Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.1, 2015

Detection of Diseases, Identification and Diversity of Viruses: A Review

Kumlachew Alemu
Department of Plant Science, Assosa University, P.O.Box 18, Assosa, Ethiopia
E-mail of corresponding Author: kum.alemu@gmail.com

Abstract
Plant viruses are known to cause considerable losses in crop yield, quality of plants and plant products around the world. They have great potential for inducing several economically important crop diseases as the fungal and bacterial plant pathogens, in spite of their extremely small size and elementary structure. They can infect whole plants, seeds and vegetatively propagated plant materials causing immense quantitative and qualitative losses. Hence, application of effective methods of detection, differentiation, quantification and identification has become necessary to prevent the incidence and spread of the diseases through infected plants and plant materials and through their natural vectors. To achieve this goal several methods based on the biological, physico-chemical, immunological and nucleic acid characteristics of these pathogens have been employed with different levels of sensitivity, specificity and reliability. To date an increasing numbers of diagnostic laboratories are adapting molecular methods for routine detection of pathogens. With the advances in molecular biology and biosystematics, the techniques available have evolved significantly in the last decade, and besides conventional polymerase chain reaction (PCR) other technologically advanced methodologies such as the second generation PCR known as the real time PCR and microarrays which allows unlimited multiplexing capability have the potential to bring pathogen detection to a new and improved level of efficiency and reliability. However, these diagnostic tools should be complemented with other techniques, either traditional culture-based methods or the newly emerged proteomic, a promising tool for providing information about pathogenicity and virulence factors that will open up new possibilities for crop disease diagnosis and crop protection.
Keywords: Detection, Diagnostic tool, Pathogen, Proteomic, Virus

1. Introduction

Plant viruses are known to cause considerable losses in crop yield, quality of plants, and plant products around the world. They pose a particular risk because they are difficult to detect and identify (Hadidi *et al*., 2004). 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 diseases. Hence, methods for detection and identification of viruses play a critical role in the management disease (Naidu and Hughes, 2001).

Accurate diagnosis of diseases is a first important step for any crop management system. With virus diseases, plant treatment after infection often does not lead to an effective control. Accordingly, the diseases are managed most effectively if control measures are applied before infection occurs. The use of healthy plant propagation material is among the most effective approaches to adopt by farmers. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive diagnostic methods (Makkouk and Kumari, 2006).

Traditional approaches to disease diagnosis generally first involve the interpretation of visual symptoms. This may be followed by laboratory identification, using for example selective media and microscopy, to confirm the diagnosis. In some cases these methods are still the cheapest, simplest and most appropriate. Conventional methods do, however, have a number of drawbacks, which has prompted the search for alternative diagnostic techniques. Traditional methods generally require skilled and specialized microbiological expertise, which often takes many years to acquire. There is a need to use more generic techniques that can be taught quickly and easily to relatively unskilled staff Ward *et al*., 2004). Methods that involve culturing can often take days or weeks to complete and this is not acceptable when rapid, high throughput diagnosis is required. The results are not always conclusive, for instance, where similar symptoms can be caused by different pathogens or physiological conditions. Closely related organisms may be difficult to discriminate on the basis of morphological characters alone. It may also be necessary to discriminate between populations of the same pathogen that have specific properties. Traditional methods may not be sensitive enough and as such much effort has been devoted to the development of novel methods for detecting and identifying plant pathogens over the last decade (Ward *et al*., 2004).

Advances in molecular biology and biotechnology over the last three decades were applied to develop rapid, specific and sensitive techniques for the detection of plant pathogen (Makkouk and Kumari, 2006). Many methods have been developed for the detection and identification of plant pathogens. A single diagnostic test or assay may provide adequate information on the identity of a particular disease causing organism, but a combination of methods is generally needed for unequivocal diagnosis. Optimally, methods for detection of

plant pathogens are sensitive, specific, and can be completed within a relatively short period of time and are inexpensive (Naidu and Hughes, 2001). This paper presents the various methods available for the detection and identification of plant diseases caused by virus.

2. Detection and Identification of Virus

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 (Narayanasamy, 2011). Strategies aimed at plant virus disease management are largely directed at preventing virus infection. 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. The detection methods are based on their biological properties, their coat protein or based on their nucleic acid (Narayanasamy, 2011; Naidu and Hughes, 2001).

2.1. Detection and Identification Methods Based on Biological Properties

2.1.1. Symptomatology

The ability to infect one or more plant species and induce characteristic symptoms indicates the pathogenic potential of the virus under investigation. Symptoms on plants commonly are used to characterize a disease having viral etiology and for rouging 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 non-viral pathogens, damage caused by insect/mite/nematode pests, air pollution, and pesticides. 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 (Naidu and Hughes, 2001). 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).

2.1.2. Transmission tests

Plant viruses differ from fungal and bacterial pathogens in the methods of dissemination from infected plants to healthy plants for infection to be initiated. Infection by viruses cannot occur through unwounded plant surfaces. Plant viruses have to be introduced and deposited inside the susceptible cells of host plants by grafting, budding or mechanical inoculation or through parasitic dodders (Narayanasamy, 2011). 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, 1993). Although host-range may not be a precise guide for virus identification, 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 (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 (Naidu and Hughes, 2001).

2.1.3. Physical properties

Physical properties of a virus such as thermal inactivation point, dilution end point, and longevity in vitro are 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).

2.1.4. Biochemical techniques

Virus infection induces several changes in physiological activity of the host plants, although the viruses themselves do not have any physiological functions. Hence, by studying variations in the physiological functions of healthy and virus infected plants, infection by viruses can be detected. Virus infection could be detected by differences in the isozyme patterns of healthy and infected leaves determined by sodium dodecyl sulfate

polyacrylamide gel electrophoresis technique. Eight dominant chitinase izozymes were detected in tobacco extracts. One of the isozymes was present only in the *Tobacco mosaic virus* (TMV)-infected leaves, while another isozyme was present in significantly higher concentration in TMV-infected leaves than in mock-inoculated leaves (Pan *et al*. 1991). Induction of synthesis of several proteins both structural and nonstructural proteins of the virus is observed in several virus-infected plants. Presence of a unique protein band (32–34 kDa) in the leaves infected by *Wheat yellow head virus* (WYHV) was detected by employing SDS-PAGE technique. This protein band was absent in the extracts of comparable healthy plants. The amino acid sequences of this protein was most closely related to the nucleoprotein of *Rice hoja blanca virus*, suggesting that WYHV might belong to tenui virus group. The SDS-PAGE technique has been shown to be useful in determining the physical properties of the purified viruses that may indicate the identity of the virus and its strains. The molecular mass of the coat protein (CP) in purified preparations of *Peanut chlorotic streak virus* were determined to be 51 and 58 kDa by applying SDS-PAGE technique (Narayanasamy, 2011).

2.1.5. Electron microscopy

Electron microscopy provides very useful information on the morphology of the virus particles and is commonly used for virus detection when Electron microscopy facilities are readily available (Milne 1993). Filamentous and rod-shaped viruses such as potyviruses, potexviruses, and tobamo viruses 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. As Electron microscopy 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; electron microscopic examination of inclusions can assist the rapid identification and characterization of many plant viruses. Some individual viruses can be distinguished by the inclusions they induce (Edwardson and Christie, 1978).

2.2. Detection and Identification Methods Based on Viral Coat Protein

2.2.1. Precipitation and agglutination tests

Precipitin tests rely on the formation of a visible precipitate when adequate quantity of virus and specific antibodies are in contact with each other (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 diffuse through a gel matrix and a visible precipitin line is formed where the two diffusing reactants meet in the gel. 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 the need to dissociate filamentous or rod-shaped viruses to allow them to diffuse through the gelmatrix, and the need for large quantities of antibodies. In an agglutination test, the antibody is coated on the surface of an inert carrier particle 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 (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 (Naidu and Hughes, 2001).

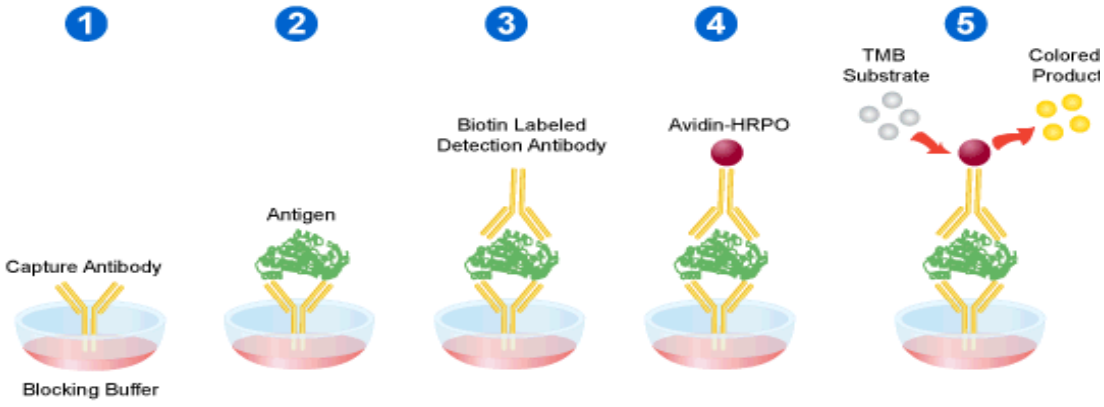
2.2.2. Immunoelectron microscopy

Immunoelectron microscopy methods have the great advantage of being able to be applied to tissue homogenates and of requiring very low quantities of virus and antiserum. Relationships can be studied in different ways: (i) by the differential trapping of virus particles on electron microscope grids coated with antisera to different viruses; (ii) by endpoint dilution of an antiserum which effectively coats (decorates) the virus particles; (iii) by the observation of a clumping reaction at different serum concentrations. Pretreatment of grids with protein A of *Staphylococcus aureus* may enhance virus adsorption (Shukla and Gough, 1979) but meaningful results occur only under certain conditions. It may be useful to follow trapping of virus particles on serum-coated grids with decoration (Milne and Luisoni, 1977), particularly as trapping may be susceptible to bias due to non-specific adsorption of virus particles to the grids (Lesemann *et aL,* 1980). Decoration of particles by antisera to distantly related viruses may be difficult to distinguish from non-specific decoration, however, especially in crude sap,

2.2.3. Enzyme-linked immunosorbent assay

The application of serology was revolutionized following the development of enzyme-linked immunosorbent assay (ELISA) technique and subsequent modification to the double-antibody sandwich technique . This method allowed the use of small amounts of reagents and test tissue, and was easily applicable to processing large numbers of samples. There was no limitation related to viral morphology, and results were available in 2-3 days. The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the antibodies antibodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognizes the first antibody is added. The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes colour, which is detected visually by a calibrated microtitre plate spectrophotometer (Webster *et al*., 2004). 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 (Van Regenmortel and Dubs, 1993) and fall into two broad categories: direct and indirect ELISA procedures.

In direct ELISA procedures, the antibodies bound to the well surface of the microtitre plate capture the virus in the test sample (Figure 1). The captured virus is then detected by incubation with an antibody-enzyme conjugate followed by addition of color development reagents. 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.



1 **a.)** Plate is coated with a suitable capture antibody. **b.)** Blocking buffer is added to block remaining protein-binding sites on plate.

2 Sample is added to plate and any antigen present is bound by the capture antibody.

3 A suitable biotin labeled detection antibody is added to the plate and also binds to any antigen present in well.

4 UltraAvidin™-HRPO *(Leinco Prod. No. A106)* is added and binds the biotin labeled detection antibody.

5 TMB substrate *(Leinco Prod. No. T118)* is added and converted by HRPO to a detectable form.

Figure 9: Direct Enzyme-linked immunosorbent assay (DAS-ELISA)

An alternative to the double-antibody sandwich method is indirect ELISA. These methods, antibodies are 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 platetrapped antigen (PTA) ELISA (Figure 2), 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

It has certain disadvantages such as competition between plant sap and virus particles for sites on the plate and high background reactions (Naidu and Hughes, 2001).

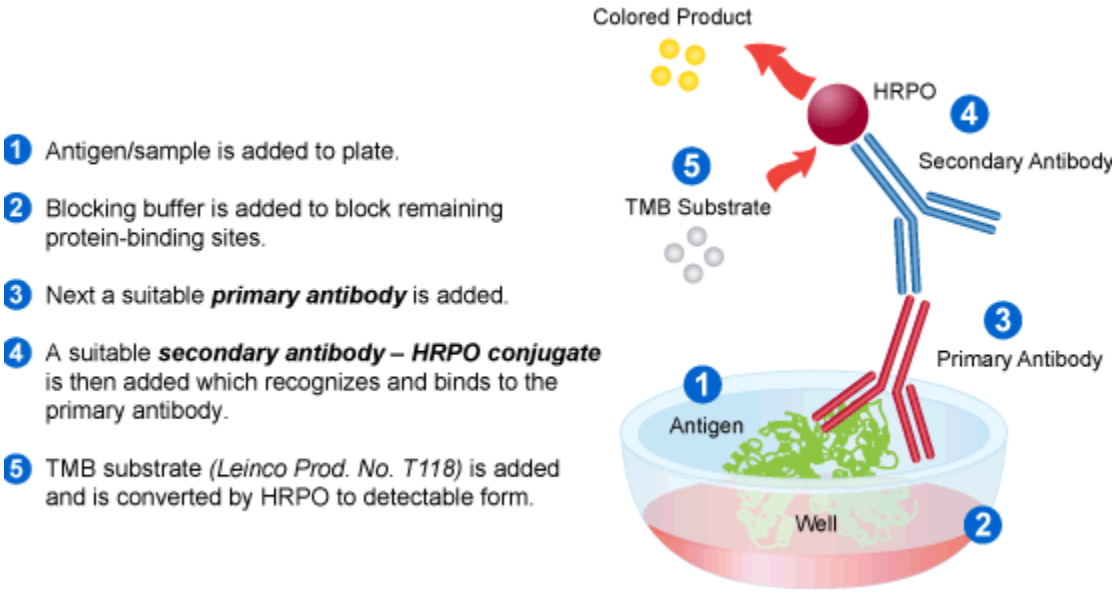


Figure 10: Indirect Enzyme-Linked Immunosorbent Assay

A second widely used approach is triple antibody sandwich (TAS) ELISA. This is similar to DAS-ELISA, except that an additional step is involved before adding detecting antibody enzyme conjugate (Figure 3). In this step, a monoclonal antibody (MAb), produced in another animal different from the trapping antibody, is used. This MAb is then detected by adding an enzyme-conjugated species-specific antibody that does not react with the trapping antibody, followed by color development reagents.

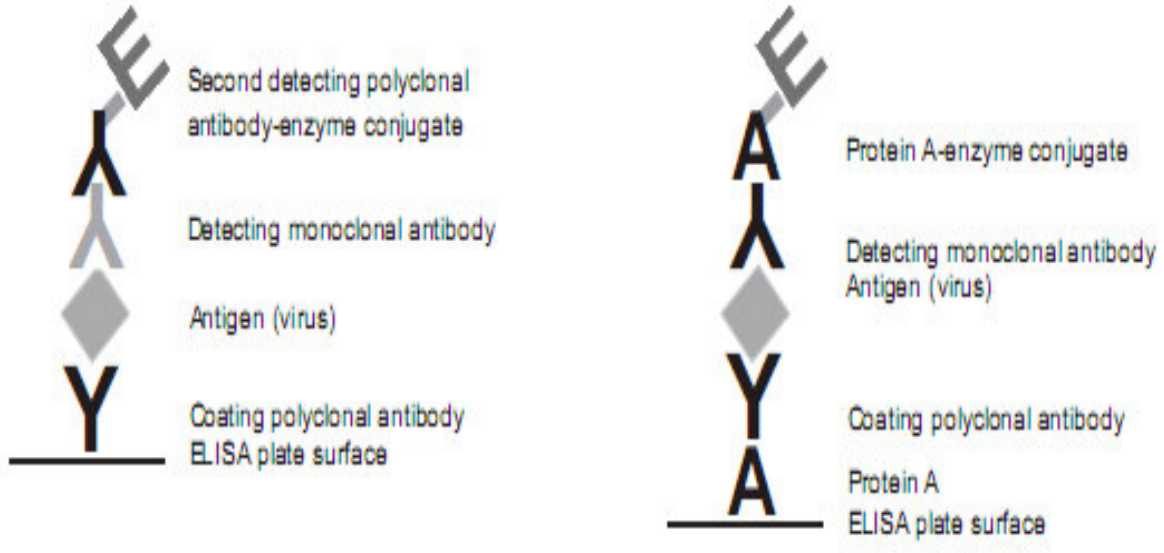


Figure 11: Enzyme-Linked Immunosorbent Assay (Triple antibody sandwich (TAS-ELISA) and protein A-sandwich (PAS-ELISA) (Naidu and Hughes, 2001)

In the third, called protein A-sandwich (PAS) ELISA, 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 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 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 (Naidu and Hughes, 2001).

2.2.4. Immunoblotting

The dot immunoblotting assay is a simple and sensitive technology which is the nucleic acid equivalent of the ELISA test (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). 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 (Makkouk *et al*. 1993). The procedure is less labour-intensive than ELISA, rapid, sensitive, simple, inexpensive, suitable for surveys of 1000 to 2000 samples per day, and the samples can be taken in the field and processed some time later (Webster *et al*., 2004).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 (Naidu and Hughes, 2001).

2.2.5. Quartz crystal microbalance immunosensors

In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies. Volt age is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. It is therefore qualitative and quantitative. The developers of the technique claim that it is as sensitive but more rapid than ELISA, and economical (Webster *et al*., 2004).

2.3. Detection and Identification 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 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 (Naidu and Hughes, 2001).

2.3.1. 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 (Naidu and Hughes, 2001).

2.3.2. Polymerase chain reaction

The polymerase chain reaction (PCR) is the most important and sensitive technique presently available for the detection of plant pathogens. 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 (Naidu and Hughes, 2001). The procedure is applicable directly to DNA plant viruses (caulimovirus, Geminivirus, and Nanovirus); 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). On completion of the reaction, the amplified DNA can be analyzed by agarose gel electrophoresis as described above (Naidu and Hughes, 2001; Makkouk and Kumari, 2006).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. 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 or for simultaneous detection of unrelated viruses in a sample by using a mixture of virus-specific primer pairs (Nassuth *et al*., 2000).

2.3.3. Multiplex PCR

Multiplex PCR allows the simultaneous and sensitive detection of different DNA or RNA targets in a single reaction (Rosenfield and Jaykus, 1999; Nassuth *et al*., 2000). On the other hand, PCR detection protocols can be designed to verify the presence of more than one pathogen in plant material by looking for common specific sequences in two or more of them, or to detect related viruses on multiple hosts. Multiplex PCR is useful in plant pathology because different bacteria and/or RNA viruses frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material. There are several examples in plant pathology of simultaneous detection of several targets and the amplification by multiplex PCR of two or three plant viruses has been reported (Saade *et al*., 2000; Sharman *et al*., 2000). Nevertheless, there are still only a few examples in which more than three plant viruses were amplified in a single PCR-based assay (Nie and Singh, 2000; Okuda and Hanada, 2001) probably due to the technical difficulties of designing a reaction involving many compatible primers. One of them is the simultaneous detection of the six major characterized viruses described in olive trees, which belong to four different genera: Cucumovirus (CMV), Nepovirus, Necrovirus and Oleavirus (Bertolini *et al*., 2003). This includes accurate design of six primer pairs for one-step RT-PCR amplification in a single closed tube and specific probes, enabling the detection of all major viruses described in olive trees, which are problematic for RNA extraction

2.3.4. Multiplex nested RT-PCR

A multiplex nested RT-PCR in a single closed tube has been developed for simultaneous and sensitive detection of the viruses CMV, CLRV, SLRSV, and ArMV using 20 compatible primers in a compartmentalized tube (Bertolini *et al*., 2003). This newly developed method combines the advantages of multiplex RT-PCR with the sensitivity and reliability of nested RT-PCR carried out in a single closed tube. It enables the simultaneous detection of several viral RNA and bacterial DNA targets in a single analysis, performed with woody plants. It also saves time and reagent costs because it can be performed in a single reaction, although accurate design of compatible primers is needed. The compartmentalization of a single Eppendorf tube with a pipette tip (Olmos *et al*., 2003) allowed multiplex-PCR and nested PCR to be combined effectively. During the first amplification reaction there is no interference of the external with internal primers because they are physically separated from the initial reaction cocktail. Once the multiplex RT-PCR ends, the internal primers are mixed with the products of the first reaction before proceeding to the nested multiplex. Because the concentration of internal primers is very high compared with that of the external primers, the nested multiplex can be performed with minimal interference. Consequently, sensitivity is increased at least 100-fold over that of multiplex RTPCR for the detection of viruses.

2.3.5. Real-time PCR

Recently, a novel real-time quantitative PCR assay was developed for the detection and quantification of plant viruses. This approach has provided insight into the kinetics of the PCR reaction and it is the foundation of “real time” PCR. The monitoring of accumulating amplicon in real time PCR has been possible by the labeling of primers, probes or amplicon with fluorogenic molecules. The increased speed of real time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluoregenic labels and sensitive methods of detecting their emissions. The reduction in amplicon size generally recommended by the inventors of commercial real-time assays may also play a role in this speed, but decreased product size does not necessarily improve PCR efficiency (Makkouk and Kumari, 2006**).**

Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. A probe is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5’ end with a reporter fluorochrome and a quencher fluorochrome added at the 3’ end. The probe is designed to have a higher Tm than the primers, and

during the extension phase, the probe must be 100% hybridized for success of the assay. As long as fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Similar to the conventional PCR, in case of RNA viruses, amplification can be measured after extraction of total RNA and preparation of a cDNA by a reverse transcription (RT) step. Real time PCR has proven increasingly valuable diagnostic tool for plant viruses (Makkouk and Kumari, 2006). The versatile application of real time PCR for detection and identification of plant virus is reviewed by Narayanasamy (2011).

2.3.6. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is used in combination with PCR to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites. After PCR amplification, the amplicon is digested with a restriction enzyme(s) and the fragment sizes analysed by gel electrophoresis. RFLP is a method that can be used to differentiate isolates of viruses without the expenses of cloning and sequencing. Its effectiveness relies on polymorphisms within restriction enzyme-recognition sites (Webster *et al*., 2004).

2.3.7. DNA microarrays

DNA microarrays or biochips are made of a surface on which are linked multiple capture probes, each one being specific for a DNA or RNA sequence of the targets. Their purpose is the detection of numerous sequences in a single assay. Various supports are currently in use for the elaboration of microarrays, including glass, nylon and different polymers. Up to 30,000 DNA probes (gene sequences) can be arrayed onto a single chip. The probes arrayed can be PCR products amplified to high concentrations or relatively short (30–50 bp) oligonucleotide probes. Once arrayed, the chip can be exposed to fluorescently labelled DNA/RNA from the sample to be tested (Lopez *et al*., 2003).The detection system uses one or several fluorophores, that can be read with laser technology to reveal the targets present in the sample. Extraction of nucleic acids from the sample, labelling and hybridization can be achieved with standard laboratory facilities. Another possibility is to use the nanochip technology developed by Nanogen (San Diego,USA), based on the combination of microelectronics with microarray technology in a solid support covered with streptavidin to increase the power of union with biotin-labelled DNA. Detection of four olive viruses by multiplex nested RT-PCR. A Multiplex nested RT-PCR products separated on a 3% agarose gel, stained with ethidium bromide and visualized under UV light.

Until now, the protocols used in the different laboratories involved in developing microarrays for detection of phytopathogens require a prior step of PCR amplification and reach low levels of sensitivity. Consequently, their use for routine detection is still far from being common, in contrast with their widespread use for functional genomics studies. Microarrays can generate fast results for several pathogens but their cost is still very high. They also generate significant amounts of data requiring expert interpretation. It is likely that microarrays will follow a path similar to that of PCR, which spent several years as a research tool before being used in diagnostics (Lopez *et al*., 2003).

3. Conclusion

The accurate and rapid identification of the organisms that cause plant disease is essential for effective disease control. It enables more informed decisions to be made about cultivar choice and how and when chemicals can be used most effectively to control disease epidemics. Recent developments in molecular detection technology led to the development of more convenient, effective, and specific assays and permitted the use of these tests for detecting plant pathogens, including viruses. Such assays will help growers, crop agronomists, and plant-health professionals not to rely exclusively on symptomatology and permit early detection of disease infection. These new techniques are effective management tools to be used in parallel with knowledge of the crop, understanding the biology of the pathogen and the ecology of the disease. Thus, these tools can be excellent tool to determine the point in time at which control measures should be implemented. In addition, such diagnostic assays are essential tools for programs devised to produce virus-free plant propagation materials. Generally, despite their specificity and sensitivity molecular diagnostic tools should be complemented with other techniques, either traditional culture-based methods or the newly emerged proteomic, a promising tool for providing information about pathogenicity and virulence factors that will open up new possibilities for crop disease diagnosis and crop protection.

References

Bertolini, E., Olmos, A., Lo´pez, M.M., and Cambra, M. (2003). Multiplex nested reverse-transcription polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses

and *Pseudomonas savastanoi* pv. savastanoi in olive trees. *Phytopathology* 93:286–292.
Bock, K.R. (1982). The identification and partial characterization of plant viruses in the tropics. *Tropical Pest Management* 28: 399–411.
Edwardson, J. R and Christie, R. G. (I 978). Use of virus-induced inclusions in classification and diagnosis. *Annual Review of Phytopathology* 16: 31-55.
Francki, R.I.B. (1980). Limited value of the thermal inactivation point, longevity in vitro and dilution end point as criteria for the characterization, identification, and classification of plant viruses. *Intervirology* 13: 91–98.
Hadidi, A., Czosnek, H. and Barba, M. (2004). DNA microarrays and their potential applications for the detection of plant viruses, viroids, and phytoplasmas. *Journal of Plant Pathology* 86 (2): 97-104.
Heide, M. and Lange. L. (1988). Detection of potato leaf roll virus and potato viruses M,S,X, and Y by dot immune binding on plain paper. *Potato Research* 31: 367–373.
Horvath, J. (1993). Host plants in diagnosis. Pp 15–48. *In:* Matthews, R.E.F.(ed) Diagnosis of plant virus diseases. CRC Press, Boca Raton, Florida, USA.
Hughes, J.d'A. and. Ollennu. L.A (1993). The virobacterial agglutination test as a rapid means of detecting cocoa swollen shoot virus. *Annals of Applied Biology* 122: 299–310.
Jones, A.T. (1993). Experimental transmission of viruses in diagnosis. Pp 49–72 *In:* Matthews, R.E.F.(ed) Diagnosis of plant virus diseases. CRC Press, Boca Raton, Florida, USA.
Lo´Pez, M. M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M. T.,Llop, P., Penyalver, R. and Cambra, M. (2003). Innovative tools for detection of plant pathogenic viruses and bacteria. *Int Microbiol* 6: 233–243.
Leong, M.M.L., Milstein, C. and Pannell. R. (1986). Luminescent detection method for immunodot, western, and southern blots. *Journal of Histochemistry and Cytochemistry* 34: 1645–1650.
Lesemann, D. E., Bozartrt, R. F. and Koenig, R. (1980). The trapping of tymovirus particles on electron microscope grids by adsorption and serological binding. *Journal of General Virology* 48: 257-262.
Makkouk, K.M. and Kumari. S.G. (2006). Molecular Diagnosis of Plant Viruses. *Arab J. Pl. Prot*. 24: 135-138.
Makkouk, KM., Hsu, H.T. and Kumari, S.G. (1993). Detection of three plant viruses by dot-blot and tissue-blot immunoassays using chemiluminescent and chromogenic substrates. *Journal of Phytopathology* 139: 97–102.
Martelli, G.P. (1993). Leaf roll. Pp 37–44. *In:* Martelli. G.P. Graft-transmissible diseases of grapevines. Handbook for detection and diagnosis. ICVG/FAO, Rome, Italy.
Matthews, R.E.F. (1980). Host plant responses to virus infection. Pp 297–359. *In:* H. Fraenkel-Conrat and Wagner. R.R. (eds.). Comprehensive virology, vol. 16, virus-host interaction, viral invasion, persistence, and diagnosis, Plenum Press, New York, USA.
Milne, R.G. (1993). Electron microscopy of in vitro preparations. Pp. 215–251. *In:* Matthews, R.E.F.(ed) Diagnosis of plant virus diseases. CRC Press, Boca Raton, Florida, USA.
Milne, R. G. and Luisoni, E. (1977). Rapid immune electron microscopy of virus preparations. *Methods in Virology* 6: 265-281.
Naidu, R.A. and Hughes J.d'A (2001). Methods for the detection of plant virus diseases. *In*: Hughes, J.d'A and BO Odu (eds.). *Plant virology in sub-Saharan Africa.* Proceedings of a Conference Organized by IITA. International Institute of Tropical Agriculture. Nigeria. p.233-260.
Narayanasamy, P., (2011). Microbial Plant Pathogens-Detection and Disease Diagnosis Viral and Viroid Pathogens, Volume 3. Springer Dordrecht Heidelberg, London. 343pp.
Nassuth A, Pollari E, Helmeczy K, Stewart S, and Kofalvi S.A. (2000). Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. *J Virol Methods* 90:37–49.
Nie, X. and Singh, R.P. (2000). Detection of multiple potato viruses using an oligo(dT) as a common cDNA primer in multiplex RT-PCR. *J Virol Methods* 86:179–185.
Okuda, M. and Hanada, K. (2001). RT-PCR for detecting five distinct Tospovirus species using degenerate primers and dsRNA template. *J Virol Methods* 96:149–156.
Olmos, A., Esteban, O., Bertolini, E, and Cambra, M. (2003). Nested RT-PCR in a single closed tube. In: Bartlett J.M.S., Stirling, D. (eds) Methods in molecular biology, 2nd ed, vol 226. PCR Protocols. Humana Press, Ottowa, pp 156–161.
Pan, S.Q., Ye X.S, and Kuć, J. (1991). A technique for detection of chitinase s-1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectro focusing. *Phytopathology* 81: 970–974.
Rosenfield, S.I., and Jaykus L.A.A. (1999). Multiplex reverse transcription polymerase chain reaction method for the detection of foodborne viruses. *J. Food Prot* 62:1210–1214.
Saade, M., Aparicio, F., Sanchez-Navarro, J.A., Herranz, M.C., Di-Terlizzi, A.M.B., Pallas, V., (2000).

Simultaneous detection of the three ilarviruses affecting stone fruit trees by non isotopic molecular hybridization and multiplex reverse-transcription polymerase chain reaction. *Phytopathology* 90:1330–1336.

Sharman, M., Thomas, J. and Dietzgen, R.G. (2000). Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J Virol Methods* 89:75–88.

Shukla, D. D. and Gough, K. U. (1979). The use of protein A, from *Staphylococcus aureus,* in immune electron microscopy for detecting plant virus particles. *Journal of General Virology* 45: 533-536.

Van Regenmortel, M.H.V. (1982). Serology and immunochemistry of plant viruses. Academic Press, New York, USA.

Van Regenmortel, M.H.V. and Dubs, M.C. (1993). Serological procedures. Pages 159–214. *In:* Matthews, R.E.F.(ed) Diagnosis of plant virus diseases. CRC Press, Boca Raton, Florida, USA.

Walkey, D.G.A., Lyons, N.F. and Taylor. J.D. (1992). An evaluation of a virobacterial agglutination test for the detection of plant viruses. *Plant Pathology* 41: 462–471.

Ward, E., Foster, S. J., Fraaije, B. A. and Mccartney, H. A. (2004). Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology* 145:1-16.

The IISTE is a pioneer in the Open-Access hosting service and academic event management. The aim of the firm is Accelerating Global Knowledge Sharing.

More information about the firm can be found on the homepage:
http://www.iiste.org

CALL FOR JOURNAL PAPERS

There are more than 30 peer-reviewed academic journals hosted under the hosting platform.

Prospective authors of journals can find the submission instruction on the following page: http://www.iiste.org/journals/ All the journals articles are available online to the readers all over the world without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. Paper version of the journals is also available upon request of readers and authors.

MORE RESOURCES

Book publication information: http://www.iiste.org/book/

Academic conference: http://www.iiste.org/conference/upcoming-conferences-call-for-paper/

IISTE Knowledge Sharing Partners

EBSCO, Index Copernicus, Ulrich's Periodicals Directory, JournalTOCS, PKP Open Archives Harvester, Bielefeld Academic Search Engine, Elektronische Zeitschriftenbibliothek EZB, Open J-Gate, OCLC WorldCat, Universe Digtial Library , NewJour, Google Scholar