Molecular diagnostic techniques and their potential role in stone fruit certification schemes

Pallás V., Sanchez-Navarro J.A., Canizares M.C.

in

Di Terlizzi B. (ed.), Myrta A. (ed.), Savino V. (ed.).
Stone fruit viruses and certification in the Mediterranean countries: problems and prospects

Bari : CIHEAM
Options Méditerranéennes : Série B. Etudes et Recherches; n. 19

1998
pages 191-208

Molecular diagnostic techniques and their potential role in stone fruit certification schemes

Vicente PALLÁS
Jesús A. SÁNCHEZ-NAVARRO
Paloma MÁS
M. Carmen CAÑIZARES
Federico APARICIO
Jose F. MARCOS
Departamento de Mejora y
Patología Vegetal
CEBAS-CSIC
Murcia (Spain)

SUMMARY - Stone fruit trees are affected by a large number of viruses that cause important economic losses. At the moment, unlike bacterial and fungal diseases, no chemical exist to be applied directly to control viral or viroid diseases. So, the early detection by means of sensitive diagnostic methods is the main way to control them. The extraordinary progress made in the nucleic acid research recently and the application of recombinant DNA technology in plant virology have permitted the use of diagnostic methods based on the genomic component of viruses and viroids. Among them, molecular hybridisation and polymerase chain reaction (PCR) have received great interest lately and have been incorporated in the diagnostic field of plant virology. This review focuses on the molecular basis of these two techniques, their application to the diagnosis of stone fruit viruses and viroids and their potential incorporation into the certification schemes.

Key words: stone fruit viruses, viroids, non radioactive molecular hybridisation, PCR, diagnostic methods.

RESUME - *Les essences à noyaux sont affectées par bon nombre de virus qui occasionnent des pertes économiques importantes. Actuellement, à l'opposé des maladies bactériennes èt cryptogamiques, il n'existe aucun produit chimique qui puisse être utilisé dans la lutte directe contre les viroses ou les maladies à viroïdes. Par conséquent, le dépistage précoce à travers des méthodes de diagnostic sensibles représente la voie principale pour les combattre. Ces dernières années, les progrès extraordinaires faits*

dans l'étude des acides nucléiques et l'application de la technologie du DNA recombinant ont permis d'utiliser des méthodes de détection basées sur la composante génomique des virus et des viroïdes. Parmi ces techniques, l'hybridation moléculaire et l'amplification de séquence (PCR) ont suscité un grand intérêt et elles ont été adoptées pour le diagnostic en virologie végétale. Le présent travail illustre les bases moléculaires de ces deux techniques, leur application au diagnostic des virus et des viroïdes des essences à noyaux et leur possible introduction dans des programmes de certification.

Mots-clés: *virus des essences à noyaux, viroïdes, hybridation moléculaire non radioactive, PCR, méthodes de diagnostic.*

I - Introduction

Stone fruit trees are affected by a large number of viruses that exhibit very different biological properties as well as structural characteristics and genome expression strategies. They belong to different genera such as ilarvirus (ApMV, PDV, PNRSV), nepovirus (ArMV, RRSV, SLRV, TBRV, MLRSV, CLRV), trichovirus (ACLSV), tombusvirus (PeAMV), and potyvirus (PPV). Economic losses caused by these viruses vary from slight (as in the case of ACLSV) to extremely severe (PPV) (Uyemoto and Scott, 1992). Two different viroids are known to infect stone fruit trees, hop stunt viroid (HSVd), originally detected as causing a stunt disease of hops, and found in plum, peach, apricot, and almond (Shikata, 1990; Astruc *et al.*, 1996). HSVd cause of dapple fruit disease in plums and peaches (Shikata, 1990). Another viroid, peach latent mosaic (PLMVd) (Hernández and Flores, 1992) is the causal agent of the peach latent mosaic disease (Desvignes, 1980). Both PLMVd and HSVd occur in high incidence in several Mediterranean areas in varieties of peach and apricot, respectively (Flores *et al.*, 1992; Cañizares *et al.*, 1997; Badenes and Llácer, 1997; Loretti *et al.*, 1998). In addition, PLMVd has occasionally been detected in cherry, plum, and apricot germplasm from countries in Europe or Asia (Hadidi *et al.*, 1997).

At the moment, unlike for bacterial and fungal diseases no chemicals exist that could be used as a direct field control of viral or viroid diseases, and therefore the early detection by means of sensitive diagnostic methods is the main way to control them (Mathews, 1991; Hull, 1993). Plant virus composition relies on both traditionally called informative molecules (nucleic acids) and functional molecules (proteins). Methods for plant virus diagnosis have evolved in a parallel way to the progress in the knowledge of these components. Until very recently 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 easy use, sensitivity and automation. However, one disadvantage of serology lie in the fact that only 2-5% of the genetic information of viral genome occur as antigenic determinants on the surface of the coat protein (Hull, 1986). However, serological techniques can not be applied to viroid diagnosis because viroids lack of specific-encoded proteins. Therefore, viroid detection must rely on bioassays or by direct detection of the genomic viroid RNA. Equally,

bioassays are not appropriate for screening large populations. Likewise, gel electrophoresis techniques, used on the basis of the distinct mobility of small circular viroid RNAs, would not be suitable for large sample numbers. The extraordinary progress made on the nucleic acid research, during the last years, and the application of recombinant DNA technology to plant virology have permitted using diagnostic methods based on the nucleotide sequences of the genome component of viruses and viroids. Among them, molecular hybridisation and polymerase chain reaction (PCR) were recently incorporated into the diagnostic field of plant virology. This is a review of these two molecular techniques, their application to the diagnosis of stone fruit viruses and viroids, and their potential incorporation into the certification schemes. For additional information, excellent reviews on molecular hybridisation (Hull, 1993; Miller and Martin, 1988) and PCR technologies, (Henson and French, 1993) have been published.

II - Molecular hybridisation techniques

1. Basic methodology

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). Molecular hybridisation, based on specific interaction between complementary purine and pyrimidine bases forming A-T and G=C base pairs, result in a stable hybrid formed by part (or the totality) of the nucleic acid sequence of the pathogen to be detected (target molecule) and the labelled complementary sequence (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 hybridisation, the dot-blot hybridisation technique, involve the direct application of a nucleic acid solution to a solid support, such as nitrocellulose or nylon membranes, and subsequent detection with appropriate specific probes.

Several aspects affecting the different steps of the molecular hybridisation technique (which include the synthesis of the labelled probe, sample preparation, hybridisation and detection) will be discussed below. Detailed protocols for all these steps can be found in previous reviews (Hull, 1993; Pallás *et al.*, 1998).

2. Synthesis of the Probe

Use of non-radioactive precursors to label nucleic acids, made the molecular hybridisation technique more accessible, and currently being used in an increasing number of virus-host combinations. 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. The main disadvantage of this system occur when sap extracts were 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 to non-radioactively labelled nucleic acids 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.

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 (Fig. 1A). To check the success and/or the yield of the riboprobe the 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 (Fig. 1B). Alternatively, transcription products may be serially diluted and spotted on nylon membranes which developed as described below (Fig. 4). Nonradioactive riboprobes for detecting viruses and viroids affecting stone fruit trees have been obtained for CLRV (Más *et al.*, 1993; Más and Pallás, 1995), PNRSV (Heuss-LaRosa *et al.*, 1995; Sánchez-Navarro *et al.*, 1998), PPV (Nemichov *et al.*, 1996), CLSV (Kummert *et al.*, 1995), PLMVd (Ambrós *et al.*, 1995; Badenes and Llácer, 1998; Hadidi *et al.*, 1997), and HSVd (Romero-Durbán *et al.*, 1995; Astruc *et al.*, 1996).

3. Sample preparation

There are no universal sample processing conditions for nonradioactive molecular hybridisation analysis. Choise of process protocol will depend on the virus being detected, the host, and the method used for detecting the digoxigenin-labelled nucleic acids. For instance, when clarified sap extracts are used, the natural green-brownish colour of leaves on the membranes interferes directly with the colorimetric detection, probably due to the reduction of the nitroblue tetrazolium by components of the plant sap, while the light emission may not be altered by the presence of these components (Más *et al.*, 1993; Pallás *et al.*, 1998). An extraction buffer that works well for most of the stone fruit 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 only slightly the sensitivity limit. However, it is necessary for viroids due to their high degree of self-complementarity (McQuarie *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. Recently, an extraction method that avoids the use of phenolics, previously described for obtaining plant genomic DNA (Dellaporta *et al.*, 1983), to enrich partially purified extracts in viroid-like RNAs (Pallás *et al.*, 1987) or in the purification of double stranded viral RNAs (De Paulo and Powell, 1995) was adapted for viroid detection (Astruc *et al.*, 1996; Cañizares *et al.*, 1998). For outline of the method, see Fig. 2.

For routine analysis in scheme certification programs, sample manipulation must be reduced to a minimum. This was achieved by using the tissue-imprinting technique that avoids sample extraction and only require the direct transfer of the plant material (stem, cutting, leaf) onto a nylon or nitrocellulose membrane. This technique was first described to detect proteins by immunocytolocalization (Cassab and Varner, 1987), later, applied to RNA detection (McClure and Guilfoyle, 1989), and then, adapted for detection and localisation of plant viruses (Mansky *et al.*, 1990; Chia *et al.*, 1992). Regarding viruses and viroids of stone fruit trees, the nonisotopic tissue-imprinting hybridisation has been applied to CLRV (Más and Pallás, 1995, see Fig. 3), PNRSV (Sánchez-Navarro and Pallás, unpublished results), and HSVd (Romero-Durbán *et al.*, 1995; Astruc *et al.*, 1996). Immuno-tissue imprinting was used to detect ACLSV and PPV (Knapp *et al.*, 1995). Alternatively, samples may be applied to membranes by using sap-impregnated cotton buds and uniformly pressing them until lateral diffusion occur (Fig. 4 and Sánchez-Navaro *et al.*, 1996).

4. Hybridisation and nucleic acid detection

Samples (nucleic acids) must be fixed onto a membrane by baking 2 h at 80°C, or at 120°C for 30 min, or by uv cross-linking (in the last two cases only Nylon membranes positively charged can be used). The last method result in a 5- to 10-fold increase in sensitivity over the baking methods.

The hybridisation process depend 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 (Tm). The stringency of the hybridisation conditions and the stability of the formed hybrid complexes determine the specificity of hybrid formation. In general, high temperatures and low salt increases

stringency. The presence of formamide in the hybridisation solution, also increases stringency, favours correct base pairing and reduces 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 an ELISA reaction using conjugates composed of high-affinity DIG-specific antibodies coupled to alkaline phosphatase (AP). After three washing steps, a reaction is obtained by subsequent addition of AP substrates, either the colour substrates BCIP and NBT or with the chemiluminescent substrate CSPD®.

III - Polymerase chain reaction techniques

1. Basic methodology

The polymerase chain reaction (PCR) as detection method is rapid, versatile, specific, and sensitive. PCR has been broadly used in plant pathology for the detection and diagnosis of pathogens as viroids, viruses, bacteria, phytoplasma, fungi, and nematodes (reviewed by Henson and French, 1993).

Principles, applications, and protocols for PCR have been detailed in numerous reviews. For this reason, this section will summarise the general guidelines, useful in the potential application of the technique in certification programs of stone fruit trees. The PCR method utilises an enzymatic and an exponential amplification of specific DNA sequences (Fig. 5). This goal may be achieved through multiple cycles of three steps performed at different temperatures to: (i) denaturate the DNA, (ii) anneal two oligonucleotide primers to the denatured DNA strands, and (iii) primer extension by thermostable DNA polymerases to synthesise the target sequence whose ends are defined by the primers. The presence of amplified DNAs can be determined by gel electrophoresis analysis. In the case of RNA pathogens viroids and most plant viruses, a previous reverse transcription step (RT) must be included to copy the target RNA into cDNA prior to being amplified.

2. Considerations, advantages and disadvantages of PCR

PCR, a very specific and sensitive molecular technique suitable for diagnosis, is depended upon the design of specific primers to initiate DNA synthesis. Primer sequences are obtained from the pathogen genome. Oligonucleotide primers must be 18-25 (annealing) nucleotide residues in length, with a 50 % G+C content, no annealing 3′ ends, 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 depend on primer length, its G+C content, and primers around 20 nucleotides require increases of up to 2°C for every A or T and 4°C for G or C. With stone fruit viruses, RT-PCR was applied in detecting of PPV, CLRV, ACLSV, PDV, ApMV, PNRSV, and TomRV (Wetzel *et al.*, 1992; Borja and Ponz, 1992; Rowhani *et al.*, 1995; Griesbach, 1995; Nemichov *et al.*, 1995; Parakh *et al.*, 1995; Rosner *et al.*, 1997; MacKeinze *et al.*, 1997; Sánchez-Navarro *et al.*, 1998)

Other factors affecting specificity are the incubation times for the different steps and concentrations of salts (KCl and $MgCl_2$), primers and enzyme. Size of the amplified product is also important; e.g. average of 200-500 residues (no longer than 1000) to maximise efficiency. Recently, Rosner *et al.* (1997), by using two different pairs of primers, yielding a short (200 base pairs, bp) or long product (785 bp), have shown that PNRSV was detected better in plant tissues with a low virus concentration (e.g. dormant trees) by amplification of the short PCR product, whereas the long product was produced at higher virus titters. Since several factors affected the PCR reaction process, a series of detailed experiments to optimise the assays must be performed. The influence of different sample treatments and concentrations should also be tested in a later stage at the design of the diagnosis approach.

Theoretically, the sensitivity of the technique allows amplification of a single nucleic acid molecule from a complex mixture. Because of this extreme property, every caution must be taken in order to minimise contamination risks and 'one-use' aliquots should be prepared from 'mother' stocks of primers, reagents, and enzymes. Similarly, positive and negative controls must be included with every group of PCR assays performed.

Initially, reasonable pure nucleic acid preparations may be used to check primers and optimise PCR parameters and buffer composition. However, the procedures for obtaining those pure preparations are laborious, time-consuming and may require specialised equipment. Less-purified preparations, like those obtained with the non-phenolic extraction method described above (Fig. 2), may be used to determine their suitability for routine use. An important point to consider will be the design of experiments to detect possible inhibitors of the PCR reaction (i.e., serial dilutions of samples added to positive control mixtures). Stone fruit trees are rich in polysaccharides, known to inhibit polymerase activity. Removal of such inhibitors from test samples may be achieved by using cation-exchange resins or polyvinil pyrrolidone (PVP), which binds polyphenolic compounds. Gene Releaser™ polymeric matrix was used to eliminate inhibitors in assays of viroids (e.g. Hadidi and Yang, 1990; Levy *et al.*, 1994). A method for the extraction of high-quality RNA from woody plants that employs commercially available spin-column matrices and mitigates the inhibitory effects of plant polysaccharides and polyphenolic compounds has been recently described (MacKeinze *et al.*, 1997).

Although unquestionable in advantages, PCR is very expensive requiring costly accessories such as thermocycler, and cost of enzymes and other molecular biology grade reagents. In

addition, PCR-based techniques are prone to render false positives due to the extreme sensitivity and with the ease of contamination by aerosols, hair, skin, gloves, contaminated reagents, commercial preparations of Taq DNA polymerase, or even autoclaved material containing target sequences (Dwyer *et al.*, 1992; Henson and French, 1993). Therefore, it might not be a 'first choice' for large scale indexing. However, the sensitivity and rapid response make PCR a convenient approach for testing 'mother' plants.

3. PCR for diagnosis of viroids

RT-PCR was successfully applied in the detection of viroids from pome (Hadidi and Yang, 1990) and stone fruits (Shamloud *et al.*, 1995; Hadidi *et al.*, 1992; 1997), citrus (Yang *et al.*, 1992), grapevine (Rezaian *et al.*, 1992; Staub *et al.*, 1995; Wan Chow Wah and Symons, 1997), and avocado (Schell *et al.*, 1997). In most cases, the nature of the tissue to be analysed and/or the low titer of the viroid made necessary the use of laborious and time-consuming sample extraction protocols. Good PCR reactions are obtained with standard viroid extraction methods (Fig. 5B). Recently, efforts were made conducted to simplify these extraction methods, either with the use of commercial products (Levy *et al.*, 1994), or alternative protocols not requiring organic solvents (Wan Chow Wah and Symons, 1997). Our work showed that a simple non-phenolic extraction protocol (Fig. 2) (Astruc *et al.*, 1996) may be used, coupled with RT-PCR to detect HSVd in apricot (Amari *et al.*, unpublished). Presumably, this procedure will be useful with other stone fruit species.

In primers designed for viroid detection, it should be noted that intra-molecular base pairing of viroid molecules (due to the high degree of self-complementarity of viroid RNAs) compete for primer annealing. To partially overcome this problem, longer oligonucleotides with higher annealing temperatures are recommended. In our laboratory, viroid PCRs are usually done at annealing temperatures no lower than 60°C. In order to maximise the detection of all sequence variants, primers should be located in the well known conserved sequences which includes the central conserved region in HSVd and the hammerhead region in PLMVd (Astruc *et al.*, 1996; C. Hernández and R. Flores, personal communication). It is not necessary to amplify complete viroid sequence and equally good results are obtained with shorter amplified products. It was demonstrated that different primer combinations resulted in different yields and sensitivity of the PCR reaction (Wan Chow Wah and Symons, 1997).

IV - Combined techniques

Serological and molecular techniques differ not only in the viral component to be detected but 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 amplified by PCR (Jansen *et al.*, 1990; Wetzel *et al.*, 1992; Nolasco *et al.*, 1993). This attractive technique, called immunocapture-PCR (IC-PCR) was 250 times more sensitive than direct PCR. IC-PCR avoids using the level of purification of the extract usually required in order to eliminate the interfering compounds that affect the PCR-based methods. More recently, it was shown that the immunocapture step may be substituted by direct virus immobilisation, not requiring antiserum (direct binding-PCR; DB-PCR; 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 (see 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 PPV and ACLSV (Olmos *et al.*, 1996).

V - Concluding remarks and future prospects.

Diagnostic methods for plant viruses are being continuously improved. In recent years, considerable progress on nucleic acid research had advanced newer methodologies in detecting the genomic components of plant viruses. Although molecular hybridisation and PCR have gained new levels of sensitivity compared to serological ones, an acceptable level of automation is lacking. For a stone fruit certification programme, a compromise between simplicity of automation and sensitivity must be chosen. As a general rule, certified or certifiable material may be assayed by serological or nonradioactive molecular hybridisation methods, whereas more sensitive techniques, but also less affordable, such as those derived from the PCR approach could be used for primary sources or pre-basic material as well as for imported, dormant budwood during postentry quarantine or sanitation purposes.

The simplicity and sensitivity of new molecular methods have been sufficiently improved to detect most of plant viruses at levels below economic thresholds. The goal for the coming years will rely on making these methods more accessible to non specialised laboratories.

Acknowledgements

Work from V.P. laboratory is supported by Grants BIO96-0459 from the Dirección General de Investigación Científica y Técnica de España and by contract CHRX-CT94-0635 from the European Union. J.A. S-N, P.M., M.C.C. and F.A. were recipients of pre-doctoral fellowships from the Ministerio de Educación y Ciencia (MEC) from Spain and J.F.M. was the recipient of a post-doctoral fellowship from MEC.

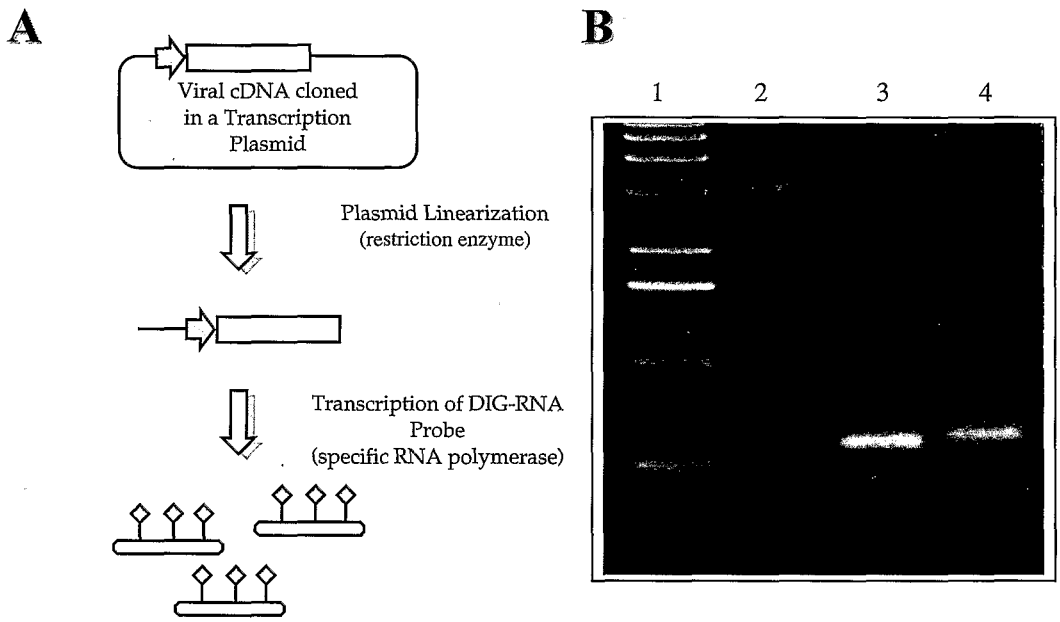


Fig. 1. **(A) Schematic diagram of the procedure used for the synthesis of a viral-specific digoxigenin-labelled RNA probe.** The cloned viral cDNA sequence is represented by an open box under the control of an RNA polymerase promoter (arrow) specific of T3, T7 or SP6 phage polymerase. Plasmid is linearised downstream of the viral sequence and then in vitro transcribed to produce the digoxigenin-labelled RNA (the digoxigenin hapten is represented by diamonds). **(B) Agarose gel electrophoresis for the analysis of digoxigenin-labelled RNA probes.** Lane 1, DNA molecular weight marker; lane 2, linearised plasmid DNA; lane 3, RNA synthesised from the plasmid DNA shown in (2) in the presence of non-labelled UTP; lane 4, RNA synthesised from the plasmid DNA shown in (2) in the presence of DIG-UTP (note the slight slower mobility due to the presence of the digoxigenin hapten).

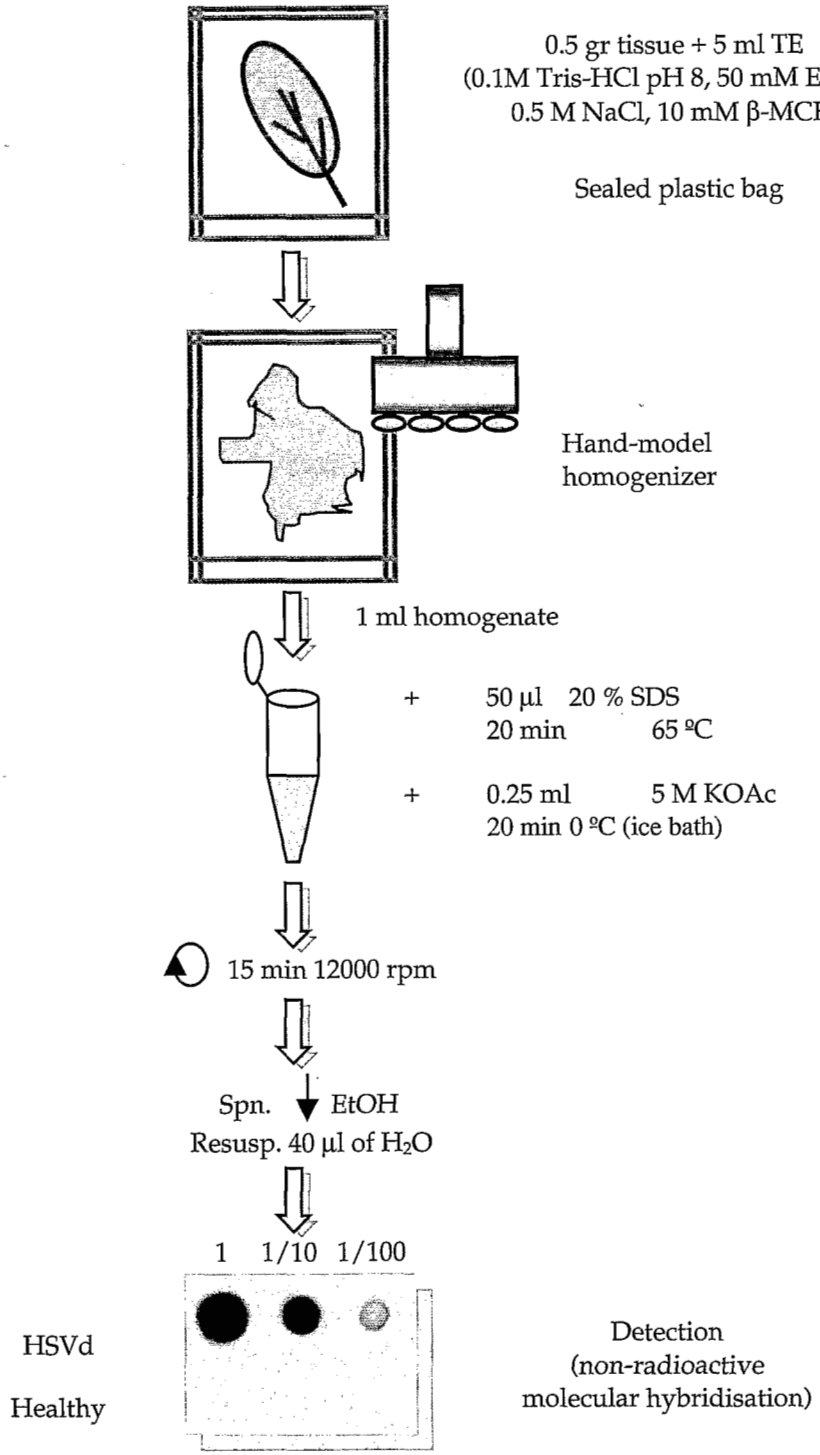


***Fig.* 2. Schematic diagram of the non-organic sample processing procedure used for viroid detection.** The procedure has been adapted for processing small volumes and managing a large number of samples. Aliquots of the samples resuspended in H_20 are finally dotted onto Nylon membranes, fixed by uv cross-linking, and hybridised and developed as outlined in Fig. 4.

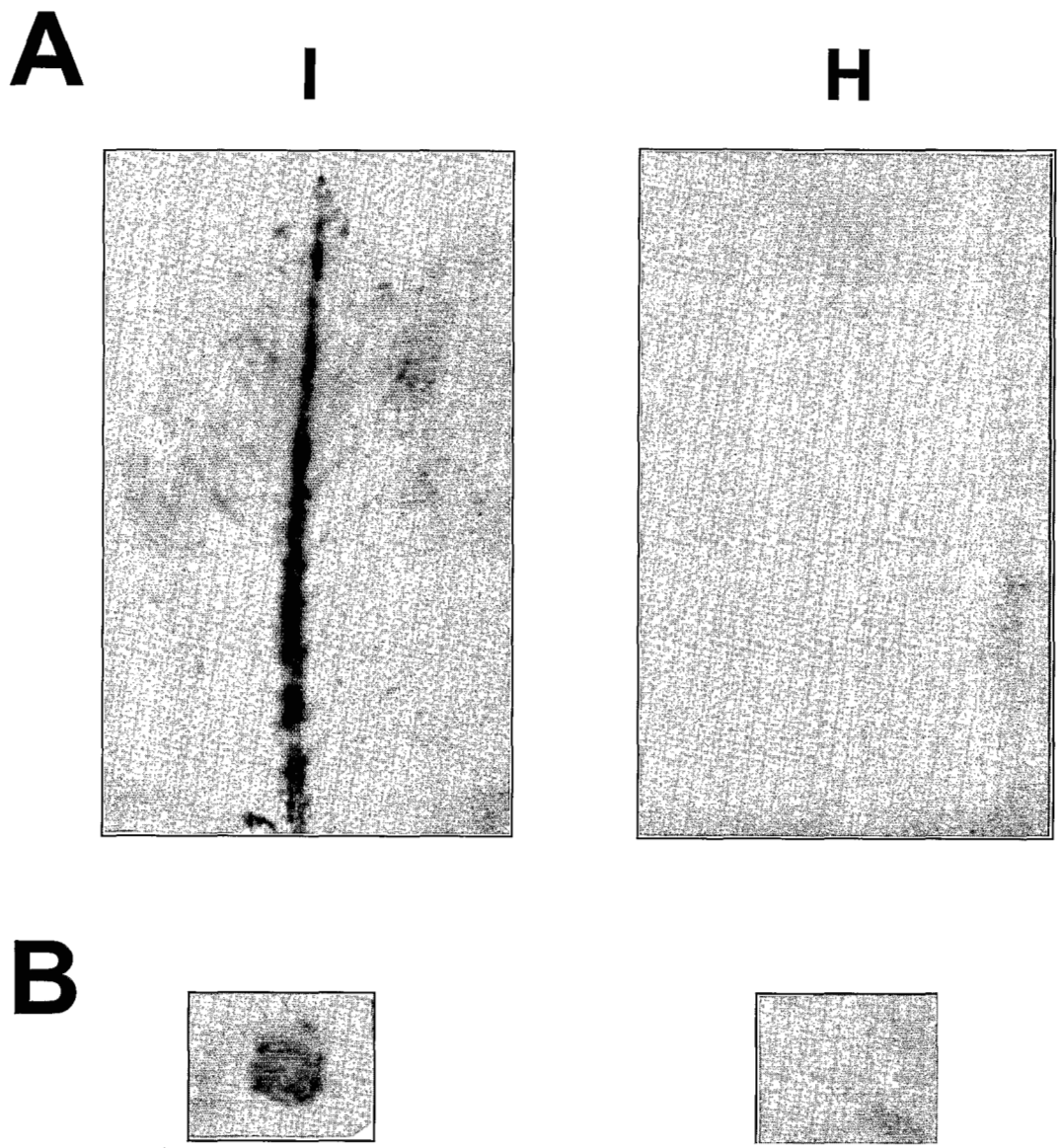


Fig. 3. **Viral RNA detection by hybridisation of tissue prints to a digoxigenin-labelled specific RNA probe.** Leaves from either cherry leaf roll nepovirus-infected (I) or healthy (H) walnut (var. MBT-231) were tissue-printed directly onto Nylon membranes. Leaves were either directly printed onto the membrane (A), or tightly rolled, cross-sectionally cut with a razor blade, and the section printed onto the membrane (B). Membranes were analysed and developed as outlined in Fig. 4.

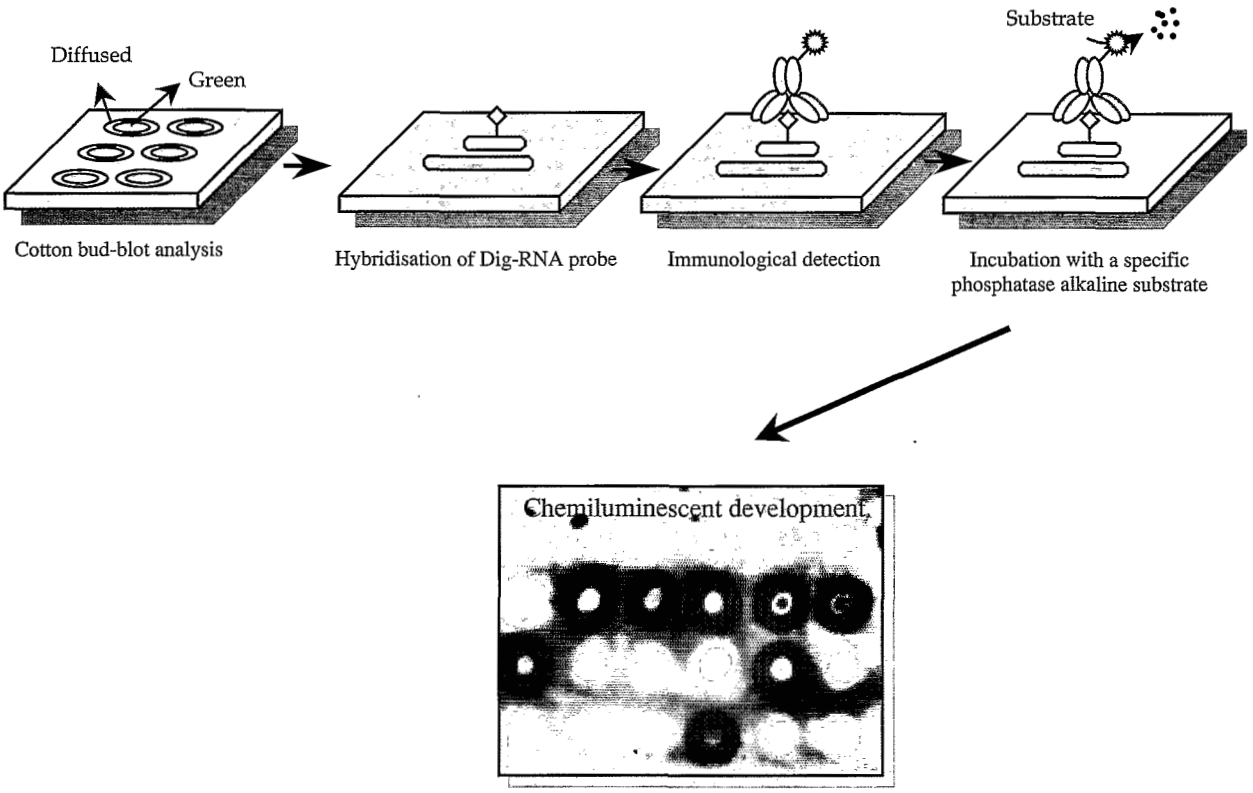
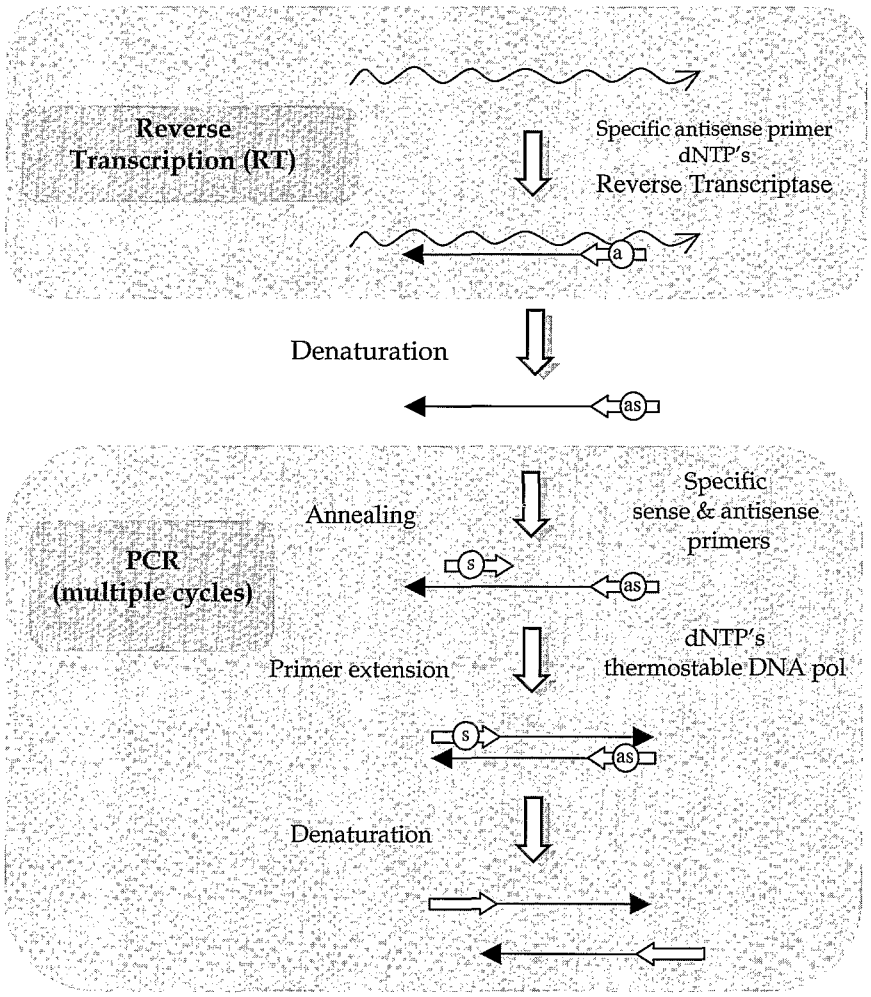


Fig. 4. **Schematic diagram of the procedure used for the analysis of samples by nonradioactive molecular hibridisation.** The samples on the membranes are hybridised to a specific digoxigenin-labelled RNA probe, overnight at 50-68 ºC in the presence of 50% formamide; the hybridised probe is detected by immunological binding to an anti-DIG antibody conjugated to either alkaline phosphatase (in this example) or horseradish peroxidase; finally, the reaction is developped with a substrate specific for the conjugated enzyme, either chemiluminiscent (in this case, CSPD®) or colorigenic. In this example, samples were applied onto the membrane by the 'cotton-bud' technique (see text).



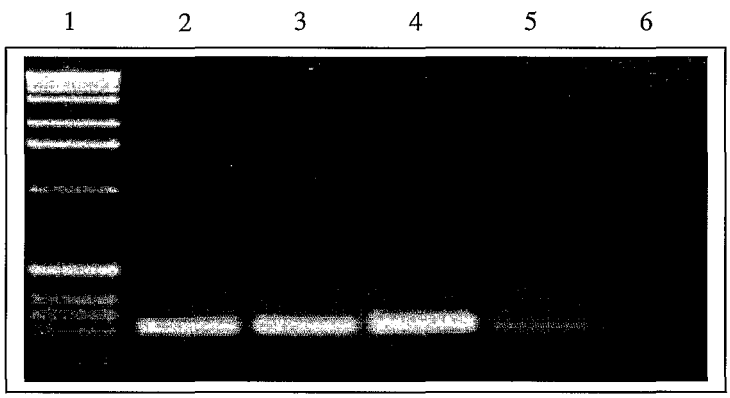


Fig 5. **(A) Schematic diagram of the RT-PCR procedure used for the detection of plant viruses and viroids.** The pathogen RNA (wavy line) is first reverse transcribed to cDNA using a specific antisense (as) oligonucleotide primer, and then exponentially amplified through multiple cycles of PCR using specific sense (a) and antisense (as) oligonucleotides. **(B) Agarose gel electrophoresis of the RT-PCR products obtained in the analysis of field samples for the presence of HSVd.** Lane 1, DNA molecular weight marker; lane 2, control sample from HSVd-infected peach GF-305; lanes 3 to 6, samples from different apricot trees (samples 3 to 5 were found infected with HSVd while sample 6 was viroid-free).

References

- AMBRÓS, S., DESVIGNES, J.C., LLÁCER, G. and R. FLORES (1995). Peach latent mosaic and pear blister canker viroids: Detection by molecular hybridization and relationship with specific maladies affecting peach and pear trees. *Acta Horticulturae,* 386: 515-521.
- ASTRUC, N., MARCOS, J. F., MACQUAIRE, G., CANDRESSE, T. and V. PALLÁS (1996). Studies on the diagnosis of hop stunt viroid in fruit trees: Identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. *European Journal of Plant Pathology,* 102: 837-846.
- BADENES, M.L. and G. LLÁCER (1998). Occurrence of peach latent mosaic viroid in American peach and nectarine cultivars growing in Valencia, Spain. *Acta Horticulturae,* 472 (1): 565-571
- BORJA, M.J. and F. PONZ (1992). An appraisal of different methods for the detection of the walnut strain of cherry leafroll virus. *Journal of Virological Methods,* 36: 73-83.
- CAÑIZARES, M.C., MARCOS, J.F. and V. PALLÁS (1998). Studies on the incidence of hop stunt viroid in apricot trees (*Prunus armeniaca*) by using an easy and short extraction method to analyze a large number of samples. *Acta Horticulturae,* 472 (1): 581-587.
- CASSAB, G.I. and J.E. VARNER (1987). Immunocytolocalization of extensin in developing soybean seed coats by immunogoldsilver stainig by tissue printing on nitrocellulose paper. *Journal of Cell Biology,* 105, 2581-2588.
- CHIA, T.F., CHAN, Y.S. and N.H. CHUA (1992). Detection and localization of viruses in orchids by tissue-print hybridization. *Plant Pathology,* 41: 355-361.
- DWYER, D.E. and N. SAKSENA (1992). Failure of ultra-violet irradiation and autoclaving to eliminate PCR contamination. *Molecular and Cellular Probes,* 6: 87-88.
- DE PAULO, J. and C. POWELL (1995). Extraction of double stranded RNA from plant tissues without the use of organic solvents. *Plant Disease,* 79: 246-248.
- DELLAPORTA, S., WOOD, J. and J. HICKS (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter,* 1: 19-21.
- DESVIGNES, J.C. (1980). Different symptoms of the peach latent mosaic. *Acta Phytopathologica Academiae Scientiarum Hungaricae,* 15: 183-190.
- FLORES, R. (1986). Detection of citrus exocortis viroid in crude extracts by dot blot hybridization: conditions for reducing spurious hybridization results and for enhancing the sensitivity of the technique. *Journal of Virological Methods,* 13: 309-319.
- FLORES, R., HERNÁNDEZ, C., AVINENT, L., HERMOSO, A., LLÁCER, G., JUAREZ, J., ARREGUI, J.M., NAVARRO, L. and J.C. DESVIGNES (1992). Studies on the detection, transmission and distribution of peach latent mosaic viroid in peach trees. *Acta Horticulturae,* 309: 325-330.
- GARGER, S.J., TURPEN, T., CARRINGTON, J.C., MORRIS, T.J., DODDS, J.A., JORDAN, R.L. and L.K. GRILL (1983). Rapid detection of plant RNA viruses by dot blot hybridization. *Plant Molecular Biology Reporter,* 1: 21-25.
- GRIESBACH, J.A. (1995). Detection of tomato ringspot virus by polymerase chain reaction. *Plant Disease,* 79: 1054-1056.
- HADIDI, A., GIUNCHEDI, L., SHAMLOUL, A.M., POGGI-POLLINI, C. and M.A. AMER (1997). Occurrence of peach latent mosaic viroid in stone fruits and its transmission with contaminated blades. *Plant Disease,* 81: 154-158.

- HADIDI, A., TERAI, Y., POWELL, C.A., SCOTT, S.W., DESVIGNES, J.C., IBRAHIM, L.M. and L. Levy (1992). Enzymatic cDNA amplification of hop stunt viroid variants from naturally infected fruit crops. *Acta Horticulturae*, 309: 339-344.
- HADIDI, A. and X. YANG (1990). Detection of pome fruit viroids by enzymatic cDNA amplification. *Journal of Virological Methods*, 30: 261-270.
- HENSON, J.M. and R. FRENCH (1993). The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology*, 31: 81-109.
- HERNÁNDEZ, C. and R. FLORES (1992). Plus and minus RNAs of peach latent mosaic viroid self cleave *in vitro* via hammerhead structures. *Proceedings National Academy of Science USA*, 89: 3711-3715.
- HEUSS-LAROSA, K., HAMMOND, R., CROSSLIN, J.M., HAZEL, C. and F.A. HAMMERSCHLAG (1995). Monitoring of Prunus necrotic ringspot virus infection by hybridization with a cRNA probe after *in vitro* micrografting. *Journal of American Society for Horticultural Science*, 120: 928-931.
- HULL, R. (1993). Nucleic acid hybridization procedures. *In*: Diagnosis of plant virus diseases. R.E.F Matthews (Ed.). CRC Press, Inc. Boca Raton.
- HULL, R. (1986). The potential for using dot-blot hybridisation in the detection of plant viruses. *In*: Developments and applications of virus testing. R.A.C. Jones and L. Torrance (Eds.). Suffok, Lavenham, 3-12 pp.
- JANSEN, R.W., SIEGL, G. and S.M. LEMON (1990). Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proceedings National Academy of Science USA*, 87: 2867-2871.
- KNAPP, E., DA CÂMARA MACHADO, A., PÜHRINGER, H., WANG, Q., HANZER, V., WEISS, H., WEISS, B., KATINGER, H. and M. LAIMER DA CÂMARA MACHADO (1995). Localization of fruit tree viruses by immuno-tissue printing in infected shoots of *Malus* sp. and *Prunus* sp. *Journal of Virological Methods*, 55: 157-173.
- KUMMERT, J., COLINET, D. and P. LEPOIVRE (1995). Detection of plant viruses by molecular hybridization using non-radioactive probes. *Bulletin OEPP/EPPO Bulletin*, 25: 301-313.
- LEVY, L., LEE, I.M. and A. HADIDI (1994). Simple and rapid preparation of infected plant tissue extracts for PCR amplification of virus, viroid and MLO nuclec acids. *Journal of Virological Methods*, 49: 295-304.
- LORETTI, S., FAGGIOLI, F., BARRALE, R. and M. BARBA (1998). Occurrence of viroids in temperate fruit trees in Italy. *Acta Horticulturae*, 472 (1): 555-561.
- MACKENZIE, D.J., MCLEAN, M.A., MUKERJI, S. and M. GREEN (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease*, 81: 222-226.
- MACQUAIRE, G., MONSION, M., MOUCHES, C., CANDRESSE, T. and J. DUNEZ (1984). Spot hybridization: application to viroid identification. *Annales of Virology (Inst. Pasteur)*, 135E: 219-230.
- MANSKY, L.M., ANDREW, R.E., DURAND, D.P. and J.H. HILL (1990). Plant virus location in leaf tissue by press blotting. *Plant Molecular Biology Reporter*, 8: 13-17.
- MÁS, P. and V. PALLÁS (1995). Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. *Journal of Virological Methods*, 52: 317-326.

- MÁS, P., SÁNCHEZ-NAVARRO, J.A., SÁNCHEZ-PINA, M.A. and V. PALLÁS (1993). Chemiluminescent and colorigenic detection of cherry leaf roll virus with digoxigenin-labelled RNA probes. *Journal of Virological Methods,* 45: 93-102.
- MATHEWS, R.E.F. (1991). Plant Virology, 3rd ed. Academic, San Diego. 835 pp.
- MAULE, A.J., HULL, R. and J. DONSON (1983). The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. *Journal of Virological Methods,* 6: 215-224.
- MCCLURE, B.A. and T.J. GUILFOYLE (1989). Tissue print hybridization. A simple technique for detecting organ- and tissue-specific Mic gene expression. *Plant Molecular Biology,* 12: 517-524.
- MILLER, S.A. and R.R. MARTIN (1988). Molecular diagnosis of plant disease. *Annual Review of Phytopathology,* 26: 409-432.
- NEMICHOV, L., HADIDI, A., CANDRESSE, T., FOSTER, J.A. and T. VERDEREVSKAYA (1995). Sensitive detection of apple chlorotic leaf spot virus from infected apple or peach tissue using RT-PCR, IC-PCR or multiplex IC-RT-PCR. *Acta Horticulturae,* 386: 51-62.
- NEMICHOV, L., HADIDI, A., MAISS, E., CAMBRA, M., CANDRESSE, T. and V. DAMSTEEGT (1996). Sour cherry strain of plum pox potyvirus (PPV): molecular and serological evidence for a new subgroup of PPV strains. *Phytopathology,* 86: 1215-1221.
- NOLASCO, G., DE BLAS, C., TORRES, V. and F. PONZ (1993). A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *Journal of Virological Methods,* 45: 201-218.
- OLMOS, A., DASI, M.A., CANDRESSE, T. and M. CAMBRA (1996). Print-capture PCR: a simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues. *Nucleic Acids Resarch,* 24: 2192-2193.
- OWENS, R.A. and T.O. DIENER (1981). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science,* 213: 670-672.
- PALLÁS, V., MÁS, P. and J.A. SÁNCHEZ-NAVARRO (1998). Detection of plant RNA viruses by non-isotopic dot-blot hybridization. In Plant Virus Protocols: From Virus Isolation to Transgenic Resistance. Ed. Foster, G. and Taylor, S. Humana Press, Totowa, 461-468.
- PALLÁS, V., NAVARRO, A. and R. FLORES (1987). Isolation of a viroid-like RNA from hop different from hop stunt viroid. *Journal of General Virology,* 68: 3201-3205.
- PARAKH, D.R., SHAMLOUL, A.M., HADIDI, A., SCOTT, S.W., WATERWORTH, H.E., HOWELL, H.E. and G.I. MINK (1995). Detection of prune dwarf ilarvirus from infected stone fruits using reverse transcription-polymerase chain reaction. *Acta Horticulturae,* 386: 421-430.
- REZAIAN, M.A., KRAKE, L.R. and D.A. GOLINO (1992). Common identity of grapevine viroids from USA and Australia revealed by PCR analysis. *Intervirology,* 34: 38-43.
- ROMERO-DURBÁN, J., CAMBRA, M. and N. DURÁN-VILA (1995). A simple imprint-hybridization method for detection of viroids. *Journal of Virological Methods,* 55: 37-47.
- ROSNER, A., MASLENIN, L. and S. SPIEGEL (1997). The use of short and long PCR products for improved detection of prunus necrotic ringspot virus in woody plants. *Journal of Virological Methods,* 67: 135-141.
- ROWHANI, A., MANINGAS, M.A., LILE, L.S., DAUBERT, S.D. and D.A. GOLINO (1995). Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. *Phytopathology,* 85: 347-352

- SÁNCHEZ-NAVARRO, J.A., APARICIO, F., ROWHANI, A. and V. PALLÁS (1998). Comparative analysis of ELISA, non-radioactive molecular hybridization and PCR for the detection of prunus necrotic ringspot virus in herbaceous and prunus hosts. *Plant Pathology,* 47: 780-786.
- SÁNCHEZ-NAVARRO, J.A., CANO, E. and V. PALLÁS (1996). Non-radioactive molecular hybridization detection of carnation mottle virus in infected carnations and its comparison to serological and biological techniques. *Plant Pathology,* 45: 375-382.
- SCHNELL, R.J., KUHN, D.N., RONNING, C.M. and D. HARKINS (1997). Application of RT-PCR for indexing avocado sunblotch viroid. *Plant Disease,* 81: 1023-1026.
- SHAMLOUL, A.M., MINAFRA, A., HADIDI, A., WATERWORTH, H.E., GIUNCHEDI, L. and E.K. ALLAM (1995). Peach latent mosaic viroid: nucleotide sequence of an Italian isolate, sensitive detection using RT-PCR and geographic distribution. *Acta Horticulturae,* 386: 522-530.
- SHIKATA, E. (1990). New viroids from Japan. *Seminars in Virology,* 1: 107-115.
- STAUB, U., POLIVKA, H. and H.J. GROSS (1995). Two rapid microscale procedures for isolation of total RNA from leaves rich in polyphenols and polysaccharides: application for sensitive detection of grapevine viroids. *Journal of Virological Methods,* 52: 209-218.
- UYEMOTO, J.K. and S.W. SCOTT (1992). Important diseases of Prunus caused by viruses and other graft-transmissible pathogens in California and South Carolina. *Plant Disease,* 76: 5-11.
- VARVERI, C., RAVELONANDRO, M. and J. DUNEZ (1987). Construction and use of a cDNA probe for the detection of plum pox virus in plants. *Phytopathology,* 77: 1221-1224.
- WAN CHOW WAH, Y. F. and R. H. SYMONS (1997). A high sensitivity RT-PCR assay for the diagnosis of grapevine viroids in field and tissue culture samples. *Journal of Virological Methods,* 63: 57-69.
- WETZEL, T., CANDRESSE, T., MACQUAIRE, G., RAVELONANDRO, M. and J. DUNEZ (1992). A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods,* 39: 27-37.
- YANG, X., HADIDI, A. and S. GARNSEY (1992). Enzymatic cDNA amplification of citrus exocortis and cachexia viroids from infected citrus hosts. *Phytopathology,* 82: 279-285.