ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 4, Issue, 12, pp. 430-437, December, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

A REVIEW PAPER ON DIAGNOSTICS OF POTATO VIRUSES

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ARTICLE INFO

Article History:
Received 25th September, 2012
Received in revised form
20th October, 2012
Accepted 23rd November, 2012
Published online 28th December, 2012

Key words:
Potato,
Virus,
Viroids.

ABSTRACT

Potato is a vegetatively propagated crop and ranks fourth in production after wheat, rice and corn. Potato is a host to many fungal and viral diseases. More than three dozen plant viruses, a viroid and phytoplasmas can infect potato crop. Viruses known to infect potato in India include PVX, PVS, PVA, PVM, PVY, *Potato leaf roll virus* (PLRV), *Tomato leaf curl New Delhi virus* (ToLCNDV), *Potato spindle tuber viroid* (PSTVd) and *Groundnut bud necrosis virus* (GBNV. PVX, PVS, PVA, PVM and PVY occur commonly. Present review article describes various techniques for identification and detection of these viruses and viroids.

INTRODUCTION

Potato is a vegetatively propagated crop and ranks fourth in production after wheat, rice and corn and provides wholesome food (Rhodes, 1982). Nevertheless, it holds a great potential as food for the ever increasing developing-world population on account of its higher dry matter and protein production per unit time and area as well as its versatility to adapt to a wide range of climates. It provides carbohydrates, proteins, minerals, vitamin C, a number of B group vitamins and high quality dietary fibers (Swaminathan and Pushkarnath, 1962). Potato is grown as short duration crop and was introduced in Indian sub-continent in early 17th century. Potato is a host to many fungal and viral diseases. More than three dozen plant viruses, a viroid and phytoplasmas can infect potato crop (Khurana and Singh, 1986; Jeffries, 1998). A lower incidence (say upto 5-10%) of the viruses, either singly or even upon combined infections, in the current season or coming from the previous season/crop, hardly reduces the yields (Khurana and Singh,1988). But a higher virus incidence coupled with early or severe infection causes serious depressions in the tuber yield (Hooker, 1981; Garg, 1987; Khurana and Singh, 1986). Accurate estimates about the economic losses caused by potato viruses are lacking. However, based on rough estimate, they may cause losses up to 50% in tuber yield (Pushkarnath,1976).Viruses known to infect potato in India include PVX, PVS, PVA, PVM, PVY, *Potato leaf roll virus* (PLRV), *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Groundnut bud necrosis virus* (GBNV) (Garg and Khurana, 2003). PVX, PVS, PVA, PVM and PVY occur commonly; PLRV is prevalent in areas where vector pressure is high round the year; ToLCNDV is a problem in the early planted seed crop in the north Indian plains while GBNV occurs only in the early planted crop in the central and western parts of India (Garg et al., 2001; Jain et al., 2004). ToLCNDV causes potato apical leaf curl disease while GBNV causes potato stem necrosis disease. GBNV is not transmitted through tubers. ToLCDNV is transmitted by the whitefly *Bamisia tabaci* and GBNV by *Thrips palmi* (Khurana et al., 1998; Garg et al., 2001). Unlike bacterial and fungal diseases, viruses can not be economically controlled with chemicals. Their management involves early detection by means of sensitive diagnostic methods (Mathews, 1991; Hull, 1993).

Until very recently only methods based on the coat protein of the virus were routinely used in plant virus detection. Among them, enzyme linked immunosorbent assay (ELISA) has been extensively used due to its high throughput, easy in use, sensitivity and automation. There has been tremendous progress in nucleic acid research during the last couple of decades which has also revolutionized molecular diagnostics of plant viruses. Of these, molecular hybridization (reviewed by Hull, 1993; Miller and Martin, 1988) and polymerase chain reaction (PCR) (reviewed by Hensonj and French, 1993) have been of particular significance in plant virology. Serology has been combined with the PCR technique in what is called as immunocapture-PCR (IC-PCR). It is more sensitive and economical than conventional PCR, as it does not involve viral RNA purification. A simpler method is direct virus immobilization on a solid phase like NCM (direct binding-PCR (DB-PCR) (Rowhani et al., 1995). Detection levels achieved by the DB-PCR are generally lower than those of IC-PCR. Another attractive PCR-based alternative was described

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by Olmos et al. (1996); it involved direct tissue-imprinting on NCM. This technique, called print-capture PCR (PC-PCR) avoids the need for grinding the samples. Complementary DNA libraries have been prepared by using the genomic RNAs of *Potato Leaf Roll Virus* (PLRV) and *Beet western yellows virus* (BWYV), by random as well as oligo (dT) priming of poly-adenylated RNA. Selected clones have been used to demonstrate specific virus detection in total nucleic acid preparations of PLRV or BWYV infected *Physalis floridana* Rydb (Prill et al., 1988). Mukherjee et al. (2003) had cloned and sequenced coat protein (CP) gene of an Indian isolate of PLRV. It was the first report of PLRV coat protein sequence from India. They further used run-way transcripts of the cloned CP gene to detect PLRV in tissue imprints and tissue dilutions.

Detection and Identification of Viruses In Potatoes

At the moment, unlike for bacteria and fungal diseases, no chemicals exist that could be used as a direct field control of viral or viroid disease in plants. Prevention of infection spreading in the field, therefore, is the most viable method of plant virus disease management. The first requirement for this is the reliable detection of virus infection in the seed stock and elimination of the infected tubers (Mathews, 1991; Hull, 1993). Plant virus particle comprises nucleic acids and a capsid (coat) consisting of coat protein molecules. Methods for plant virus diagnosis have evolved in parallel to the progress in the knowledge of these components. Earlier, only methods based on the protein component of the viral particle were routinely used in plant virus detection. Among them, the serological ones (ELISA) were used due to their ease in use, sensitivity and automation. However, one disadvantage of serology lies in the fact that only 2-5% of the genetic information of viral genome encodes for antigenic determinants on the surface of the coat protein (Hull, 1986). Moreover, serological techniques can not be applied to viroid diagnosis because viroids do not encode coat proteins. Therefore, viroid detection must rely on bioassays or by direct detection of the genomic viroid RNA. Bioassays are not appropriate for screening large populations. Similarly, reverse gel electrophoresis technique, used for the detection of viroid RNAs, would not be suitable for large sample numbers. The extraordinary progress made on the nucleic acid research, during the last more than two decades, and the application of recombinant DNA technology to plant virology have permitted using diagnostic methods based on the nucleotide sequence of the genome component of viruses and viroid. Among them, molecular hybridisation and polymerase chain reaction (PCR) were incorporated into the diagnostic field of plant virology.

Test Plants

In the field, symptoms give the first clue to the identity of a virus. In the laboratory and greenhouse, symptoms produced in a range of test plants may be of considerable diagnostic value but insufficient to allow positive identification. However, visual inspection for symptoms is often not adequate, since the symptom expression can be highly variable. Experimental host plants, under standardized conditions, will exhibit consistent and characteristic disease symptoms when infected with a particular virus. Knowledge of the host range of a particular virus and the symptoms it elicits are usually essential for studying new viruses or virus strains and may provide useful clues to the identity of an unknown virus. Several herbaceous plants are susceptible to a large number of viruses. Some *plant* species react to mechanical inoculation with potato viruses by exhibiting local lesions or systemic symptoms. The following test plants can be used for detecting, and in many instances, diagnosing potato virus infections. *Physalis floridana*, *Gomphrena globosa*, *Datura stramonium*, *D. metel*, *Chenopodium amaranticolor*, *Lycopersicon esculentum*, *Solanum demissum x* S. *tuberosum* and *Nicotiana glutinosa.* These methods are, however, not suitable for processing a large number of samples in a short time and have the disadvantage that strains of the same virus may produce different symptoms on the same host and they are less sensitive (Ball1974; Hunnius, 1982; Lal and Khurana, 1983).

Serology

It has been shown that booster immunization with a partially purified *Potato virus Y* preparation, after priming with SDS-PAGE coat protein, enhances the binding capacity of capture antibodies as measured by ELISA (Gera et al., 1999). Antibodies produced by a hybrid cell (hybridoma) formed by fusion of a B lymphocyte cell with a mouse myeloma cell, are called monoclonal antibodies (Mabs). Since the first production of Mabs against *Tobacco mosaic virus*, they have been prepared against 50 different plant viruses, including important viruses infecting potato, such as PLRV, *Potato virus A, Potato virus M, Potato virus* S, *Potato virus X* and PVY. These antibodies are valuable for detecting viruses that are difficult to purify. In practice, however, production of Mabs is often labor-intensive. Old immunological test procedures included chloroplast agglutination, microprecipitation tests and gel immunodiffusion (Hampton et al., 1990). In order to increase the sensitivity of serological tests, the use of solid phase to adsorb an antibody or antigen with subsequent attachment of an antigen or antibody and the use of an antibody-enzymes conjugate to detect the antigen (ELISA) has become an important procedure in plant virus detection. In this chapter only methods which are commonly used for potato virus detection will be reviewed.

Enzyme-linked Immunosorbent Assay (ELISA): Plate ELISA

The ELISA technique was first applied to plant virus detection by Clark and Adams (1977). It has significantly increased the ability to detect and study plant viruses, and is currently the most widely used method for the detection of potato viruses due to its simplicity, adaptability, rapidity, sensitivity and accuracy. ELISA has earlier been reviewed by Converse and Martin (1990). The double antibody sandwich (DAS-ELISA) test on a solid phase (usually plastic) is most commonly used. Virus is first selectively trapped by a specific antibody adsorbed on a solid surface, a specific enzyme-labeled antibody (conjugate) is added to the immobilized virus, and the reaction is measured visually or spectrophotometrically, after adding a suitable enzyme substrate. A variation of the above method is the indirect ELISA, in which plates are coated with antigen, and the primary antiviral antibody of one animal species (e.g. rabbit) is added. A secondary commercial antibody (e.g. goat anti-rabbit) enzyme conjugate which reacts with the first antibody is then added. DAS-ELISA is

especially useful for detecting antigens in complex mixtures. This is because the bound antibody specifically traps the antigens of interest, while non-specific antigens are removed in the wash step. Although the indirect ELISA is considered less strain-specific, the test is simple to perform and the same enzyme-antibody conjugate can be used for detecting many different viruses. Commercial kits for PLRV, PVM, PVS, PYX, PVY, and other viruses are available, and give reliable results when potato leaves are tested. For routine testing it is preferable to use polyclonal antisera. Care has to be taken to include both positive and negative controls in ELISA tests. The minimum level of virus detection by ELISA is about 2ng/ml. Torrance and Robinson (1989) have reported that the Swiss now routinely test 20,000 seed potato tubers per day for PVY and PLRV by ELISA under certification program. Thousands of microplants and polyhouse grown potato plant samples are routinely tested for virus freedom with ELISA in India at CPRI, Shimla under the breeder seed production programme.

Dot-ELISA (or NCM -ELISA)

Assays in which antibodies or antigens are bound to nitrocellulose or nylon membranes have been used to detect PVS, PYX and PVY (Banttari and Goodwin, 1985) and PLRV (Smith and Banttari, 1987). In principle, solutions containing purified antigen or crude sap from infected plants are spotted directly onto a membrane and air dried. The membrane surface is then saturated with bovine serum albumin (BSA) and a specific (primary) viral antibody is added. Finally, a secondary antibody-enzyme conjugate and substrate are added. The enzyme reacts with the soluble substrate to form an insoluble colored product at the site of the reaction. The method is readily adaptable to field application.

Tissue Blotting and Tissue Squashes

Tissue blotting is a serological technique similar to ELISA (Lin et al. 1990). Blots are made by pressing the freshly cut tissue surface gently but firmly on a nitrocellulose membrane. Antigens in tissue blots are detected by enzyme-labeled probes. The technique has been used to detect both PYX and PVY from tubers in the field (Bravo-Almonacid, et al. 1992).Nucleic acids of plant viruses were also detected specifically and sensitively by hybridization of infected plant tissue squashed onto a nylon membrane (squash blot) with a specific radioactive probe (Navot et al. 1989). The method provides a specific, rapid and simple tool for large-scale diagnosis of plant viruses.

Electron Microscopy

Plant viruses in infected tissues often exist in sufficient concentrations that they can be extracted and examined by standard electron microscopy (EM). Observation of the shape and size of a virus particle is a basic step towards virus identification. In many cases, EM provides information on virus morphology to be obtained within minutes after sampling a diseased plant. EM is used to examine viruses in crude extracts from infected plants (Hill, 1984).

Immunosorbent Electron Microscopy (ISEM)

Techniques involving the detection and identification of plant viruses by combining electron microscopy and serology are highly sensitive. ISEM was introduced by Derrick (1973) for the detection of plant viruses, has been subsequently further improved (Milne and Lasemann, 1984; Garg et al., 1989) and is as or more sensitive than ELISA for some viruses (Roberts et al., 1980; Garg and Khurana 1991) and thousand times more sensitive than conventional electron microscopy (Roberts and Harrison, 1979; Garg et al., 1989). *Potato Leaf Roll Virus* (PLRV) is a small, phloem-restricted virus occurring in very low concentration and poses problem in detection with conventional electron microscopic detection. Optimum parameters were determined for the reliable and sensitive immune electron microscopic diagnosis of *potato leaf roll virus* along with other important potato viruses and PLRV was best detected when the virus and its antibodies interacted in liquid phase followed by trapping on the grid coated with protein A and homologous antibodies (Garg and Khurana 1991). PLRV can be detected in potato leaves by serology, using commercial ELISA kits. However, concentration of PLRV varies and, in plants grown at temperatures of 30ºC or in older plants, ELISA may not always detect infection. It is also difficult to detect PLRV by ELISA in unsprouted tubers (Hill and Jackson, 1984). Various methods based on PCR or nucleic acid probes are now being developed and evaluated. Thus, PLRV could be detected in tubers within one day by Immunocapture and a flurogenic 5' nuclease RT-PCR assay (Schoen et al., 1996; Russo et al., 1999). Using a digoxigenin-labeled cRNA probes, PLRV is easily detected in dormant tubers (Loebenstein et al., 1997). The limit of PLRV detection with this probe was 1pg/ml compared with 2ng/ml by ELISA. Such methods, if adopted by testing laboratories will eliminate the present necessity of sprouting dormant tubers thereby saving time and glasshouse space.

Polymerase Chain Reaction Techniques

The polymerase chain reaction (PCR) as detection method is rapid, versatile, specific, and sensitive. PCR employs an enzymatic and an exponential amplification of a specific segment of DNA. This goal may be achieved through multiple cycles of three steps performed at different temperatures to: (i) denature the DNA, (ii) anneal two oligonucleotide primers to the denatured (opened) DNA strands, and (iii) primer extension by thermostable DNA polymerase to synthesize the target sequence whose ends are defined by the primers. The presence of amplified DNAs can be determined by gel electrophoresis analysis. PCR has been widely used in plant pathology for the detection and diagnosis of pathogens such as viroids, viruses, bacteria, phytoplasma, fungi, and nematodes (reviewed by Henson and French, 1993). Since PCR needs a DNA segment for amplification and most of the plant virus posses RNA genomes, it is absolutely essential to first convert the RNA genome into a cDNA through reverse transcription (RT). RT-PCR is known to be at least 1000 times more sensitive than ELISA in terms of detection sensitivity for potato viruses (Leone et al., 1997; Jeon, 1997). Large scale testing of both pre harvest & post harvest samples is possible through NASH and RT-PCR as described by (Singh et al., 1999). PCR specificity and success in pathogen diagnosis depends upon the design of specific primers used to initiate DNA synthesis. Primer sequences are designed from the pathogen genome sequences available in gene bank (NCBI). Oligonucleotide primers must be 18-25 nucleotide residues in length, with a 50 % G+C content, no annealing 3' end, no

secondary structures, and high G+C content at the 3' ends. Primers may be targeted either to conserved regions (to amplify sequences from groups of pathogens) or to variable regions (to discriminate between strains). The annealing temperature of primers will affect specificity of PCR and successful reaction depends on primer length, its G+C content. Primers around 20 nucleotides require increase of up to 2°C for every addition of A or T and 4°C for G or C. With potato viruses, RT-PCR was applied in detecting *Potato virus Y* (PVY), *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX), *Beet western yellow virus* (BWYV), *Sugarcane yellow leaf virus* (ScYLV), *Potato virus A* (PVA) , *Potato virus S* (PVS), *Potato virus M* (PVM) and *Potato apicl leafcurl virus* (PALCV) (Shalaby et al., 2002; Suluja et al., 2005; Prill et al.,1988; Maia et al., 2000; Singh and Singh, 1998; Kaushal et al., 2007; Xianzhou et al., 2008 and Nagata et al., 2004). RT-PCR has been applied for cloning, molecular detection and sequence analysis of CP gene of PLRV. PLRV CP gene of different isolates was cloned in sequencing vectors e.g., an Indian isolate was cloned in pGEM-T vector (Mukherjee et al., 2003), Fujian isolate in pGEM-T vector (XingQuan et al., 2006) and complementry DNA of PLRV in pUC9 (Smith et al., 1988). Polymerase chain reaction linked automated sequencing was used to compare the relatedness of Brazilian potato leafroll luteovirus (PLRV) isolates amongst themselves with each other (Souza-Dias et al., 1999).

Nine Tunisian isolates of PLRV from potato cv Spunta were readily transmitted by *Myzus persicae* (Sulzer) from potato plants to *Physalis floridana* test plants inducing symptoms of differing severities. In order to investigate the genetic variability between the PLRV isolates, the ORF0 region of the Tunisian PLRV isolates was sequenced and compared with that of a French isolate (Fr5) that induces mild symptoms and other 24 ORF0 sequences available in the Genbank. A tentative correlation between symptom expression in P. *floridana* and specific changes in the ORF0 sequences was detected (Djilani- Khouadja et al., 2005). Although unquestionable in advantages, PCR is very expensive requiring costly equipment (thermocycler) and costly molecular biology grade consumables. In addition, PCR-based techniques are prone to render false positives due to its extremely high sensitivity coupled with the ease of contamination by aerosols, hair, skin, gloves, contaminated reagents, commercial preparations of TaqDNA polymerase, or even autoclaved material containing target sequences (Dwyerd et al., 1992 and reviewed by Henson and French, 1993). Moreover, only a limited number of samples can be tested in one run. Therefore it might not be a 'first choice' for large scale indexing. Nontheless, high sensitivity and rapid response make PCR a convenient approach for testing 'mother' seed stocks/plants.

Standardized RT-PCR procedures were developed and validated for detection of *Alfalfa mosaic virus* (AMV), *Impatiens necrotic spot virus* (INSV), *Tobacco rattle virus* (TRV), *Tomato spotted wilt virus* (TSWV), *Potato leaf roll virus* (PLRV), *Potato mop top virus* (PMTV), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato spindle tuber viroid* (PSTVd). Under the same conditions the O, N, NTN, and N:O strains of PVY could be differentiated using previously published multiplex primers. (Crosslin and Hamlin, 2011).

Molecular Hybridisation Techniques

Molecular hybridisation as a diagnostic tool in plant virology was first used to detect viroids (Owens and Diener, 1981) and later, applied to plant viruses (Maule et al., 1983; Garger et al., 1983; Salazar and Querci, 1992). Molecular hybridization, based on specific interaction between complementary purine and pyrimidine bases forming A=T and G≡C base pairs, results in the formation of a stable hybrid between the target sequences and those of the probe. The stability of the hybrid depends on the number of hydrogen bonds formed and on both electrostatic and hydrophobic forces. Electrostatic forces rely on the phosphate molecules of the nucleic acid backbone whereas hydrophobic interactions are maintained between the staggered bases. The most common method for molecular hybridization, the dot-blot hybridization technique involves the direct application of the target nucleic acid preparation solution to a solid support, such as nitrocellulose or nylon membranes, and subsequent detection with appropriate specific probes (Hull, 1993; Pallas et al., 1998).

Preparation of Probe

Radioactive and non radioactive labels can be added to double stranded cDNA by nick-translation (Rigby et al., 1977; Vivian, 1992). Single stranded cDNA in two orientations (plus and minus) have also been produced for PSTVd (Salazar et al., 1988**).** The insertion of the required cDNA sequence in to vectors flanked by both SP6 and T7 polymerase promoters allows the construction of probe specific for plus or minus sequence (Melton et al., 1984; Salazar et al., 1988). Use of non-radioactive precursors to label nucleic acids, made the molecular hybridization technique more accessible, and currently being used in an increasing degree of virus-testing.
Among non-radioactive precursors, those derived from biotin and digoxigenin molecules are most widely used. The biotinyl labelled nucleic acids are recognised with great efficiency by avidin or its microbial analogue, streptavidin, taking advantage of the exceptionally high affinity of the avidin-biotin complex. Biotin and glycoproteins having affinity to avidin are commonly present in plants.The main disadvantage of this system is encoutered when sap extracts are used, where the endogenous biotin may cause false positives or, alternatively, the presence of glycoproteins that bind avidin or biotin-binding proteins give rise to unworkable high background.

Another widely used molecule is the hapten digoxigenin which is bound via a spacer arm (eleven carbon residues) to uridin-nucleotides and incorporated enzymatically into nucleic acids by standard methods of transcription. Viroids and most of the plant viruses, including the totality of viruses affecting stone fruit trees have RNA genomes. RNA-RNA hybrids are more stable than RNA-DNA hybrids; therefore more stringent hybridisation conditions can be selected in the case of RNA-RNA hybrids that will help to increase specificity and lower nonspecific background. Hence, RNA probes are preferred over DNA ones to detect stone fruit viruses. Nonradioactive RNA probes (riboprobes) are synthesised by incorporating the digoxigenin hapten into a cRNA by means of an in vitro transcription reaction from cloned viral cDNA. To check the

success and/or the yield of the riboprobe, electrophoretic mobility in TBE-agarose gels of the transcription products obtained in the presence and absence of the precursor DIG-UTP must be compared. If the digoxigenin was incorporated into the cRNA, the electrophoretic mobility of the transcript will be slower than that of unlabelled transcript. Alternatively, transcription products may be serially diluted and spotted on nylon membranes which are then developed. Nonradioactive (biotinylated) riboprobes for detecting viruses and viroids affecting potato have been obtained for PSTVd (Verma et al., 2006), PVS (Eweida et al., 1989) and PVX (Eweida et al., 1990). Hopp et al. (1988) reported the use of specific biotinylated probes for the simultaneous detection of *Potato virus X, Potato virus Y*, PLRV and PSTVd. Incase of PLRV, DIG was ued to prepare the nucleic acid probe (Seo-Hyo et al., 1998). ^{32}P-labelled run way transcripts of the cloned CP gene were used to detect PLRV tissue imprints and tissue sap dilutions (Mukherjee et al., 2003). Specificity of the amplification was validated by northern blot analysis with a specific ^{32}P-labelled probe (Leone et al., 1997).

Sample Preparation

No universal sample processing protocols are available for molecular hybridisation analysis. Choise of the protocol will depend on the virus being detected, the host, and the type of labeled probe. For instance, when clarified sap extracts are used, the natural green-brownish colour of leaves on the membranes interferes with the colorimetric detection, probably due to the reduction of the nitroblue tetrazolium by components of the plant sap while the radiation emission may not be altered by the presence of these components in case of chemiluminiscence/radioactive emission detection (Mas et al., 1993; Pallas et al., 1998). An extraction buffer that works well for most of the virus assays is the one that was applied for PPV (Varveri et al., 1987) and consists of 50 mM sodium citrate pH 8.3, containing 20 mM diethyldithiocarbamate (DIECA) and 2% (w/v) polyvinilpyrrolidone (PVP). Samples are homogenised, clarified by centrifugation at 5000 g for 5 min at 4 °C, and denatured by heating at 60°C for 15 min in the presence of formaldehyde. This last ‘step is optional for viruses since it increased the sensitivity limit only slightly. However, it is necessary for viroids due to their high degree of self-complementarity (Macquarie et al., 1984; Flores, 1986; Astruc et al., 1996). Most methods used for (viroid) RNA extraction require use of phenol or other toxic organic solvents, making them undesirable for diagnostic laboratories that process large number of samples.

Hybridisation and Nucleic Acid Detection

Samples (nucleic acids) must be fixed on to a membrane by baking for 2 h at 80°C, or at 120°C for 30 min, or by UV cross-linking (in the last two cases only positively charged Nylon membranes can be used). The last method results in a 5- to 10-fold increase in sensitivity over the baking methods. Hybridisation is influenced by on several factors such as the complexity (length and composition of the nucleic acid), concentration of the probe, the temperature, salt concentration, base mismatches and hybridisation accelerators. The temperature at which half of the strands separate is the melting temperature (*T*m). The stringency of the hybridisation conditions and the stability of the formed hybrid complex determine the specificity of hybrid formation. In general, high temperatures and low salt increase stringency. The presence of formamide in the hybridisation solution also increases stringency by favouring correct base pairing and reducing background noise. For plant RNA virus detection, hybridisations are often carried out at 65-68°C. For viroids, good signal to background ratio is achieved at 70-72°C in 50% formamide. Hybridised filters can be either processed immediately or stored dry. The labelled hybrids are detected by signals obtained on X-ray film or by an ELISA reaction using conjugates composed of high-affinity DIG-specific antibodies coupled to alkaline phosphatase (AP). After three washing steps, result is obtained either by subsequent addition of AP substrates (BCIP and NBT) or the chemiluminescent substrate CSPDB (Verma et al., 2006; Seo-Hyo et al., 1998).

Combined Techniques for Detection of Viruses

Serological and molecular techniques differ not only in the target viral component to be detected but also in their specificity, sensitivity, and facility of automation. Recently, the specificity and facility of automation for serological methods were combined with the sensitivity of the PCR technique in a single assay in which viral particles were initially antibody-captured and then their nucleic acid amplified by PCR (Jansen et al., 1990; Wetzel et al., 1992; Nolasco et al., 1993). This attractive technique, called imrnunocapture-PCR (IC-PCR) was 250 times more sensitive than direct PCR. IC-PCR avoids purification of the viral nucleic acid required to eliminate the interfering plant cell components that affect the PCR-based methods .This technique was successfully applied to PLRV detection (Leone et al., 1997). More recently, it was shown that the immunocapture step may be substituted by direct virus immobilization (direct binding-PCR; DB-PCR) not requiring antiserum (Rowhani et al., 1995), and the detection levels achieved by the DB-PCR were generally lower than those of IC-PCR. Another attractive PCR-based alternative was the one described by Olmos et al. (1996) in which the simplicity of the tissue-imprinting technique (as mentioned above) was combined with the specificity and sensitivity of the IC-PCR. This technique, called print-capture PCR (PC-PCR) avoids the need for grinding the samples without lost of sensitivity. This technique was applied for detecting *Plum pox virus* (PPV) and *Potato apical leaf curl virus* (PALCV) (Olmos et al., 1996; Gawande et al., 2007).

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