the same.

In "direct" ELISA procedures (Fig. 1a), the antibodies (usually as an immuno-γ-globulin or IgG fraction of the antiserum) bound to the well surface of the microtitre plate capture the virus in the test sample. The captured virus is then detected by incubation with an antibody-enzyme conjugate followed by addition of color development reagents (substrate or substrate/dye combination). The capturing and detecting antibodies can be the same or from different sources. Since the virus is sandwiched between

two antibody molecules, this method is called the double antibody sandwich (DAS) ELISA. In practice, DAS-ELISA is highly strain-specific and requires each detecting antibody to be conjugated to an enzyme.

There are several alternative “indirect” forms of ELISA available for virus detection. In these methods, antibodies raised in two different animal species and alternative ways of immobilizing the virus in the wells of the ELISA plate have been used. One approach, known as direct antigen-coating (DAC), antigen-coated plate (ACP), or plate-trapped antigen (PTA) ELISA (Fig. 1b), is to allow the virus, in the absence of any specific virus trapping layer as in DAS-ELISA, to adsorb on the plate surface by adding the test sample directly to the wells. In the second step, virus antibody (usually called primary antibody) is added either as IgG or crude antiserum. The primary antibody is then detected with antispecies antibodies (secondary or detecting antibody) conjugated to an enzyme, followed by addition of color development reagents. The detecting antibody binds specifically to the primary antibody since the former is produced against IgGs from the animal in which virus antibodies are raised (e.g., if virus antibodies are produced in rabbits, antirabbit IgGs are produced in a second species such as goats). It has certain disadvantages such as competition between plant sap and virus particles for sites on the plate and high background reactions.

A second widely used approach is triple antibody sandwich (TAS) ELISA (Fig. 1c). This is similar to DAS-ELISA, except that an additional step is involved before adding detecting antibody–enzyme conjugate. In this step, a monoclonal antibody (MAb), produced in another animal (usually mice) different from the trapping antibody, is used. This MAb is then detected by adding an enzyme-conjugated species-specific antibody (e.g., rabbit antimouse IgG), that does not react with the trapping antibody, followed by color development reagents.

In the third, called protein A-sandwich (PAS) ELISA (Fig. 1d), the microtitre wells are usually coated with protein A before the addition of trapping antibody. The protein A keeps the subsequently added antibodies in a specific orientation by binding to the Fc region so that the F(ab’)2 portion of the antibodies traps virus particles. This can often increase the sensitivity of the ELISA by increasing the proportion of appropriately aligned antibody molecules. The trapped virus is then detected by an additional aliquot of antibody (the same antibodies that were used for trapping) which in turn is detected by enzyme-conjugated protein A and subsequently color development reagents. Thus, in this method the antibody–virus–antibody layers are sandwiched between two layers of protein A. As a result, different orientations of the IgG in the trapping and detecting

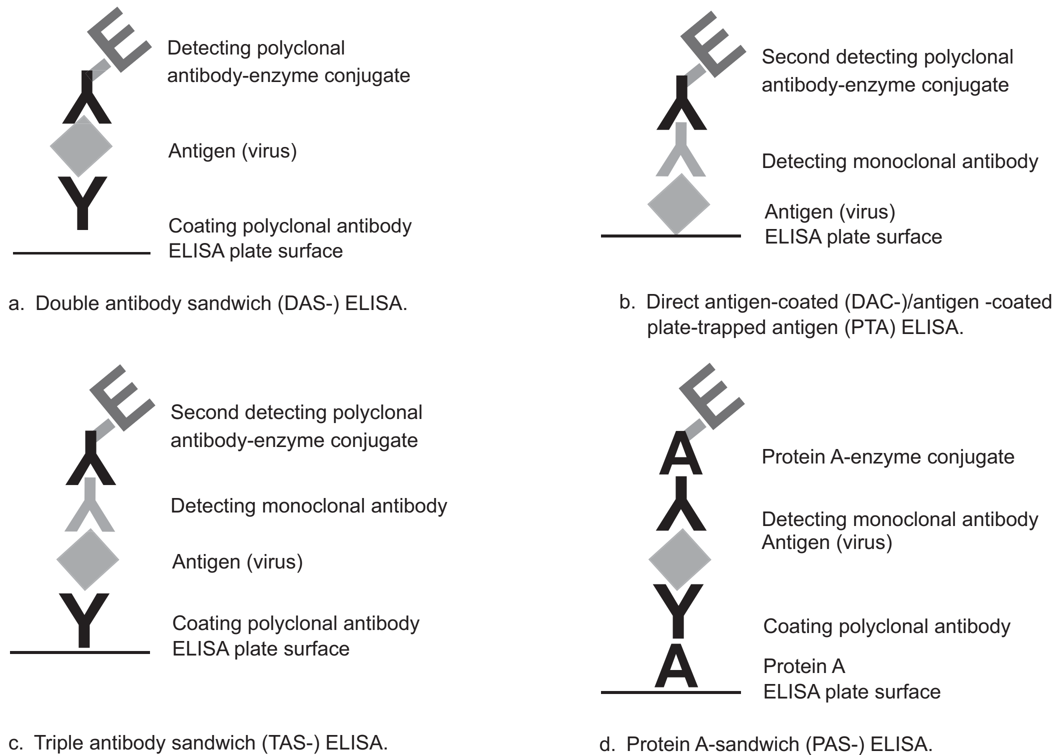

Figure 1(a,b,c,d). Four types of enzyme-linked immunosorbent assay commonly used for plant virus detection.

layers of antibodies enable the protein A to conjugate to discriminate between them. This permits use of unfractionated antisera.

Thus, in indirect ELISA procedures, the virus is detected by using a heterologous antibody conjugate that is not virus-specific, but specific for the virus antibody or primary antibody. As a result, a single antibody-conjugate (such as goat antirabbit, rabbit antimouse, or protein A enzyme conjugates) can be used in indirect assays to detect a wide range of viruses. Indirect ELISA procedures are more economical and therefore suitable for virus detection in a range of situations that include disease surveys and quarantine programs.

Although many enzymes have been suggested as the reporter molecules in antibody–enzyme conjugates, most of the ELISA assays described employ alkaline phosphatase or horseradish peroxidase. The antibody conjugates with these enzymes are available commercially or can be prepared by covalent coupling of antibodies to enzymes (Avrameas 1972; Farr and Nakane 1974). Penicillinase was reported to be a useful alternative to alkaline phosphatase for virus detection in developing countries (Sudarshana and Reddy 1989). Although penicillinase is cheaper, readily

available in many developing countries, and the detection limits are similar to alkaline phosphatase (Singh and Barker 1991), this method is not widely used due to inherent difficulties in quantification of results. Additionally, penicillinase–antibody conjugates are not available commercially and laboratories in many developing countries lack adequate facilities for the safe handling of glutaraldehyde, a hazardous chemical used in enzyme–antibody conjugation procedures. To overcome these problems and as a trade-off between the human safety and cost factors, it is preferable to use well-defined and high-quality antibody–enzyme conjugates (e.g., alkaline phosphatase conjugates) that are available commercially. Nevertheless, the penicillinase system can be used in qualitative tests since the positive reactions can be monitored by the change in color of indicator dye and the results assessed visually.

The sensitivity of virus detection can be increased by amplifying the colorimetric signal generated in alkaline phosphatase-based immunoassays using enzyme cycling systems (Self 1985). This technique has the potential to be at least 500 times more sensitive than the classical one employing ρ-nitrophenyl-phosphate as a substrate (Van Weemen 1985), thus making it possible to use these systems for detection of the virus in individual vectors (Torrance 1987; Van den Heuvel and Peters 1989; Smith et al. 1991). But the reagents used in this assay are expensive thus making it less favorable.

Factors that influence ELISA results

Although ELISA is versatile and individual steps are simple, the assay is complex in that several steps with different reagents are involved. Many factors can therefore influence the sensitivity and reliability of the assay that include quality of antibodies, preparation and storage of reagents, incubation time and temperature, selection of appropriate parts of plant samples, and use of suitable extraction buffers (McLaughlin et al. 1981; Hewings and D'Arcy, 1984). It is critical that positive and negative controls are included in each assay to define a threshold for differentiating between "infected" and "noninfected" samples. Generally a sample is regarded as positive if the absorbance value exceeds the mean value of a negative control by 2–3 standard deviations. In some cases, the simple arithmetic cut-off of twice the absorbance value of the average of the negative controls is used.

Preparation of reagents

The quality of the ELISA results depends on the quality and proper use of reagents. Therefore, all reagents must be prepared with "good quality" water, either distilled or at least deionized. The molarity and pH of the reagents, purity of chemicals, and clean glassware also contribute to the final results of the assay. Reagents, stock solutions, and

antibodies must be stored appropriately to prevent contamination by microorganisms and from introducing unwanted reagents through the use of contaminated glassware and micropipette tips.

Tissue extraction

Since extraction of plant samples is probably the most time consuming stage of the ELISA, a suitable procedure for the extraction of a large number of samples in as short a period as possible must be used. If a large number of plant samples is involved, it is preferable to keep plant extracts at low temperatures in order to avoid possible denaturation of antigens. Clarification of plant extracts by low speed centrifugation before adding to the ELISA plate wells is useful to avoid nonspecific binding of plant materials. In many cases, additives like polyvinylpyrrolidone (1–2% w/v) to bind polyphenols or diethyldithiocarbamate (DIECA; 0.1 M) as an antioxidant may be added to the virus extraction buffer to prevent plant extracts turning brown during the extraction process, thereby minimizing detrimental effects on the antigens in the plant samples (Clark and Adams 1977; McLaughlin et al. 1981; Scott et al. 1989).

Nonspecific reactions

Nonspecific reactions in ELISA may be caused by adsorption of plant proteins to sites in the ELISA plate well, antibodies binding to normal plant antigens, or by nonimmunological binding of enzyme conjugates. These problems can be eliminated by using appropriate sample dilutions and/or by addition of immunologically inert substances to the dilution buffer and washing solutions. These substances may include nonionic detergents (such as Tween 20), which at a low concentration allow interaction of antigen and antibody, or a high concentration of a blocking agent (e.g., polyvinylpyrrolidone, ovalbumen, nonfat milk powder) to prevent adsorption of nonspecific substances. Often a combination of both detergents and blocking agents are used in the extraction and/or conjugate buffers. Proper washing and emptying of the ELISA plate wells after each incubation step helps separate unbound (free) from bound reagents and reduces or eliminates nonspecific reactions. Washing is generally done three times with phosphate-buffered saline (pH 7.4) containing 0.05% (v/v) Tween 20 in order to maintain isotonicity, since most antigen–antibody reactions are optimal under such conditions.

Virus distribution

Viruses are known to be unevenly distributed in many host plants and seeds (Adams 1978; Kolber et al. 1982; Torrance and Dolby 1984; Latvala et al. 1997; Hughes and Ollennu 1994; Dahal et al. 1998) thus making the sampling strategies critical for virus

detection. Where the distribution of the virus is not known, the use of composite samples from different parts of the plant or seed will help to avoid this problem.

Quality of antibodies

Of all the variables, antiserum quality is the most important factor in ELISA procedures (Clark 1981). A virus coat protein will elicit a specific immune response when injected in an appropriate manner into a warm-blooded animal (rabbits are usually used for this purpose, although mice, chicken, sheep, and goats can be used) resulting in the production of virus-specific antibodies in the animal's blood. The basis for the range of serological assays described above is due to the availability of polyclonal and monoclonal antibodies. Polyclonal antibodies are a heterogeneous mixture of antibodies directed towards different antigenic determinants or epitopes of the protein and with varying affinities. Monoclonal antibodies (MAbs) are produced using hybridoma technology (Köhler and Milstein 1975) and, unlike polyclonal antibodies, each MAb is produced from a clonal population of cells derived from a single hybridoma cell line. Therefore, each MAb preparation consists of homogeneous antibody molecules with the same specificity and affinity for an epitope.

Polyclonal antibodies are widely used for detection of viruses in several ELISA procedures. Two important aspects that need to be kept in mind while using polyclonal antibodies are their quality and variability. In many cases, polyclonal antibodies contain antibodies against contaminating host-plant material in the virus preparation and they react with host-plant components giving nonspecific results. To minimize such reactions, host proteins can be cross-adsorbed by preincubation of antiserum with healthy leaf extract before use in ELISA. The polyclonal antibodies also show variability between different batches of antisera due to differences in antigenic response between animals as well as possible differences in the antigen preparations injected into the animals. The specificity and titre of antisera may even vary between different bleedings from the same animal (van Regenmortel 1982). In recent years, a number of strategies are emerging to overcome these problems by using cloned viral coat protein, DNA-based immunization methods (Hinrichs et al. 1997), and single-chain variable fragment (scFv) antibodies from a synthetic phage display library (Ziegler et al. 1995; Harper et al. 1997; Susi et al. 1998).

MAbs are often considered superior to polyclonal antibodies in virus diagnosis (van Regenmortel 1986; Torrance 1992; Van Regenmortel and Dubs 1993; Torrance 1995). Since the MAb-secreting hybridoma cells are immortal, they can be stored for long periods at low temperature and regenerated when needed, thereby achieving a continued supply of antibodies with constant specificity and titre. Many of the problems associated

with polyclonal antibodies can thus be overcome by using Mabs, allowing detection and discrimination of an increasing number of viruses in infected plants and vectors at the strain, species, and genus level (Torrance et al. 1986; D'Arcy et al. 1989; Jordan and Hammond 1991; Smith et al. 1991; Macintosh et al. 1992; Konaté et al. 1995; Franz et al. 1996; Naidu et al. 1997). There are, however, certain limitations to MAbs as diagnostic reagents. Most importantly, some MAbs may be too specific, recognizing only a rare or narrow range of isolates/strains of a particular virus (Oxford 1982). This is a particularly important limitation in disease surveys and quarantine diagnostics. In such cases, a cocktail of several MAbs may be needed to detect all known strains of a virus (Gugerli and Fries 1983).

Incubation conditions

Successful results in ELISA depend on the incubation conditions, mainly the temperature and duration of incubation. This in turn depends on whether the ELISA plates are incubated under constant shaking or stationary conditions. Shaking the plates during incubation ensures that the reactants are continuously in contact with each other. This allows assays to be performed under short periods of incubation independent of temperature considerations. Stationary incubations, on the other hand, require longer periods to allow maximum reaction between reactants through the diffusion of molecules and are thus dependent on temperature. Since stationary conditions are used in most laboratories, standardization of incubation conditions is critical. Most steps in stationary plate assays require incubation for 1–3 hrs at 37 °C. However, any of the incubation steps can be carried at 4 °C, usually overnight. Where incubations at room temperature are done, seasonal variation in laboratory temperature should be taken into account since temperature fluctuations are greater in tropical environments. Plates should be covered during incubations either with Saran wrap (clingfilm) or kept in a closed, moist container to prevent evaporation of reagents and drying of the wells. If multiple plates are processed at the same time, they should be handled identically during all steps and kept separated and not stacked during incubations.

Immunoblotting

Dot immunoblotting assay (DIBA) can be used to detect viruses in both plants and vectors (Rybicki and Von Wechmar 1982; Banttari and Goodwin 1985; Graddon and Randles 1986; Lange and Heide 1986; Heide and Lange 1988; Makkouk et al. 1993). The technique is similar to ELISA except that the plant extracts are spotted on to a membrane rather than using a microtitre plate as the solid support matrix. Unlike in ELISA, where a soluble substrate is used for color development, a precipitating (chromogenic) substrate is used for virus detection in the DIBA. Hydrolysis of chromogenic substrates results

in a visible colored precipitate at the reaction site on the membrane. Chemiluminescent substrates, which emit light upon hydrolysis, can also be used and the light signal detected with X-ray film as with radiolabelled probes (Leong et al. 1986). An optimized DIBA is as sensitive as ELISA, simple, relatively inexpensive, and the results can be scored visually.

Tissue immunoblotting assay (TIBA) is a variation of DIBA in which a freshly-cut edge of a leaf blade, stem, leaf, tuber, root or an insect is blotted on the membrane, followed by detection with labelled antibodies as described above (Navot et al. 1989; Hsu and Lawson 1991; Polston et al. 1991; Makkouk et al. 1993). This method is also simple, does not require elaborate sample preparation or extraction, and provides information on the distribution of viruses in plant tissues (Lin et al. 1990; Hu et al. 1997).

The disadvantages of DIBA and TIBA are possible interference of sap components with the subsequent diagnostic reactions. Sometimes the color of the sap will prevent weak positive reactions from being observed and the results cannot be readily quantified. Nevertheless, their sensitivity, the relatively short time required to assay large numbers of samples, the need for minimum laboratory facilities for the assay, the ability to store blotted membranes for extended periods, and low costs favor TIBA and DIBA as useful diagnostic techniques. The other advantage is that the samples can be blotted onto the membranes right in the field and such membranes can be carried or shipped by mail for further processing at a central location either within the country or in a different country.

Detection methods based on virus nucleic acid

Although widely used for virus detection, serological methods have certain disadvantages. They are based on the antigenic properties of the virus coat protein, which represents only about 10% of the total virus genome (Gould and Symons 1983) and thus does not take into account the rest of the virus genome. Nucleic acid-based detection methods, on the other hand, have the advantage that any region of a viral genome can be targeted to develop the diagnostic test. In addition, there are situations where immunological procedures have limited application in particular for the detection of viroids, satellite RNAs, viruses which lack particles (e.g., *Groundnut rosette virus* (GRV) genus *umbravirus*, the NM-form of tobacco rattle virus), viruses which occur as extremely diverse serotypes (e.g., Indian and African *Peanut clump virus* and *Tobacco rattle virus*) and viruses that are poor immunogens or are difficult to purify. Consequently nucleic acid-based diagnostic assays may be the methods of choice.

Nucleic acid hybridization assays

The affinity of one strand of DNA for its complementary sequence is one of the strongest and most exquisitely specific interactions found in nature. This specificity has been exploited in developing nucleic acid hybridization assays, which are based on the homology between two strands of nucleic acid (DNA:DNA, DNA:RNA or RNA: RNA). In these assays, a single-stranded complementary nucleic acid (either DNA or RNA), which has been "labelled" with a reporter molecule is used as a probe to form a hybrid with the target nucleic acid. The double-stranded probe-target hybrid molecules are then detected by several methods, depending on the reporter molecule used.

The dot- or spot-blot hybridization assay is a commonly used technique in plant virus diagnostics (Maule et al. 1983; Garger et al. 1983; Owens and Diener 1984; Rosner et al. 1986; Baulcombe and Fernandez-Northcote 1988; Palukaitis 1984). The whole process involves solid–liquid hybridization, wherein (i) the target nucleic acid (i.e., viral nucleic acid in the sample to be tested) is spotted and immobilized onto nitrocellulose or positively charged nylon membrane, (ii) the free binding sites on the membrane are blocked with a nonhomologous DNA (usually salmon sperm or calf-thymus DNA) or protein (usually bovine serum albumin or nonfat dried milk), (iii) hybridization is allowed to take place between the bound viral nucleic acid and the probe (which is free in the hybridization solution), (iv) the nonhybridized probe is removed from the membrane by a series of washing steps at defined stringency, and (v) the target sequences are assayed by detecting the reporter molecule in the hybridized probe.

Complementary DNA (cDNA) clones, specific to any region of the viral genome, are commonly used as a probe to detect virus in plant extracts. To produce cDNA clones, the viral RNA is usually converted to double-stranded DNA and cloned into suitable vectors (Sambrook et al. 1989). The major advantages of using cloned DNA are purity and unlimited supply of the probe. In addition, cloning of DNA into vectors immortalizes the cDNA, so that such clones are available for use at any time and can be supplied to different labs for use in virus diagnostics, thereby offering uniform test results.

The choice of labelling method is dictated by the nature of the probe to be used. DNA probes may be generated by nick translation, random primed labelling, and by polymerase chain reaction (PCR), whereas RNA probes are prepared by in vitro transcription (Palukaitis 1984). Unlike DNA probes, single-stranded RNA probes can hybridize only with the target sequence without re-annealing and RNA:RNA hybrids are more stable than DNA:RNA hybrids. However, the potential risk of degradation of RNA probes due to RNAase contamination during hybridization and high costs of generating such probes make the use of DNA probes more common in virus detection assays.

Radioactive isotopes like ^{32}P are used for labelling nucleic acid probes and the signal detected by autoradiography. Radioisotopes have a short half-life (causing supply problems), can be hazardous to health if improperly handled, and require extensive and costly procedures to meet safety regulations. In recent years, these problems have been overcome by nonradioactive labelling and detection methods (Eweida et al. 1990; LeClerc et al. 1992; Fouly et al. 1992; Mas et al. 1993; Dietzgen et al. 1994; Singh and Singh 1995; Wesley et al. 1996) using either biotin/streptavidin (Langer et al. 1981) or digoxigenin (DIG)/antiDIG systems (Höltke et al. 1995). There are certain disadvantages of the biotin/streptavidin system, such as the presence of endogenous biotin in the samples and the tendency of streptavidin to stick nonspecifically to solid phases like nylon membranes, resulting in severe "background" problems. Therefore, the DIG/antiDIG system has been widely employed for detection of several viruses. In this system the membranes are exposed, subsequent to hybridization, to antiDIG antibodies coupled to an enzyme (alkaline phosphatase or horseradish peroxidase), and the signal is generated by adding a suitable substrate that results in either a precipitated product (chromogenic detection) or chemiluminescence (chemiluminescent detection) which is detected by autoradiography.

Polymerase chain reaction

The sensitivity of nucleic acid-based detection systems was greatly improved following the development of the polymerase chain reaction (PCR) procedure (Mullis et al. 1986). PCR is an in-vitro method for amplifying target nucleic acid sequences. The speed, specificity, sensitivity, and versatility of PCR made it suitable in many areas of research in biology. Since PCR has the power to amplify the target nucleic acid present at an extremely low level and form a complex mixture of heterologous sequences, it has become an attractive technique for the diagnosis of plant virus diseases (Henson and French 1993; Hadidi et al. 1995; Candresse et al. 1998a).

PCR consists of three steps: (i) denaturation at high temperature (usually 94–95 °C) to separate the two complementary strands of the double-stranded DNA, (ii) annealing of two oligonucleotide primers to their complementary sequences in the opposite strands of the target DNA (annealing temperature depends on the nucleotide composition and length of the primer, usually anywhere between 35 and 65 °C), and (iii) extension of each primer through the target region (usually at 72 °C) using a thermostable DNA polymerase (e.g., Taq polymerase). Each DNA strand made in one cycle will serve as a template for synthesis of a new DNA strand in the next cycle. This results in an exponential increase in PCR product as a function of cycle number. The three step cycles are repeated many times (between 30 and 40 cycles) in an automated thermal cycler

until sufficient product is produced. Thus a single template molecule will be amplified 2^n times after n cycles, i.e., approximately 3.4×10^{10} times in 35 cycles if it is assumed that the reaction proceeds with 100% efficiency. However, the efficiency typically spans the range of 65–85% and one can expect that the total amount of product synthesized would be between 1.65^n and 1.85^n (Krawetz 1989). Thus in a few hours, the target sequence is amplified to greater quantities and the results can be analyzed by agarose gel electrophoresis of the PCR reaction mixture followed by ethidium bromide staining to reveal the presence of amplified DNA. A number of automated thermal cycler machines are commercially available which can be used to analyze many samples concurrently, rendering these machines suitable for routine diagnostics.

This procedure is applicable directly to DNA plant viruses (caulimo, gemini, and badnaviruses); however, for diagnosis of plant viruses with RNA genomes, the RNA target has to be "converted" to a complementary DNA (cDNA) copy by reverse-transcription before PCR is begun. The cDNA provides a suitable DNA target for subsequent amplification. During the initial cycles of PCR, a complementary strand of DNA will be synthesized from the cDNA template, and thereafter the reaction will proceed as for double-stranded DNA described above. This process of amplification is called reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 2). On completion of the reaction, the amplified DNA can be analyzed by agarose gel electrophoresis as described above.

Besides its usefulness as a detection technique, PCR can also be used in conjunction with techniques like restriction fragment length polymorphism (RFLP) or sequencing of the amplified DNA to study the variability of viruses at the molecular level (Almond et al. 1992; Tenllado et al. 1994; Candresse et al. 1995). Based on the nucleotide sequence information of several different viruses, specific oligonucleotide primers can be designed and used in PCR to detect and differentiate viruses at the family, genus, or strain level (Robertson et al. 1991; Omunyin et al. 1996), or for simultaneous detection of unrelated viruses in a sample by using a mixture of virus-specific primer pairs ("Multiplex" PCR; Bariana et al. 1994; Minafra and Hadidi 1994; Smith and Van de Velde 1994; Hauser et al. 2000; Nassuth et al. 2000). The potential of PCR technology can be effectively exploited in epidemiological studies and in breeding programs for virus resistance, and especially in situations where detection is otherwise difficult with other techniques (Rush et al. 1994; Harrison et al. 1997; Candresse et al. 1998b).

Although the advantages of RT-PCR can outweigh its disadvantages, considerable care must be taken while carrying out PCR reactions, because of its exquisite sensitivity and tremendous amplification power, in order to avoid false positives due to cross-contamination or "carryover". Some of these problems can be overcome

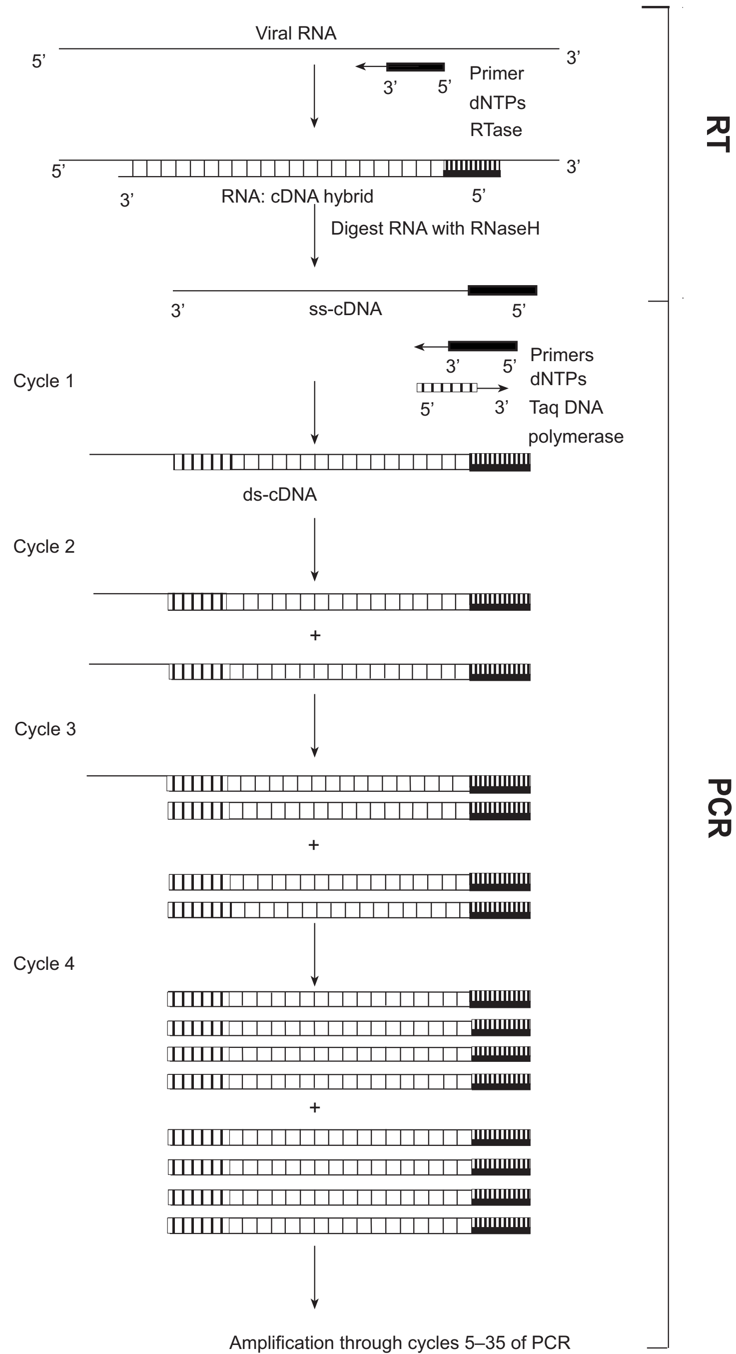

Figure 2. Diagrammatic representation of reverse transcription-polymerase chain reation (RT-PCR). Each cycle of PCR consists of denaturation, annealing, and extension. RTse = reverse transcriptase, ss–and ds-cDNA = single- and double-stranded cDNA, respectively.

dNTPs = deoxynucleotide triphosphates.

with forethought and adequate care by guarding the solutions and samples against accidental contamination with exogenous DNA via aerosols, running negative controls simultaneously with the test samples during each and every PCR reaction, having a dedicated laboratory area for pre- and post-PCR work, and using separate positive displacement micropipettes in the two areas (Kwok and Higuchi, 1989; Candresse et al. 1998a).

A technique that combines the technical advantages of PCR with the practical advantages of ELISA, called immunocapture (IC)-PCR, was developed for the detection of several different plant viruses (Wetzel et al. 1992; Nolasco et al. 1993). In this assay, the virus particles are first "concentrated" by trapping onto a solid surface (either microcentrifuge tube or ELISA plate) using virus specific antibodies. The trapped virus particles are disrupted and the released viral nucleic acid amplified by RT-PCR. This results in greater sensitivity, and problems encountered with RNA extraction are minimized and inhibitors of RT-PCR washed away prior to amplification. Thus IC-PCR is a very useful alternative for RT-PCR in virus detection from plant material and insect vectors (James et al. 1997; Latvala et al. 1997, Mumford and Seal, 1997; Candresse et al. 1998a; Jain et al. 1998).

Recently, a novel real-time quantitative PCR assay (TaqMan technology) was developed for the detection and quantification of plant viruses (Dietzgen et al. 1999; Mumford et al. 2000; Eun et al. 2000; Roberts et al. 2000). In addition to sensitivity and specificity, this technique has certain advantages over RT-PCR; it reduces the risk of cross-contamination, obviates post PCR manipulations, provides higher throughput, and enables quantification of virus load in a given sample. However, this technology requires expensive and special equipment and reagents compared with conventional PCR technology.

Future outlook for sub-Saharan Africa

A wide range of techniques, as discussed above, is currently available for the detection and identification of plant viruses. These techniques are useful in surveys for virus diseases, disease monitoring in crops, epidemiological studies, quarantine systems, and breeding programs to incorporate host plant resistance. The use of a range of different detection methods results in increased sensitivity and specificity, and expands the range of applications of the diagnostics in developing effective virus disease management strategies to mitigate the effects of many of the devastating virus diseases (Martin et al. 2000).

Virus detection based on biological properties and serological assays are by far the most widely used methods in many of the national programs of SSA. Unlike other

pathogens, diseases caused by viruses are particularly prone to erroneous diagnosis when made entirely on symptoms (Bock 1982). Thus, diagnostics based on serology are more important for virus diagnosis as they can be performed under the variety of situations in most laboratory facilities in SSA.

While nucleic acid-based assays provide an excellent opportunity for rapid and sensitive detection of viruses, their success largely depends on good laboratory facilities and personnel with adequate technical skills. These requirements can not always be met and the many advantages afforded by nucleic acid-based diagnostic assays have to be weighed against the costs of establishing and maintaining an effective laboratory facility for carrying out these assays. Alternative options could be to arrange for shipping the nitrocellulose membrane blots for dot blot hybridization assays, and/or nucleic acid extracts for RT-PCR, to a central laboratory within or outside SSA, where facilities and necessary reagents (cDNA probes, primers for RT-PCR etc.) are available, to complete the assays.

However, it is important to bear in mind that in instances where both nucleic acid and serology-based methods provide similar information through detection sensitivity and specificity, serology is the preferred method of diagnosis on the basis of cost and the need for specialized facilities for nucleic acid-based diagnostics.

In the era of "globalization" of agriculture, application of phytosanitary standards are likely to play a significant role in seed exchange and international testing of germplasm and improved varieties in SSA (Olembo 1997). Obviously, good seed health procedures should be followed to assure shipment of "virus-free" seed and vegetative propagules (Frison et al. 1990; Spiegel et al. 1993). It is important, however, to note that a positive result in either serologogical or nucleic acid-based detection assays does not necessarily indicate the presence of a biologically active virus and that the virus is transmissible through seed (Konaté and Barro 1993; Johansen et al. 1994; Konaté and Neya 1996). If the situation warrants, it is appropriate and desirable to carry out additional confirmatory tests before taking a decision to reject a seed lot. This has important implications in quarantine and seed certification programs, and development of realistic and uniform phytosanitary guidelines for viruses across SSA will be valuable (Olembo 1997). This should at least be addressed on a regional basis rather than country by country.

A critical aspect, however, is the standardization and harmonization of detection methods applied for the same purpose in various labs in SSA (Raubo and Schmid 1997; Maury et al. 1998). This will ensure accuracy of testing methods and reliability of assay results leading to the establishment of uniform quality assurance systems at the continental level. One of the ways of addressing this issue is by providing diagnostic kits from a common source to scientists across SSA, so that variables associated with quality and

specificity of different reagents in assay kits are minimized. This would benefit quality assurance, phytosanitary activities, and ensure consistency across the region.

Nevertheless, all these activities require research personnel with adequate skills and experience to optimize and carry out the diagnostic assays in the many different environments of SSA and interpret results without any ambiguity. Short-term training courses should be organized at regular intervals to provide either hands-on experience on various diagnostic methods or upgrade the skills and knowledge base of the research and extension personnel working on plant virus diseases. Participation of scientists in such courses as resource persons from different institutions, both within and outside SSA, and with expertise in different areas of plant virus research is crucial to achieve this objective. In-country or regional training courses are preferable to those organized in an environment where "nothing-goes-wrong". It is important that research organizations and other donor agencies participating in crop improvement programs in SSA continue efforts to strengthen capacity in virus research in the national programs. It is also critical to have a long-term coordinated strategy to document existing virus diseases occurring on different crops in SSA and fully characterize those which are not yet studied. It is also important to assemble, validate, and distribute robust diagnostic for use by scientists in various institutions. Research institutions (both within and outside SSA) involved in agricultural improvement in SSA should continue to take a leading role in facilitating such initiatives aimed at developmental impact and achieving real gains in sustainable agricultural production.

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