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Serology Applied to Plant Virology

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1. Introduction

Virus infections affect seriously the quality and quantity of agricultural products around the world, especially in less developed countries. For this reason, the development and the adaptation of efficient and rapid techniques for diagnosis and control of plant viruses constitute an imperative and relevant necessity. Attempting to control plant diseases including those caused by viruses without sufficient information about their causal agents, their dissemination and surviving properties, usually results in inadequate control and many times, in a total failure. So, any attempt to establish a control program for a plant disease must be, always, preceded by a correct and precise laboratory diagnosis.

Several methods can be used for arriving at a correct and definitive diagnosis of plant disease caused by virus and at the beginning of the study of plant virology, the symptoms represented important characteristics for plant virus identification and characterization (Almeida & Lima, 2001; Astier et al., 2007; Mulholland, 2009; Naidu & Hughes, 2001; Purcifull et al., 2001). Nevertheless, it is often impossible to diagnose plant virus infections merely by observing host symptoms because symptoms caused by viruses vary according to the plant variety involved, the environmental conditions, the strain of the virus, the fact that sometimes different viruses can cause similar symptoms in the same plant species and sometimes the disease could result from the synergistic effect of infection caused by two different viruses. Additionally, symptoms may be very slight and inconclusive, or infected plants may be symptomless. However, bioassay through the use of a series of indicator plants remains an indispensable tool for detection and identification of plant viruses and the original symptoms are still of great importance for plant virus denomination (Astier et al., 2007; Lima et al., 2005; Mulholland, 2009).

Over the last few decades, laboratory-based methods have been developed and are now being used routinely in many laboratories for accurate diagnosis of diseases caused by plant viruses. These techniques involve physical, biological, cytological, serological, and molecular properties of viruses. Numerous laboratory methods have been developed and adapted for virus diagnosis, but serology is one of the most specific and easiest methods to

obtain a rapid and precise identification (Astier et al., 2007; Koenig et al., 2008; Lima et al., 2005; Naidu & Hughes, 2001; Purcifull et al., 2001). Serological assays have been developed and used successfully for many years for plant virus detection. Plant Disease is a journal that publishes papers that deal significantly with diagnosis and control of plant virus diseases. Of 164 papers published on plant viruses in 22 issues of this journal during 2010-2011, the authors of 115 papers (70%) utilized serology for virus diagnosis or detection. Enzyme-linked immunosorbent assay (ELISA) methods were used extensively (the authors of 105 papers utilized ELISA). Other serological techniques that were used included tissue blot immunoassays, immunoelectron microscopy (trapping and decoration), Western blots, dot blots, lateral flow rapid tests, immunocapture PCR, and double diffusion tests. Serological tests may be decisive in the final identification of an unknown virus and may be important in studying the relationship between virus species and strains. The great value of serological methods for plant virus identification is based on the specific reaction between the viral antigens and their specific antibodies. An antigen is a molecule that when injected into a vertebrate animal (usually a mammal or a bird), it can trigger an immune response in the animal which results in production of specific antibodies that can combine with the foreign antigen (Astier et al., 2007; Lima et al., 2005; Naidu & Hughes, 2001; Purcifull et al., 2001). Virus particles themselves and their proteins have several antigenic determinants (epitopes) which vary in their amino acid sequence and have the properties of inducing the production of specific antibodies. The virus particles, their coat proteins and the other types of virus induced proteins including those found in inclusion bodies can function as antigens (Astier et al., 2007; Hiebert, et al., 1984; Lima et al., 2005; Naidu & Hughes, 2001; Purcifull et al., 2001).

Antibodies are also proteins of the immunoglobulin group (Ig) produced against specific antigenic determinants and are present in the animal blood. The immunoglobulin G (IgG) is the most common type of Ig produced and, consequently most commonly involved in the serological tests for plant virus identification. This type of antibody is composed of four linked polypeptides with Y-shape of approximately 150 Kd, with two identical heavy chains and two identical light chains of polypeptides (Figure 1). The IgG has two identical combining sites specific for antigenic determinants called paratopes in the NH_2 terminal regions of the heave and light chains. These two identical combining sites have highly variable amino acid sequence, which permit the production of specific IgGs for the different virus epitopes. The C-terminal regions of the heavy chains are linked together by sulfur bridges to produce the Fc fraction of the antibody (Figure 1) which links specifically with protein A or cell membranes (Almeida, 2001; Purcifull et al., 2001).

Generally the methods that involve the antigen antibody reactions *in vitro* are simple and do not require sophisticated and expensive apparatus. The most serious limitation to using serology for plant virus identification and detection is the difficulty in producing a good virus specific antiserum. Most antisera used for plant virus identification and detection are usually prepared by immunizing mammals or birds with purified plant virus or their different types of proteins. However several other methods have been used to produce very specific antibodies, including monoclonal antibody (Mab) which consists of a single type of antibody that reacts with only one specific epitope of a virus protein. The production of Mab consists of a series of steps, including mouse immunization, collection of antibody producing cells, fusion with myeloma cells to produce a *hybridoma*, screening and selection process of *hybridoma* specific for the desired virus epitope. The *hybridoma* cells obtained can be stored in a freezer and used to produce the same monoclonal antibody according to

necessity (Cancino et al., 1995; Purcifull et al. 2001). Monoclonal antibodies have been used, for instance, to study antigenic differences and relationships among virus strains. Examples include studies of the relationships among strains of potyviruses that infect cucurbits (Baker et al., 1991; Desbiez et al., 2007)

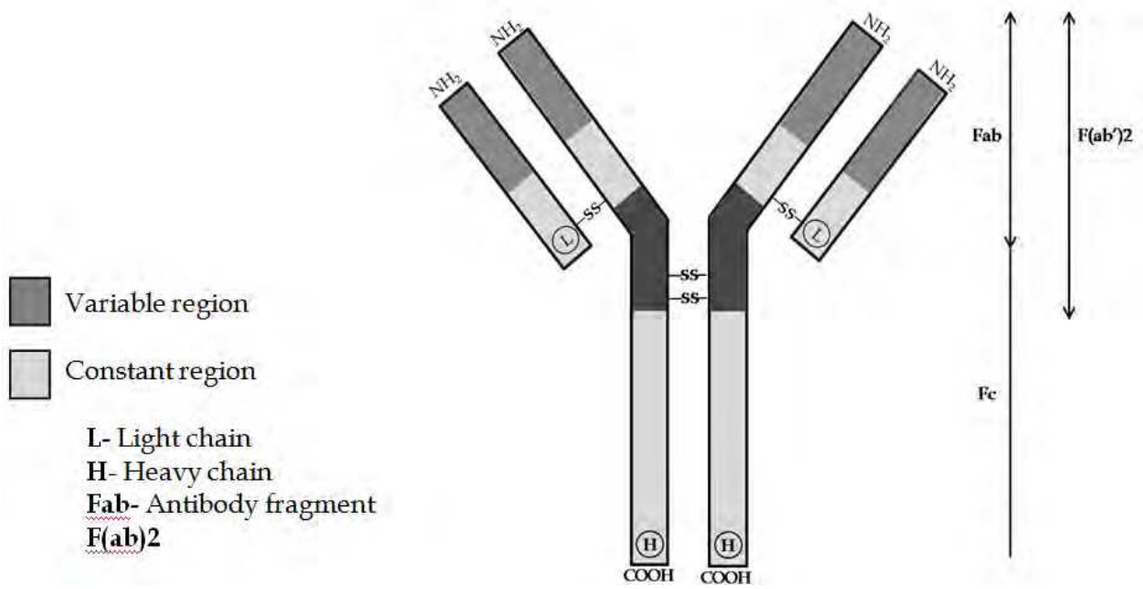


Fig. 1. Diagrammatic representation of the structure of an immunoglobulin G (IgG) molecule. Fab, F(ab)2, and Fc represent fragments obtained by enzyme cleavage of IgG.

Considering the importance of serology for diagnosing and surveying plant virus diseases, a program of polyclonal antiserum production for plant viruses was established at the Plant Virus Laboratory from the Federal University of Ceará (PVLab/UFC) in Northeastern Brazil, since the beginning of 1978. The serological plant virus survey program has been essential for establishing control programs such as roguing of virus infected plants in papaya (*Carica papaya* L.) orchards, indexing for production of virus free nurseries of banana (*Musa* spp.), melon (*Cucumis melo* L.) and watermelon [*Citrullus lanatus* (Thumb.) Matsum. & Nakai Mansf.], for production of virus-free seeds of cowpea [*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*] and for selecting sources of virus resistance in cowpea, melon and watermelon. Polyclonal antisera specific for several virus species and strains that infect tropical crops have been produced at the PVLab/UFC (Table 1).

Banana plantation is expanding in the Northeast of Brazil but surveys using enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) had shown that its productivity is still affected by *Cucumber mosaic virus* (CMV) family *Bromoviridae* genus *Cucumovirus* and *Banana streak virus* (BSV) family *Badnaviridae* genus *Caulimovirus* (Nascimento et al., 2011).

More than 20 virus species were found naturally infecting different cucurbit species, but only five species were serologically detected and isolated in Northeastern Brazil, including those that belong to the following families and genera: family *Bromoviridae* genus *Cucumovirus*: *Cucumber mosaic virus* (CMV); family *Comoviridae* genus *Comovirus*: *Squash mosaic virus* (SQMV) and family *Potyviridae* genus *Potyvirus*: *Papaya ringspot virus,* type

Watermelon (PRSV-W); *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) (Lima & Amaral, 1985; Moura et al., 2001; Oliveira et al., 2000; 2002).

Several factors affect papaya productivity in Northeastern Brazil, mainly the infectious diseases caused by viruses, which have been responsible for great losses in the crop throughout the country (Barbosa & Paguio, 1982; Lima & Gomes, 1975; Lima et al., 2001; Nascimento et al., 2010; Ventura, 2004). The most important viruses that infect papaya in Northeastern Brazil are *Papaya ringspot virus* (PRSV), family *Potyviridae*, genus *Potyvirus* (Lima & Gomes, 1975)*; Papaya lethal yellowing virus* (PLYV), possible family *Sobemoviridae*, genus *Sobemovirus* (Nascimento et al., 2010) and *Papaya meleira virus* (PMeV), which is still being characterized to be classified taxonomically by the ICTV (Marciel-Zambolim et al., 2003). Among the major virus diseases of passion fruit is the woodiness caused by *Passion fruit woodiness virus* (PWV) and *Cowpea aphid borne mosaic virus* (CABMV), both from the family *Potyviridae,* genus *Potyvirus.* These viruses cause great damage to the plant, inducing mottle, mosaic in young leaves, reduction of apical internodes, woodiness, deformation and reduction of fruit size (Bezerra et al., 1995).

Cowpea that is part of the traditional agricultural system of the semi-arid region of Brazil is largely infected with several virus species (Lima et al., 2005) including those from the following families and genera (Lima et al., 2005): family *Comoviridae* genus *Comovirus*: *Cowpea severe mosaic virus* (CPSMV); family *Potyviridae* genus *Potyvirus*: *Cowpea aphid-borne mosaic virus* (CABMV); family *Bromoviridae* genus *Cucumovirus*: *Cucumber mosaic virus* (CMV) and family *Gemniviridae* the genus *Begomovirus*: *Cowpea golden mosaic virus* (CGMV) (Florindo & Lima, 1991; Lima et al., 1979; 1991; 1993).

2. Polyclonal antiserum production

Most plant viruses are good and effective antigens which when artificially injected into a suitable vertebrate animal stimulate the production of specific antibodies that can be used in various serological tests. The rabbits are the most common animal choice in polyclonal antiserum production since they are easily housed and adapt well to being handled, but other animals such as mice, goats and chickens can be used. Nevertheless, the serious limitation in using serology for plant virus identification is the difficulty in producing a high titer virus specific antiserum. Through necessity, specific plant virus antisera have been produced in many different Plant Virus Research Laboratories throughout the world. Often, these collections have served as a major source of antisera for commercial companies that sell plant virus antisera and diagnosis services on an international basis. The PVLab/UFC in the State of Ceará, Brazil has been given an important collaboration in the production of antisera for tropical plant viruses (Table 1) and in the development and adaptation of serological techniques for plant virus identification since the 1970-decade (Lima & Amaral, 1985; Lima & Nelson, 1977; Lima et al., 1993; 1994; 2001; 2005; Nascimento et al., 2010; Oliveira et al., 2000; 2002). However, some virus isolates already identified at family and genus levels have been maintained in greenhouses for purification and subsequently antiserum production.

The production of antiserum for some plant viruses is, mainly, limited by the difficulty in purifying such viruses free from plant protein contaminants and in appropriate concentration to be used as antigen. Several other methods have been developed for plant

Crop/Virus specie/ Strain	Quantity of Antiserum (ml)	Number of samples in ELISA (1:2,000)*
Lettuce: *Lactuca sativa* L.		
Lettuce mosaic virus (LMV)	122	1,220,000
Curcubitaceae		
Cucumber mosaic virus (CMV)	422	4,220,000
Papaya ringspot virus - Watermelon (PRSV-W)	210	2,100,000
Squash mosaic virus (SQMV)	380	3,800,000
Watermelon mosaic virus (WMV)	191	1,910,000
Zucchini yellow mosaic virus (ZYMV)	62	620,000
Leguminosae		
Bean common mosaic virus (BCMV)	112	1,120,000
Blackeye cowpea mosaic virus (BlCMV)	90	900,000
Cowpea aphid-borne mosaic virus (CABMV)	429	4,290,000
Soybean mosaic virus (SoyMV)	85	850,000
Cowpea severe mosaic virus (CPSMV)		
$CPSMV_{CE}$	530	5,300,000
$CPSMV_{MC}$	311	3,110,000
$CPSMV_{CROT}$	320	3,200,000
$CPSMV_{AL}$	208	2,080,000
$CPSMV_{PB}$	125	1,250,000
Papaya: *Carica papaya* L.		
Papaya yellow mosaic virus (PLYV)	285	2,850,000
Papaya ringspot virus - Watermelon (PRSV-W)	210	2,100,000
Passionfruit: *Passiflora edulis* Sims.		
Cucumber mosaic virus (CMV)	422	4,220,000
Passion fruit woodiness virus (PWV)	42	420,000

Table 1. Polyclonal antisera specific for plant virus species that infect tropical crops produced in the Plant Virus Laboratory from the Federal University of Ceará (PVLab/UFC). * Number of samples that could be tested with available antiserum diluted 1:2,000 using 100 µl per well and replication of each sample.

virus antigen production, including the transformation of bacterial cells with the virus coat protein gene. This chapter will describe the most commonly used method to produce polyclonal antibodies specific for plant virus detection and identification. The process describes the production of polyclonal antibodies by rabbit immunization with purified virus preparations (Figure 2). Although not very specific for virus species/strains discrimination, the polyclonal antiserum is very useful and practical for virus surveys and indexing.

Several routes have been used to immunize rabbits with plant viruses, including intravenous, intramuscular and through the foot pad. The protocols of rabbit immunization vary greatly, but the following general immunization procedure has given satisfactory results for preparation of good titter plant virus antiserum. A rabbit is immunized with purified virus preparation by three weekly injections, using in each immunization an aliquot of 500 µl from the purified virus preparation (0.5-1.0 mg/ml) emulsified with equal volume of Freund incomplete adjuvant. The emulsified virus preparation is injected into the thigh

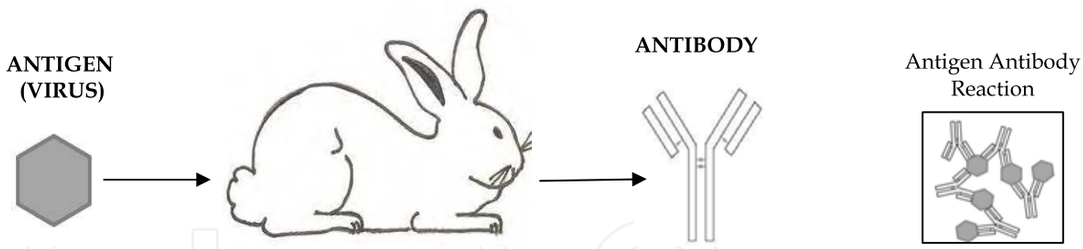


Fig. 2. Principle of polyclonal antiserum production specific for plant virus.

muscle of each hind leg or into the foot pad of the animal, and 15 days after the last injection the rabbit could be bled for antiserum production. Blood samples of 10 to up 50 ml are taken by nicking the marginal ear vein of the animal and collecting in glass centrifuge tubes. The tubes with the blood samples are maintained in a water bath at 37 ^{o}C for one hour and then centrifuged at 4,000 g for 10 min. The serum should be drained from the clots in conical-bottom tubes and centrifuged at 10,000 g for 15 min (Almeida & Lima, 2001; Purcifull & Batchelor, 1977). The clear supernatant serum from the second centrifugation is collected, evaluated by indirect ELISA and/or Ouchterlony double-diffusion tests (Almeida & Lima, 2001; Ouchterlony, 1962; Purcifull & Batchelor, 1977) and stored at -20 ^{o}C.

Antisera for some plant viruses were also obtained by oral immunization of rabbit or mouse with purified viruses or even with concentrated leaf extracts of infected plants (Florindo & Lima, 1991). Antiserum specific for CPSMV, SQMV and PLYV were obtained by immunizing rabbits with partially purified preparations or with foliar extracts of infected plants prepared in 0.15 M NaCl solution, in a proportion of 1:1 (w/v), clarified by centrifugation at 10,000 g for 10 min, using daily doses, for a period of seven days (Rabelo Filho et al., 2005).

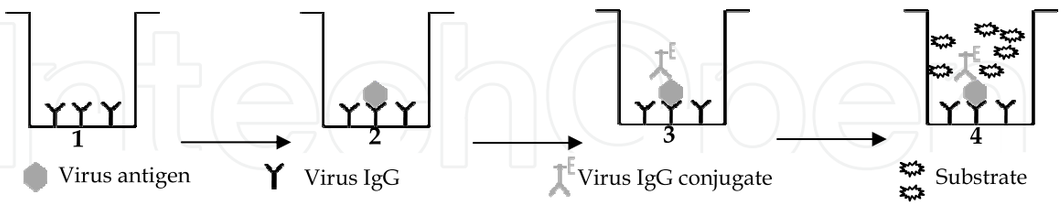
3. Serological methods

Numerous serological techniques have been developed for identification and characterization of plant viruses and the advent of the enzyme-linked immunosorbent assay (ELISA) has facilitated the use of serology for virus identification in large scale (Astier et al., 2007; Hampton et al., 1990; Lima et al., 2005; Naidu & Hughes, 2001; Nascimento et al., 2010; Purcifull et al., 2001; Van Regenmortel, 1982; Van Regenmortel & Dubs, 1993). All the serological techniques for virus identifications are based on the virus coat protein properties. The serological methods can be subdivided into two broader categories involving liquid and solid phase methods. The liquid phase methods can be represented by the double immune diffusion techniques in which the antigen and the antibodies react in agar media producing visible precipitates. In the solid phase methods, one of the reagents, usually the antibody, is trapped on a solid surface that could be nitrocellulose membrane, a microtitre plate, polystyrene or polyvinyl chloride plates. In this case the antigen-antibody reaction is detected by a labeled antibody as in the ELISA and its variations. In addition the virus particles are detected by direct visualization as with serologically specific electron microscopy (SSEM). An overview of SSEM, double immune diffusion technique, ELISA and its different variations will be provided in the following sections, including some illustrations, but detailed descriptions can be found in Van Regenmortel (1982), Hampton et al. (1990), Torrance (1992), Van Regenmortel & Dubs (1993), Purcifull et al. (2001) and Naidu & Hughes (2001).

3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a very specific and sensitive serological technique introduced to the study and identification of plant viruses in the 1970s (Clark & Adams, 1977; Voller et al., 1976). This technique is able to detect virus particles in very low concentrations and can be used with viruses of different particle morphology. Because of its adaptability, high sensitivity, and economy in the use of reagents, ELISA is used in a wide range of situations, especially for indexing a large number of samples in a relatively short period of time. The ELISA technique is based on the basic principle in which the virus antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties. The ELISA method is commonly accomplished in a 96-well polystyrene plate by adding the antigens and antibodies into the wells in an established sequence, involving several stages (Figure 3 A, B). In the final stage, the positive reactions are detected when a colorless substrate, usually p-nitrophenyl phosphate, undergoes a chemical change resulting in a yellow colored product as the result of exposure to the enzyme alkaline phosphate linked to the antibody. The degree of color change indicates the degree of reactivity that is read by an ELISA plate reader apparatus. It is always recommended to include a homologous antigen for the specific virus antibody and extracts from healthy plants to compare the absorption readings and to obtain a correct interpretation of the results. Differently from some other serological tests, the principle of ELISA techniques consists of detecting the antigen-antibody interactions by enzyme induced color reaction rather than by observing their precipitation. Although different variations of this serological technique have been developed, the direct and the indirect ELISA (Figure 3) are the most frequently used methods for diagnosis of plant virus diseases (Almeida & Lima, 2001; Clark & Bar-Joseph 1984; Cooper & Edwards 1986; Van Regenmortel & Dubs 1993). It is always recommended to include a homologous antigen (positive control) for the specific virus antibody and extracts from healthy plants (negative control) to compare the absorption readings and to obtain a correct interpretation of the results

A- Direct ELISA – DAS-ELISA



B- Indirect ELISA – PTA-ELISA

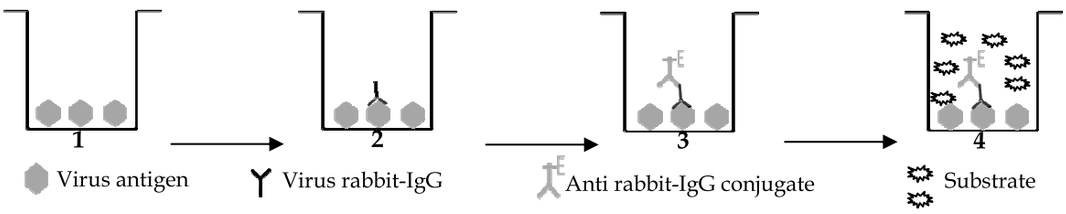


Fig. 3. Diagrammatic drawings of the most frequently used methods for detection of plant viruses: A- Direct ELISA and B- Indirect ELISA.

3.1.1 Direct ELISA

The direct ELISA (Figure 3A), also called double antibody sandwich (DAS-ELISA), is highly strain-specific and requires each detecting antibody to be conjugated to an enzyme. Typically, the enzyme is alkaline phosphatase. The first step in the test is the adsorption of virus-specific antibodies to the wells of ELISA plates. Unbound antibody is removed by washing, and the samples to be tested for virus antigen are added. Controls include extracts from known infected plants (positive control), and extracts from healthy plants (negative control). After incubation and washing, the enzyme-antibody conjugate is added. If virus attached to the coating antibody is present, the enzyme-antibody conjugate will combine with the virus. Plates are washed, and the colorless substrate (p-nitrophenylphosphate) is added. Positive wells will show a yellow reaction, due to the action of the conjugated enzyme (alkaline phosphatase) on the substrate. Negative wells will remain colorless. The colorimetric changes are measured in an ELISA reader, using a filter for 405 nm wave length. The washing procedures can be accomplished by the use of a plate washing apparatus, according to a programmed schedule. The quality of the antiserum is critical in achieving certain objectives, but a good, broad spectrum polyclonal antiserum will give satisfactory results in most virus indexing programs. On the other hand, monoclonal antibodies could be useful for identification and characterization of specific plant virus strains.

3.1.2 Indirect ELISA

Although the direct ELISA technique has high sensitivity and specificity, a method called indirect ELISA or plate-trapped antigen (PTA- ELISA) was developed to avoid the inconveniences and the difficulties of conjugating the enzyme with the IgG specific for each virus species to be used in the second layer of antibodies in direct ELISA. For this reason, the indirect ELISA or PTA-ELISA requires antibodies produced in two different animal species and the virus particles are trapped in the wells of the ELISA plate. The indirect ELISA also requires the use of a universal IgG enzyme conjugate which can be used with the antibodies of all virus species. The so called universal conjugate is composed of an IgG produced against the IgGs from the animal in which virus antibodies are raised linked to the enzyme alkaline phosphate. If the virus antibodies are produced in rabbits (e.g.), an anti-rabbit IgGs are produced in a second animal species such as goats or mice. So, the detecting antibody conjugate binds specifically to the primary virus specific antibody (Figure 3B).

In this method, the wells of the ELISA plate are, initially, covered with extracts from infected and healthy plant samples prepared in the proportion of 1:10 in carbonate buffer, pH 9.6. Following that, the virus particles are covered with a layer of virus specific antibodies produced in a rabbit, for example. The complex antigen-antibodies are covered with a universal conjugate that could be an anti-rabbit IgG produced in goats or mice linked to the enzyme alkaline phosphate. The linked anti-IgG-enzyme that react with the virus antibodies (IgG) which had reacted with the virus particles adsorbed to the bottom of the ELISA plate wells will be detected by colorimetric changes of a specific substrate that is added into the wells. Considering that a single universal antibody-conjugate can be used for detection of a wide range of plant viruses, the indirect ELISA technique is more economical, practical and suitable for virus detection in disease diagnosis and quarantine programs. For this reason, this ELISA technique is described in more detail in this chapter. Nevertheless,

this method has certain disadvantages such as competition between plant sap and virus particles for sites on the plate wells and, consequently, high background reactions. Although the indirect ELISA technique is not very specific for plant virus strain identification, it can be used for virus species differentiation by antiserum cross absorption. Using indirect ELISA, Oliveira et al. (2000) demonstrated that an isolate of *Papaya ringspot virus* type watermelon (PRSV-W) genus *Potyvirus* is serologically different from *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) also from the genus *Potyvirus* while isolates of WMV and ZYMV showed strong relationship with some differences that could be detected by reciprocal cross absorption with homologous and heterologous polyclonal antisera specific for WMV and ZYMV in indirect ELISA (Figure 4).

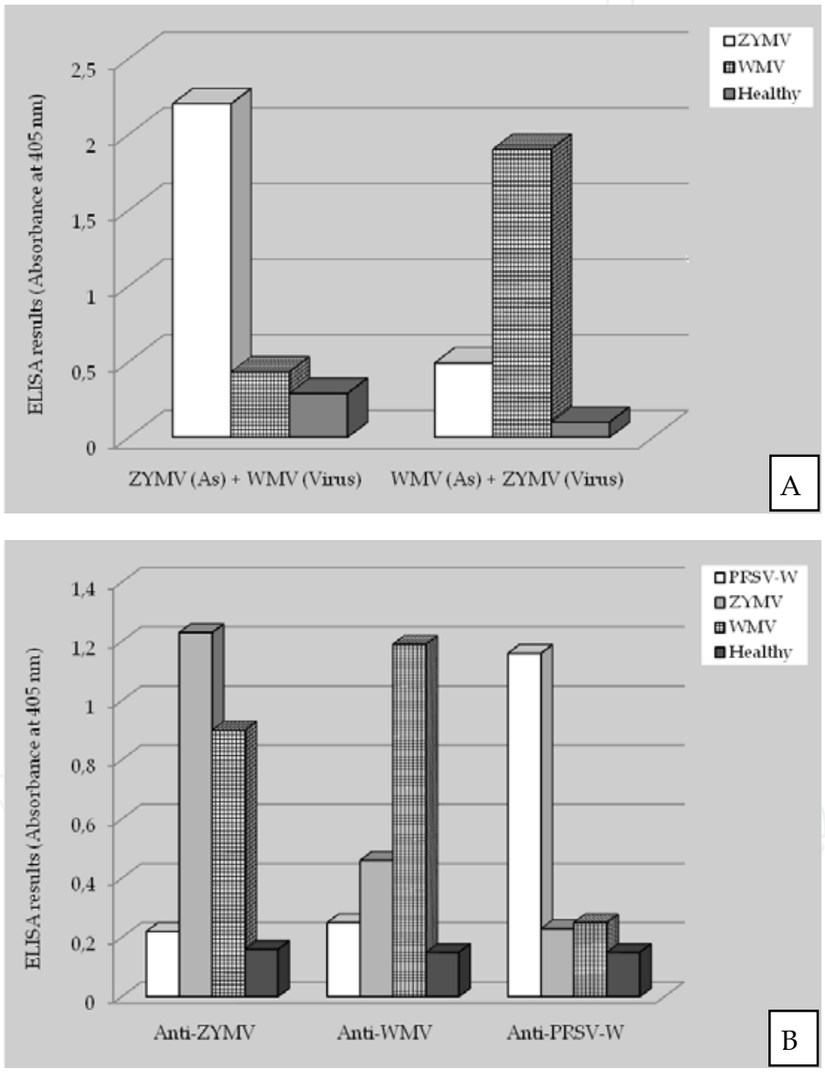


Fig. 4. Results of indirect ELISA showing serological relationship among *Papaya ringspot virus* type watermelon (PRSV-W), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV), using polyclonal antisera. A) Reciprocal serological tests with antiserum for each virus species; B) Reciprocal indirect ELISA with polyclonal antisera for WMV and ZYMV absorbed with the heterologous virus species.

The complete indirect ELISA protocol consists of, initially, covering the plate wells with extracts from infected and healthy plant tissues prepared in the proportion of 1:10 in carbonate buffer, pH 9.6 and the plates are incubated at 37 ^{0}C for 1 h. The plates are washed three times with PBS Tween buffer and 100 µl of the virus polyclonal antiserum produced in rabbit previously absorbed by extracts from healthy plants, diluted to 2,000 to 6,000 are added into the wells. The plates are incubated again at 37 ^{0}C for 1 h, after which they are washed three times with PBS-Tween. After drying, 100 µl of anti-rabbit IgG produced in goat or mouse conjugated to alkaline phosphatase, diluted in the proportion of 1:2,000 to 1:6,000 in a buffer contain 2% of polyvinylpyrrolidone, 0.2% of albumin and 0.02% of sodium azide are added into the wells. The plates are incubated once more at 37 ^{0}C for 1 h and washed again three times with PBS-Tween. The washing procedures can be accomplished by the use of a washing ELISA plates apparatus, according to programmed schedules. Finally, 100 µl of a substrate of p-nitrophenyl phosphate in the concentration of 0.5 mg/ml dissolved in a buffer containing 12% of diethanolamine and 0.25% of sodium azide, pH 9.8 are added into the wells. After 20, 40 and 60 min the plates are analyzed in the ELISA plate reader apparatus, using a filter for 405 nm wave length.

For the polyclonal antiserum absorption with extracts from healthy plants one volume of the antiserum is mixed with two volumes of concentrated healthy plant extracts and the mixture is incubated at 37 ^{o}C for 3 h. The mixture of antiserum and plant healthy extracts is centrifuged at 10,000 g for 10 min and the pellet is discarded. The polyclonal absorbed antiserum should not interfere with the results by reacting with plant proteins from extracts for healthy plants in all ELISA procedures.

3.1.3 Triple Antibody Sandwich (TAS- ELISA)

Another widely used ELISA variation is the triple antibody sandwich (TAS- ELISA), which is similar to the direct ELISA (DAS- ELISA), except that an additional antibody produced in another animal is used (Figure 5A). First, the bottom of the ELISA plate wells are coated with a virus antibody produced in a species of animal (e.g., rabbit) and the virus antigen is linked in the trapped antibodies. The virus antigen is covered with a second layer of virus specific antibody produced in another animal species (e.g., mouse or goat) and the presence of this antibody is detected by adding an enzyme-conjugated specific antibody (e.g., rabbit anti-mouse IgG), that does not react with the plate well trapped antibody, followed by colorimetric changes of a specific substrate that is added into the wells. Considering that virus specific monoclonal antibodies are usually used in the second layer of antibodies this procedure is an effective method of combining the broad reactivity of polyclonal antibodies in the virus trapping phase with the specificities of the monoclonal antibodies (Purcifull et al., 2001).

3.1.4 Protein A-Sandwich (PAS- ELISA)

This ELISA variation is based on the property of protein A combining specifically with the Fc portion of the IgG. The protein A is obtained from the cell wall of *Staphylococcus aureus* and has a molecular weight of approximately 42 – 56 Kd (Almeida, 2001). This protein is very stable at a broad pH range and it is produced commercially, including a protein A-enzyme conjugate to be used in plant virology. It is prepared by direct dilution in pure

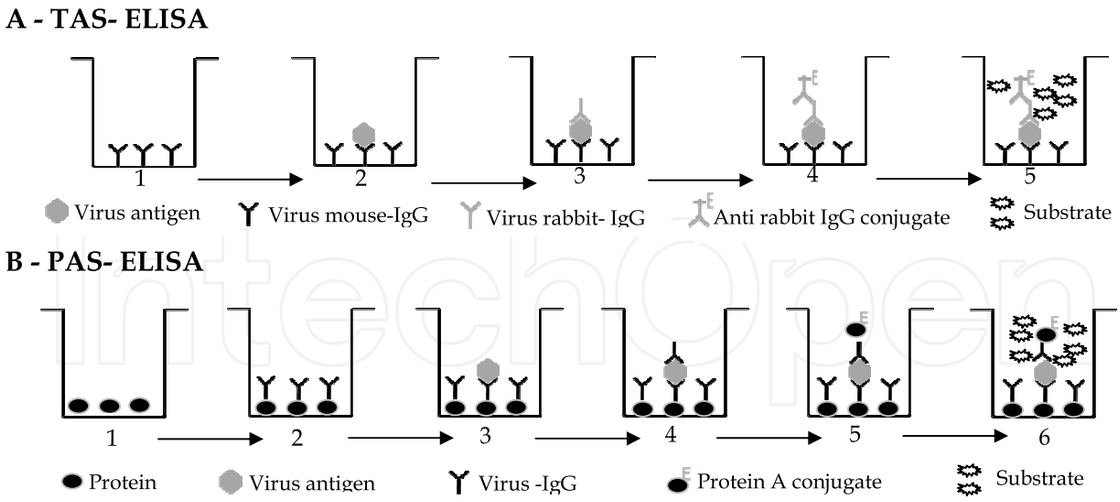


Fig. 5. Diagrammatic drawings of two ELISA variations for detection plant viruses: A) Triple antibody sandwich (TAS- ELISA) and B) Protein A-sandwich (PAS- ELISA).

water (1 mg/ml) and diluted in ELISA buffer to determine its adequate concentration for good results in PAS-ELISA. In the PAS-ELISA the antibody–virus–antibody layers which occur in the direct ELISA are sandwiched between two layers of protein A. The method consists of coating the bottom of the ELISA plate wells with a layer of protein A before the addition of the trapped virus antibody. Since the Fc region from the antibodies (IgG) has affinity to protein A, the added antibodies link specifically with the protein A trapped at the bottom of the wells keeping the virus antibodies in a specific orientation so that the F(ab´)2 portion of the antibodies will be free to trap the virus particles. The F(ab´)2 portion of the virus antibody orientation will increase the sensitivity of the PAS- ELISA by increasing the proportion of appropriately aligned antibody molecules. The exposed virus particles will link to the F(ab´)2 portion of a second added layer of the same antibodies which will be detected by an enzyme-conjugated protein A followed by colorimetric changes of a specific substrate that is added into the wells (Figure 5B).

3.1.5 Immune Precipitation ELISA (IP- ELISA)

Considering the problems with plant viruses whose particles are not well adsorbed in the ELISA plate wells, a new ELISA technique involving the immune virus particle precipitation (IP- ELISA) was developed and validated for detection of plant viruses from different families and genera, especially those from the genus *Comovirus* (Lima et al., 2011b). As for the other ELISA procedures, approximately 0.5 g of virus infected plant tissues are ground in ELISA extraction buffer and 0.5 ml from the obtained extract is mixed with an equal volume of specific antiserum diluted to 1:100 to 1:1000 (v/v). The mixture of infected plant extract and the antiserum is incubated at 37 ^{o}C for 3 h or overnight at 4 ^{o}C and centrifuged at 5,000 g for 10 min. The pellet containing the virus particles linked to the antibodies are ressuspended in ELISA extraction buffer and used as for conventional indirect ELISA (Figure 6). The IP- ELISA showed efficiency for detection of virus from different families and genera in different kinds of infected tissues. The immune virus precipitation followed by ELISA (IP- ELISA) for detection of viruses was shown to be a sensitive and practical

diagnostic technique for plant viruses, especially for *Cowpea severe mosaic virus* (CPSMV) and *Squash mosaic virus* (SQMV), family *Comoviridae*, genus *Comovirus* (Lima *et al.*, 2011b), whose virus particles do not adsorb well in the bottom of the plate wells (personal observation). This method increases the efficiency of the indirect ELISA (Figure 7) without the necessity of using protein A.

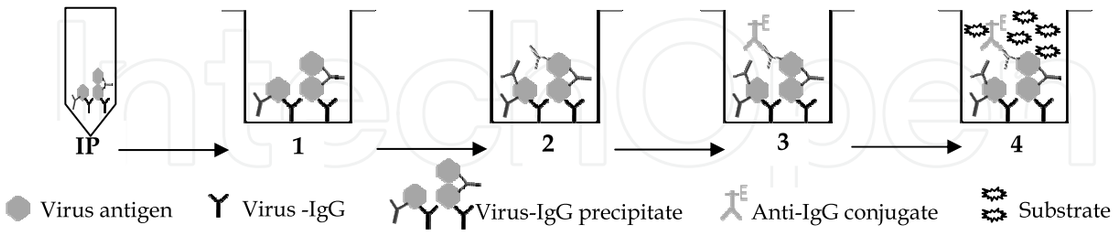


Fig. 6. Diagrammatic drawings of immune precipitation enzyme-linked immunosorbent assay (IP- ELISA) for detection of plant virus with particles that do not adhere well in the ELISA plates.

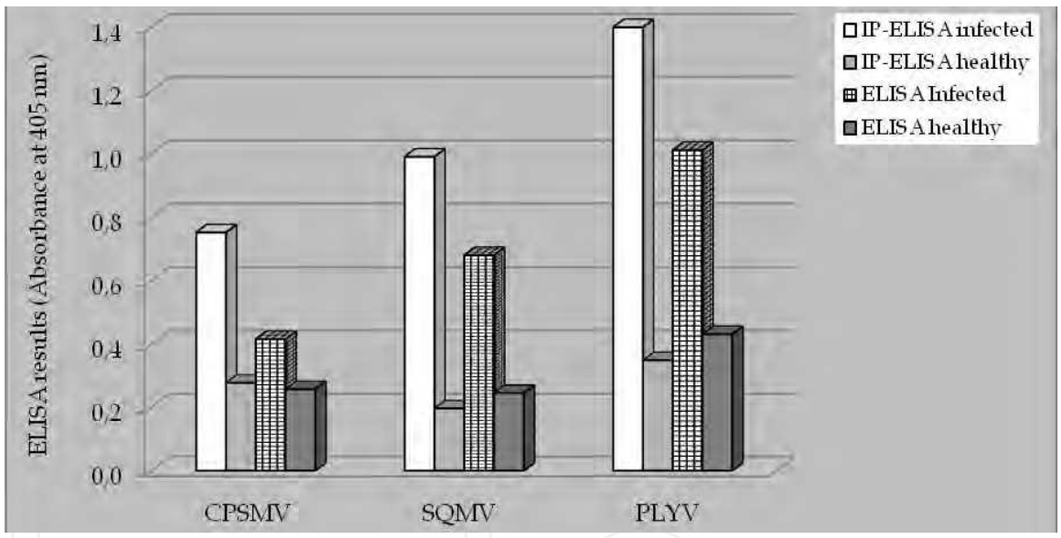


Fig. 7. Results showing the sensitivities of immune precipitation ELISA (IP- ELISA) and indirect ELISA for detection of *Cowpea severe mosaic virus* (CPSMV), *Squash mosaic virus* (SQMV) and *Papaya lethal yellowing virus* (PLYV) in infected tissues.

3.1.6 Rapid immunochromatographic tests for field and laboratory diagnosis

In recent years rapid serological tests using lateral flow immunochromatography have been developed for plant virus diagnosis (Tsuda et al., 1992; Ward et al., 2004). Samples can be analyzed in the field or the laboratory in less than 30 minutes. In one form of the test, strips containing virus-specific antibodies are placed in extracts from plants to be assayed, and the sample wicks upward. If virus is present, it reacts with specific antibody to give a visible band at the test line on the strip, which indicates a positive result. Such tests have shown good agreement with results obtained by conventional ELISA tests (Agdia, 2007a; 2007b). Variations of the immunochromatographic tests for certain plant viruses are available commercially, eg., at Agdia, Bioreba, or Forsite Diagnostics (Pocket Diagnostic).

3.1.7 Immune Capture Polymerase Chain Reaction (IC-PCR)

A technique called immune capture polymerase chain reaction (IC-PCR), which combines the technical advantages of PCR with the practical advantages of serology, was developed for the detection of several different plant viruses (Nolasco et al., 1993). First, microtiter tubes are coated with specific virus antibodies and incubated at 37°C for 2 h. After washing, the microtiter tubes coated with the antibodies will trap the virus particles which will be disrupted followed by the release of viral nucleic acid. The virus nucleic acid is amplified by polymerase chain reaction (PCR) or reverse transcription PCR (RT- PCR), and the entire procedure is carried out in a single microtiter tube (Figure 8A). According to Nolasco et al. (1993) this method also can be used with monoclonal antibodies raised to double-stranded RNA permitting the possibility of detection of satellite-RNAs or viroids. The IC-PCR has been shown to be a very useful alternative in virus detection from plant material and insect vectors (Candresse et al., 1998; Mumford & Seal, 1997; Mulholland, 2009; Wetzel et al., 1992).

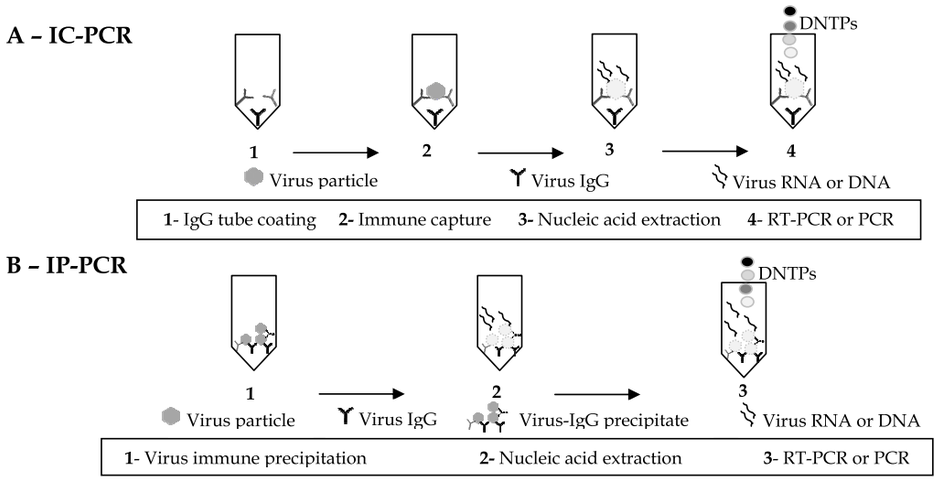


Fig. 8. Diagrammatic drawings of immune capture polymerase chain reaction IC-PCR (A) and immune precipitation polymerase chain reaction IP-PCR (B) for plant virus detection.

3.2 Immune Precipitation Polymerase Chain Reaction (IP-PCR)

A new PCR technology involving virus particles immune precipitation (IP-PCR) was developed for identification and molecular characterization of plant viruses from different families and genera (Lima et al., 2011a). The new technique is very practical, specific and minimizes problems with RNA extraction combining the serological properties and the technical advantages of virus nucleic acid amplification (Figure 8B). Approximately 0.5 g of plant tissues infected with virus are grind in 1.0 ml of extraction buffer (0.15 M Na_2CO_3, 0.035 M of $NaHCO_3$ and 0.007 M of sodium diethyldithiocarbamate, pH 9.6). The extract is obtained by straining through triple cheesecloth, and 0.5 ml was mixed with an equal volume of specific virus polyclonal antiserum diluted to 1:500 (v/v) in the antiserum buffer (PBS-Tween 20 with 0.5 M polyvinylpyrrolidone, 0.2% ovalbumin, 0.03 M sodium azide, 0.17% of sodium diethyldithiocarbamate). The mixture is incubated at 37 ^oC for 3 h or overnight at 4 ^oC, and centrifuged at 5,000 g for 10 min. The precipitated virus particles linked with their specific antibodies are disrupted for RNA extraction using Trizol® Reagent according to the manufacturer's instructions. Alternatively the virus RNA is extracted with

a RNA isolation system (Promega, Madison, WI). A first strand cDNA is synthesized from each virus RNA using their antisense specific primers and the M-MLV Reverse Transcriptase (Promega, Madison, WI). The cDNA fragments corresponding to virus RNA are amplified by PCR. This newly developed immune virus precipitation followed by PCR or RT-PCR (IP-PCR) was shown to be a practical and sensitive technique for detection of plant viruses. Similar to IC-PCR, the IP-PCR has the advantage of combining the analyses of the serological and the molecular virus properties. Additionally, the IP-PCR technique reduces the risk of cross-contamination with plant RNAs and does not require expensive equipment and reagents. The application of the new IP-PCR technology for detection of virus in infected plant tissues has been useful for all the virus-host combinations tested so far. The new IP-PCR technique provides partial virus particle purification by its specific immune precipitation, and it should be especially useful for detecting viruses that are present in low or variable titers in plant species which contain various forms of PCR amplification inhibitors. The method has the typical sensitivity of assays based on PCR combined with the virus serological properties and is not more laborious than ELISA procedures.

The IP-PCR was shown to be capable of detecting the presence of RNA of Papaya lethal yellowing virus (PLYV) up to dilution of 1:10,000 of the infected plant extracts and did not amplify any cDNA from healthy plant extracts (Figure 9). The new technique also was demonstrated to be efficient for detecting the presence of five virus species from three different families: Bromoviridae, Comoviridae, Potyviridae and Sobemoviridae (Lima et al., 2011a). In addition to its sensitivity and specificity, similar to IC-PCR (Nolasco et al., 1993; Mumford & Seal, 1997; Candresse et al., 1998), the IP-PCR has the advantage of combining the analyses of the serological and the molecular virus properties. Additionally, the IP-PCR technique reduces the risk of cross-contamination with plant RNAs and does not require expensive equipment and reagents.

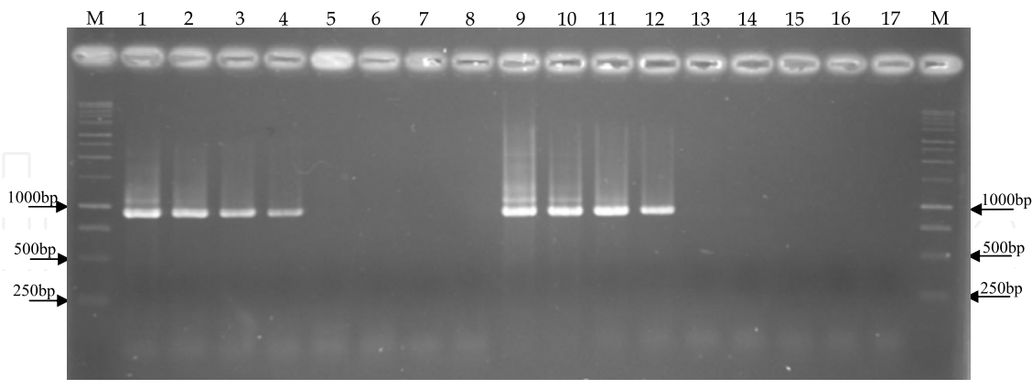


Fig. 9. Results of reverse transcription polymerase chain reaction (RT-PCR) and immune precipitation PCR (IP-PCR) of plant tissues infected with *Papaya lethal yellowing virus* (PLYV) at different dilutions of the plant extracts. Lanes 1-4: RT-PCR of PLYV infected papaya in the dilutions of 1:10 (1), 1:100 (2), 1:1,000 (3) and 1:10,000 (4); Lanes 5-8: RT-PCR of healthy papaya in the correspondent dilutions; Lanes 9-12: IP-RT-PCR of PLYV infected papaya in the dilutions of 1:10 (9), 1:100 (10), 1:1,000 (11) and 1:10,000 (12); Lanes 13-17: IP-RT-PCR of healthy papaya in the correspondent dilutions and Lane M: DNA ladder with standards of indicated length in Kb. The gels were stained with ethidium bromide analyzed under UV light.

3.3 Immunoblotting methods

Serological solid support matrix methods similar to ELISA techniques were developed in which the virus antigens are trapped onto a membrane rather than in a microtitre plate. Similar to indirect ELISA, virus particles or their proteins are immobilized on nitrocellulose or nylon membranes (Almeida, 2001; Purcifull et al., 2001). As distinguished from indirect ELISA, it is not necessary to use an ELISA reader for detecting the virus antibodies interactions and for this reason it is not possible to quantify the results by numerical absorbance values (Almeida, 2001; Astier et al., 2007). According to the process by which the virus antigens are applied in the membranes these methods can be divided into three categories: a) Western blot; b) Dot blot or dot immuno binding assay (DIBA) and c) Tissue blot immuno assay (TIBA).

3.3.1 Western blot

In this method the virus protein antigens are transferred from polyacrylamide gels in which they were previously separated by electrophoresis to nitrocellulose or nylon membranes. Several methods can be used to transfer the virus protein and the electro-blotting is the most used system. Similar to ELISA techniques, the proteins are detected in the membrane by the use of specific enzyme labeled antibodies (Almeida, 2001; Purcifull et al., 2001). Different antibody labeling systems, including biotin-avidin and chemiluminescent systems are sometimes used to increase sensitivity. The Western blot is usually used for characterization of virus proteins rather than for detection since it has the advantage of determining the serological and molecular properties of the virus protein.

3.3.2 Dot Blot or Dot Immuno Binding Assay (DIBA)

Although based on the Western blot principle, the dot immunoblotting assay (DIBA) requires a simple and easier method to prepare and apply the samples on nitrocellulose or nylon membranes (Almeida, 2001; Astier et al., 2007; Purcifull et al., 2001). The samples containing the virus antigens are prepared by grinding tissues in Tris-buffered saline and the extracts are applied directly on the membrane. The sample application on the membrane is usually accomplished through the use of a plastic mold with 96 wells which presses the membrane marking the places where the samples should be applied. Usually the spaces not occupied by the antigens on the membrane are blocked with neutral protein solution. The addition of virus IgG produced in rabbit and the anti-rabbit IgG produced in mouse follow protocols similar to indirect ELISA or PTA-ELISA (Figure 3), except that the positive reactions in DIBA are recorded as colored dots on the membrane. Considering that DIBA is a simple, less laborious and quick test, it can be used routinely for plant virus indexing and survey programs (Almeida, 2001; Astier et al., 2007; Banttari & Goodwin, 1985; Graddon & Randles, 1986; Heide & Lange, 1988; Lange & Heide, 1986; Makkouk et al., 1993; Purcifull et al., 2001; Rybicki & Von Wechmar, 1982). One disadvantage of DIBA is the possibility of sap components interfering with the antigen-antibody reactions, resulting in subsequent problems with the diagnostic results.

3.3.3 Tissue Blot Immune Assay (TIBA)

This is the simplest immunoblotting assay technique developed for virus antigen detection in different types of plant and insect tissues. It is a variation of DIBA in which the samples

consist of preparation of infected plant tissues. The tissue immunoblotting assay (TIBA) can be used to detect virus antigens in plant tissues such as leaf, stem, bulb, tuber, root and fruit or insect vectors of plant viruses. The tissues are cut with razor blades and pressed on the membrane to transfer the virus particles or protein (Hsu & Lawson, 1991; Makkouk et al., 1993; Navot et al., 1989; Polston et al., 1991; Purcifull et al., 2001). The detection of the virus antigens applied on the membrane is accomplished by protocols similar to those used for indirect ELISA or PTA-ELISA (Figure 3) and for DIBA. As with DIBA, sometimes the sap components can interfere with the diagnostic results and the color of the sap interferes with the observation of weak virus antigen antibody reactions. The TIBA technique has been demonstrated to be sensitive enough to evaluate the *in situ* distribution of plant virus species from different families and genera (Astier et al., 2007; Makkouk et. al., 1993). As with DIBA the virus-antibody interactions are not quantified since the results are not presented by numerical forms by absorbance values as in the ELISA variations (Almeida, 2001; Astier et al., 2007). The low cost and simplicity of these immunoblotting assay techniques (DIBA and TIBA) make them useful for laboratories with limited facilities (Astier et. al., 2007; Makkouk & Kumari, 2002). Another advantage of these methods is that the samples can be blotted onto the membranes right in the field or in simple laboratories and shipped for further processing at a more equipped laboratory (Naidu & Hughes, 2001).

3.4 Serologically specific electron microscopy

A technique known as serologically specific electron microscopy (SSEM) was introduced by Derrick (1973) and it has become widely used in plant virology (Derrick & Brlansky, 1976; Lima & Purcifull, 1980; Milne, 1991; Naidu & Hughes, 2001). This technique combines the specificity of serological properties with the morphology of the virus particles visualized in the electron microscope. Virus particles are selectively trapped on antibody-coated grids with little contaminating host-plant material (Lima & Purcifull., 1980). Copper electron microscope grids coated preferably with parlodion film are treated with the virus specific antiserum diluted to 1:1,000 – 1:6,000 in an appropriate buffer. The antibody coated grids are washed with buffer and floated on drops of extracts from virus infected plant tissue at room temperature for 3 – 4 h. After washing again for three times, the grids are stained with 1.0% uranyl acetate in 50% ethanol, dried and examined in the electron microscope (Figure 10).

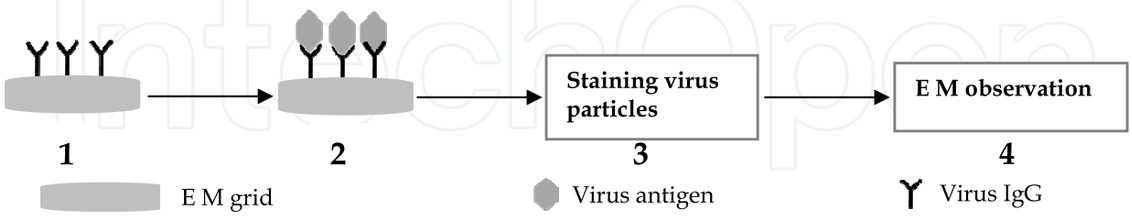


Fig. 10. Diagrammatic drawings of serologically specific electron microscopy (SSEM) for detection of plant virus.

This technique has been used to detect polyhedral and rod shaped virus (Figure 11). It has the great advantage of requiring only very small amounts of antiserum and antigens, and does not require the use of labeled antibodies. For those reason, the SSEM technique is more sensitive for detecting virus particles in leaf extracts than the leaf dip method and shows

little contaminating host-plant material (Figure 11; Lima & Purcifull., 1980). Other advantages are that polyclonal antiserum can be used without interfering in the virus particle observations and samples can be blotted onto the IgG coated grids in one small laboratory and shipped for further analysis in an electron microscope Center. Considering the requirement of an electron microscope, the SSEM is recommended for confirmatory tests using small numbers of samples. Some variations of the SSEM have been developed including the decoration of virus particles (Purcifull et al., 2001).

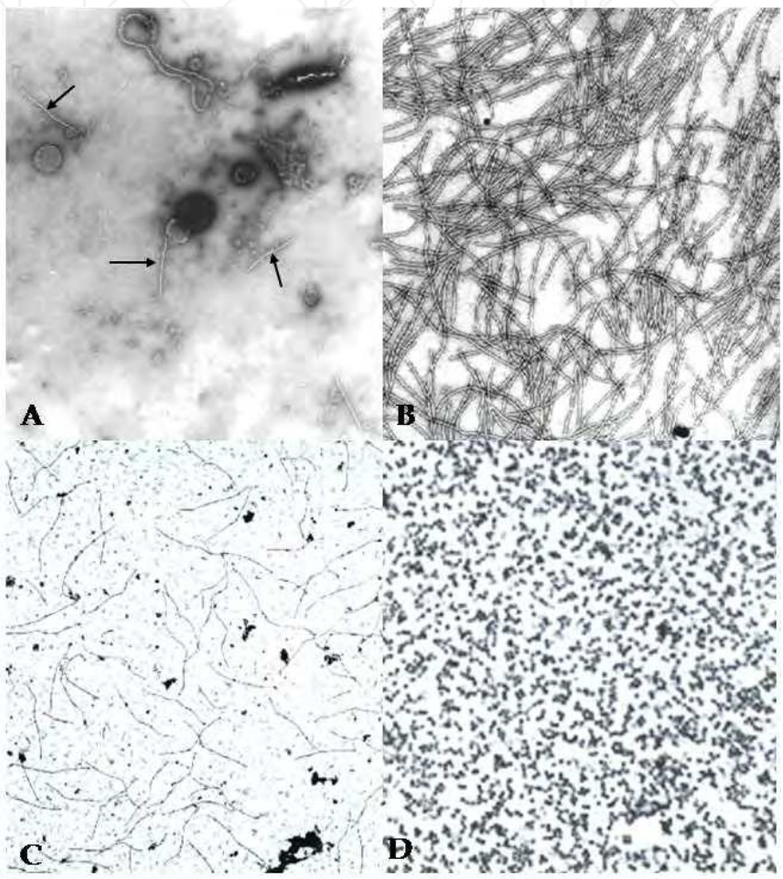


Fig. 11. Electron micrographs of virus particles in the transmission electron microscope. A) Leaf dip preparation with *Blackeye cowpea mosaic virus* (BlCMV) negatively stained with phosphotungstic acid (arrows indicate virus particles); B) Purified BlCMV particles negatively stained with phosphotungstic acid; C) BlCMV particles on grids sensitized with antibodies for BlCMV according to the method for serologically specific electron microscopy (SSEM), and positively stained with uranyl acetate and D) Hexagonal particles of *Cowpea mosaic virus* (CPMV) on grids sensitized with antibodies for CPMV according to the SSEM technique, positively stained with uranyl acetate.

3.5 Double immunodiffusion

The double immunodiffusion test in agar gel also known as the Ouchterlony test (Ouchterlony, 1962) is based in the fact that the antigens and the antibodies deposited into

wells opened in agar gels diffuse in all directions through the medium. This test is preferentially developed in Petri dishes but it can also be accomplished on microscope slides. Reactant wells are opened in the agar gel with cork borers or adjustable gel cutting device and the agar plugs are removed with glass tubing connected to a vacuum pump. A useful gel pattern consists of up to six peripheral antigen wells of 3 to 7 mm in diameter, surrounding a central serum well. Each peripheral well is 4 to 5 mm from the central well at the closest point (Figure 12). The antigens are pipetted into the peripheral wells and the antiserum into the central well. Reactions usually appear within 12 h and are complete within 24 -48 h after the addition of the reactants. The results can be viewed and recorded photographically by dark-field illumination (Purcifull & Batchelor, 1977).

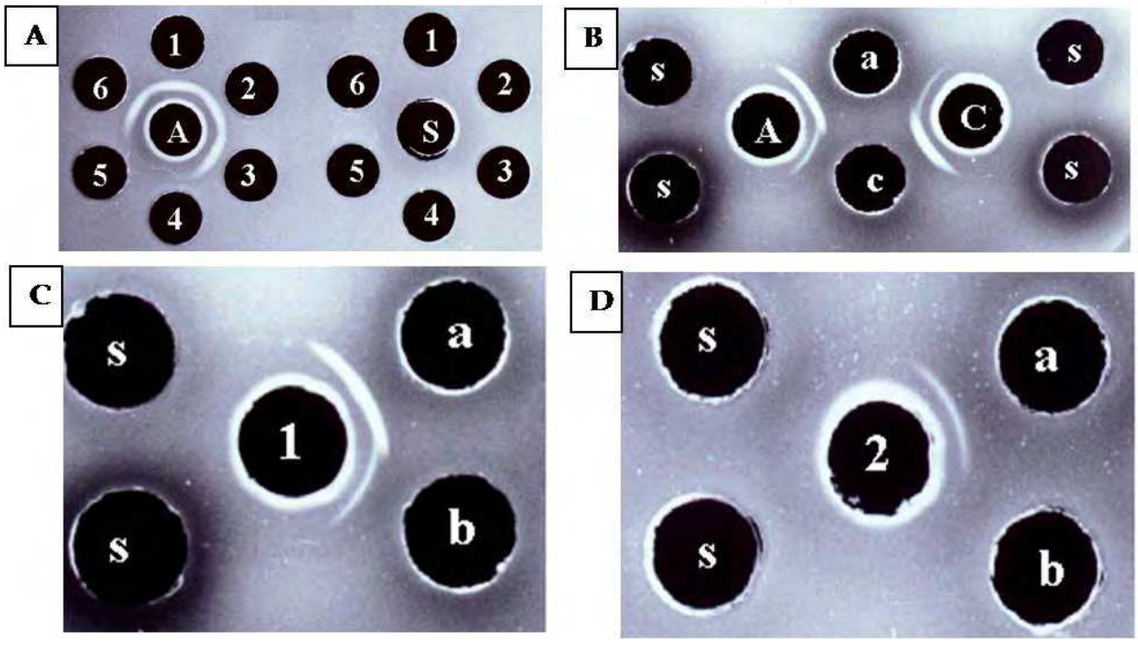


Fig. 12. Serological double immune diffusion test in agar gel: A) A hexagonal arrangement with the antigens in peripheral wells and the antiserum (A) and normal serum (S) in the central well; extracts of infected plants (1, 2, 3 and 6) and extracts from healthy plant (4 and 5); B) Reciprocal tests with antisera to: (A) *Blackeye cowpea mosaic virus* (BlCMV) and (C) *Bean common mosaic virus* (BCMV) , showing spur formation between the virus antigens; C, D) Intra-gel cross absorption tests with BlCMV (C) and with BCMV (D): (1) antiserum for BlCMV, (2) extracts of plants infected with BCMV and 12 h later antiserum for BlCMV; (a) extracts of plants infected with BlCMV, (b) extracts of plants infected with BCMV and (s) extracts of healthy plants.

Depending on the medium composition this method can be used for polyhedral or rod shaped viruses. Although the polyhedral particles of 28 to 40 nm could diffuse through the medium pores, the long rod shaped viruses over 300 nm need to be dissociated into their coat protein units to diffuse through the medium (Purcifull & Batchelor, 1977; Purcifull et al., 2001; Almeida & Lima, 2001). This can be accomplished by the use of several chemical compounds, especially sodium dodecyl sulfate (SDS). Although not very sensitive this test is still preferred to evaluate the antiserum titers, for routine diagnosis of a few samples and to

study the serological relationship among some plant viruses. Using reciprocal double immunodiffusion tests with polyclonal antisera, Oliveira et al. (2000) confirmed the type of serological relationship among PRSV-W, WMV and ZYMV demonstrated by indirect ELISA.

The intra-gel cross absorption test is a variation of the standard double immunodiffusion technique recommended to define the serological relationship among virus species or strains. It is still used to define the relationship among virus species and isolates from the genera *Potyvirus* and *Comovirus*. To develop this method, very highly concentrated extracts of heterologous viruses are deposited into the central well 12 h before the antiserum and the homologous virus are pipetted into their respective wells (Figure 12).

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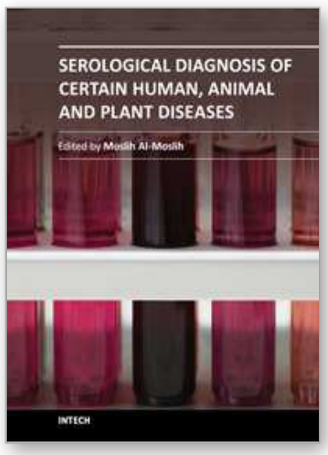

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This book explains the concept of serological methods used in laboratory diagnoses of certain bacteria, mycoplasmas, viruses in humans, animals and plants, certain parasitic agents as well as autoimmune disease. The authors present up-to-date information concerning the serological methods in laboratory diagnosis of such infectious diseases. Section one deals with the serological methods for bacteria. Section 2 deals with serological methods in human, animal and plant viruses. Section 3 is concerned with the serological laboratory diagnosis of echinococcus and human toxocariasis agents. The last section deals with serological laboratory methods in the diagnosis of coeliac disease.

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