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Development of quantitative real-time RT-PCR for the detection and quantification of *Peach latent mosaic viroid*

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Abstract *Peach latent mosaic viroid* (PLMVd) is a damaging pathogen for peach, its detection being of critical importance to both sanitary and certification programs worldwide. Here we report a quantitative real-time reverse transcription polymerase chain reaction assay (qRT-PCR) based on TaqMan® chemistry to improve the diagnosis of this pathogen. Critical to this approach is the design of a specific set of primers and of a probe, and the use of a suitable extraction method proving a reliable, sensitive and specific PLMVd diagnosis. More specifically, the sensitivity was evaluated using 12 ten-fold dilution series of an in vitro transcript of the entire PLMVd genome as a standard for quantification in infected samples. The assay detected up to 1 fg of target RNA. The protocol was also evaluated for its specificity using healthy peach, apricot, plum and pear controls and non-target viroids (*Apple scar skin viroid*, *Hop stunt viroid*, *Pear blister canker viroid*, *Apple dimple fruit viroid* and *Potato spinder tuber viroid*). None of the non-target or healthy analysed samples reacted in qRT-PCR. The efficiency and accuracy of the method was evaluated using different PLMVd peach isolates as templates, including three 'calico' variants: a strong signal was obtained from all of them. Finally, five RNA extraction methods were compared and evaluated to choose the best for detection and quantification of PLMVd by the qRT-PCR protocol.

Keywords PLMVd diagnosis · qRT-PCR · RNA extraction methods · Taq-Man chemistry

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Introduction

Viroids are plant pathogens distributed world wide, and may induce severe damage to infected plants, causing important economic losses to farmers (Tsagris et al. 2008). They are infectious, low molecular weight single-stranded circular RNA molecules of 246 to 399 nucleotides in length with autonomous replication. Their genomes do not encode any protein, so they rely exclusively on the host's proteins. They are divided into two families: the *Pospiviroidae* and the *Avsunviroidae*. The members of the first family, for which the typical representative is the *Potato spindle tuber viroid* (PSTVd), share a conserved central region, a nuclear localisation and an asymmetric rolling circle replication that appears to involve host enzymes. Members of the family *Avsunviroidae* have been shown to accumulate in the chloroplasts, possess hammerhead self-cleaving RNA motifs and replicate according to a symmetric rolling circle mechanism (Flores et al. 1998; Bussière et al. 1999).

Peach latent mosaic viroid (PLMVd) belongs to the *Avsunviroidae* family and it is the causal agent of peach latent mosaic disease, one of the most widespread and damaging peach tree diseases (Hernández and Flores 1992). PLMVd is found across the world and occurs in most commercial peach varieties (Desvignes 1986; Flores and Llacer 1988; Albanese et al. 1992; Shamloul et al. 1995; Ozaki et al. 1999; Kyriakopoulou et al. 2001; Mandic et al. 2008), where it can cause heavy losses (Faggioli et al. 2003). The main symptoms on peach trees include: irregularly shaped, discoloured and deformed fruits with cracked sutures and enlarged pits, bud necrosis, delay in bud-burst, flowering and fruit ripening, decline and reduced longevity of the plants. Some strains also induce clear-cut foliar leaf discolouration (*e.g.* calico, Malfitano et al. 2003) or yellowing. According to its incidence in peach cultivation and its worldwide distribution, many diagnostic protocols have been reported for the detection of PLMVd. The first applied diagnostic technique was a cross-protection bioassay that allowed for the study and control of the disease in France (Desvignes 1976). Subsequently, the polyacrylamide gel electrophoresis analysis of RNA extracts allowed for the identification and the molecular characterisation of the viroid (Flores and Llacer 1988; Flores et al. 1990). After complete sequencing of the PLMVd genome (Hernández and Flores 1992), different molecular methods have been applied for its detection: dot-blot hybridisation with radioactive and non-radioactive probes (Ambros et al. 1995; Loreti et al. 1995), tissue imprint hybridisation (Loreti et al. 1999; Mandic et al. 2008); single and multiplex reverse transcription polymerase chain reaction (RT-PCR) (Loreti et al. 1999; Ragozzino et al. 2004; Shamloul et al. 1995; Shamloul and Hadidi 1999), RT-PCR capture hybridisation (Shamloul et al. 2002) and loop mediated isothermal amplification (RT-LAMP) (Boubourakas et al. 2009).

Over the past few years, the development of novel chemistries and instrumentation platforms enabling the detection of PCR products on a real-time basis has led to the widespread adoption of real-time RT-PCR as the method of choice for the quantification and detection of nucleic acid targets. Recently, the quantitative real time reverse transcription method (qRT-PCR) has been applied for the detection of viroids either based on the SYBER-green (Tessitori et al. 2005; Rizza et al. 2009) or the TaqMan® chemistries (Boonham et al. 2004).

TaqMan® probes are oligonucleotides that have a fluorescent reporter dye attached to the 5′ end and a quencher moiety coupled to the 3′ end. These probes are designed to hybridise to an internal region of a PCR product (Holland et al. 1991). In the non-hybridised state, fluorescence emitted by the reporter is absorbed by the quencher (fluorescent resonance energy transfer). During amplification, the probe is cleaved separating the dyes, resulting in the release of fluorescence, the amount of which is related to the amount of amplified product. The increase in reporter fluorescence is monitored in real time during amplification, using a combined thermal cycler and fluorescence reader. As a result, TaqMan® assays are closed-tube and no post PCR manipulations (*e.g.* gel running) are required. This paper describes the development of an assay for the routine detection of PLMVd, based upon real time qRT-PCR and TaqMan® chemistry**.**

Materials and methods

Plant materials

The germplasm collection of the CRA—Plant Pathology Research Centre (CRA-PAV) supplied healthy samples (GF 305, apricot, plum, peach and pear), infected (peach) controls of PLMVd and non-target samples of plant material infected by other viroids such as: *Apple scar skin viroid* (ASSVd), *Hop stunt viroid* (HSVd), *Pear blister canker viroid* (PBCVd), *Apple dimple fruit viroid* (ADFVd) and *Potato spinder tuber viroid* (PSTVd). Moreover, in order to test the efficiency and accuracy of the protocol, 20 PLMVd-infected peach samples, previously identified and characterised (Luigi et al. 2009), have been collected from commercial orchards.

RNA target extractions

Five methods of nucleic acids extraction have been used and compared.

Extraction method no.1—Total nucleic acids (TNA) were extracted according to the protocol published by Faggioli et al. (2001). Briefly, 500 mg of tissue was powdered in liquid nitrogen and then mixed with 900 µl 0.2 M Tris–HCl buffer pH 8.2 containing 17.5 µl of 5 M NaCl, 8 µl 10% Triton

X-100 and 2 μl β-mercaptoethanol. After centrifugation at 9000 g for 20 min the pellet was discarded and the supernatant was mixed with 500 μl of water saturated phenol pH 7, 100 μl 5% SDS, 100 μl 0.1 M EDTA pH 7. Nucleic acids present in the aqueous phase were recovered by ethanol precipitation and then re-suspended in 500 μl of dietilpyrocarbonate treated water (DePC).

Extraction method no.2—Low molecular weight RNA (lmwRNA) was extracted from 70 mg of powdered tissue using a commercial kit for low molecular weight RNA species (mirPremier™ microRNA Isolation Kit–Sigma), according to the manufacturer's protocol. RNA was finally eluted with 50 μl of elution buffer.

Extraction method no.3—100 mg of tissue were processed and TNA extracted using the Tri-Reagent product (Sigma, Deisenhofen, Germany). The tissue was homogenised in a lysis solution containing 2% PVP-40, 1% PEG, 0.05% Tween 20, 0.5 M Tris–HCl pH 8.3, 140 mM NaCl. 200 μl of the homogenised samples were collected and added to 750 μl of the Tri-Reagent solution after 5 min of incubation at room temperature. 200 μl of chloroform were added to the mixture and the samples were incubated for a further 2 min at room temperature. After centrifugation at 10000 g for 15 min at 4°C, the supernatant was added to 500 μl of isopropanol and 1 μl of glycerol. After an incubation period of 10 min at room temperature, the samples were centrifuged for 10 min at 10000 g and 4°C. The supernatant was discarded and the pellet was washed with 750 μl of ethanol (70%), dried and resuspended in 35 μl of DePC.

Extraction method no.4—Total RNA (TRNA) was extracted using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany); this kit is capable of enriching RNA molecules greater than 200 nt. RNA was finally eluted with 50 μl of RNase-free water.

Extraction method no.5—TRNA were extracted according to the protocol published by Olmos et al. (2005). Fresh leaves were directly printed on positively charged nylon membranes (Roche, Mannheim, Germany) and cut into small squares. The square membranes were put in a tube and the nucleic acids were released following the addition of a Triton-X-100 0,5% solution.

RNA standard preparation for standard curve construction

PLMVd full-length amplified cDNA (obtained according to the protocol of Loreti et al. 1999) was cloned into a plasmid and the obtained construct was used to transform *E. coli* competent cells according to the manufacturer's protocol (pGEM-T Easy Vector system II—Promega, Madison, WI). The cells were plated in a Petri dish and two white colonies were chosen and transferred to a liquid medium. The plasmids obtained from the overnight growing of cultures were extracted using a Plasmid Mini-prep Kit (Bio-Rad, Hercules, CA). The orientation and the propriety of the inserted sequence were checked by means of sequencing. The plasmids were cut using the *Spe*I restriction enzyme and the in vitro transcription was performed using the Riboprobe® Combination System-*SP6/T7* RNA Polymerase kit (Promega), according to the manufacturer's protocol. The DNA was removed with *DNase* I (Promega) and the RNA was recovered by extraction using a phenol:chloroform:isoamilic alcohol (25:24:1) followed by ethanol precipitation.

The obtained RNA was quantified, serially log-diluted and used for the construction of the standard curve.

TaqMan® probe and primers design

The primers and probe were designed by means of computer analysis (Beacon Designer software—SIGMA), using a multiple sequence alignment of PLMVd sequences obtained from the National Centre for Biotechnology Information (NCBI) database and taking into account their melting temperature (*T*m—within the 58–60°C range for the primers and 10°C higher for the probe, so as to improve specificity) and the total length of each amplicon (below 100 bp so as to allow for efficient amplification). The sequences of set of the primers and probe chosen for PLMVd detection are given in Table 1. The 5′ terminal reporter dye used was FAM (6-carboxyfluorescein) and the 3′ quencher dye was TAMRA (tetra-methylcarboxyrhodamine). Probe and primers were supplied by the SIGMA.

Real-time RT-PCR (qRT-PCR)

The TaqMan® PCR reactions (total volume 25 μl) were set up in 96-well reaction plates using PCR core reagent

Table 1 Sequence and position (accession NC_003636.1) of the primers and probe for the TaqMan®qRT-PCR

Name	Sequence	POS (nt)
PLMVd-probe	5′-CTTCTGGAACCAAGCGG-3′	165–181
PLMVd-H	5′-CTCGCAATGAGGTAAGGTG-3′	137–155
PLMVd-C	5′-ACGTCGTAATCCAGTTTCTAC-3′	236–216

kits and established cycling conditions (Applied Biosystems). For this reason, comparisons were only made between probe and primer concentrations and the quality of target nucleic acids. The optimal one tube-one step reaction conditions were as follows: 2 μl of target RNA were added to following reaction mixture containing: Master Mix 1X (Applied Biosystems, Foster City, CA), RT enzyme mix 1X (Applied Biosystems), 1 μM reverse primer, 1 μM forward primer and 0.5 μM of the probe (Table 2). cDNA was synthesised for 30 min at 48°C followed by 10 min of denaturation at 95°C. PCR was performed as follows: denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min, for a total of 40 cycles. The assays were carried out on an ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems).

Results

Optimisation of the qRT-PCR assay

Different primer and probe sets were tested for this purpose. The best primers and probe set (Table 1) was chosen because of its sensitivity (higher detected number of expected positive samples) and specificity (no reaction to healthy and non target samples). Moreover, in order to optimise the reaction, different primer and probe concentrations of the chosen set were used to detect the same amount of target RNA (Table 2). The best results were obtained with mixture C, where the threshold cycle (*C*t) was found to be lowest. The *C*t is the cycle at which a significant increase in fluorescence occurs; hence, a low *C*t value means a better efficiency of the reaction.

Standard curve construction

A standard curve was constructed from RNA target of known concentration. This curve was used as a standard reference for the extrapolation of quantitative information for RNA targets of unknown concentrations. Specifically, the in vitro transcript product obtained from a cloned PLMVd full-length sequence (339 nt) was quantified using a NanoDrop™ spectrophotometer. The starting amount of target RNA was of 10 ng/μl, equal to 10^{11} copies of the viroid. This amount of RNA was 12 10-fold diluted from 10^{11} to 0 copies and the dilutions were used as targets in the qRT-PCR assays. The 12 dilutions were loaded in triplicate so as to estimate experimental errors. The graph reported in Fig. 1 shows the results of the reactions obtained by plotting *C*t values against log-transformed concentrations of the series of ten-fold dilutions of the nucleic acids target. As expected, the increase in *C*t is directly proportional to the dilution of the RNA standards. The fluorescent signal has been detected in RNA standard dilutions from 0,1 ng of RNA (10^9 copies) up to 1 fg (10^3 copies) with a slope of 3,371 and an efficiency of 98%.

Analytical sensitivity: comparisons among extraction methods and between RT-PCR and qRT-PCR

Leaves from the same PLMVd infected plants were divided into five sets. Each set was extracted using one of the five methods described and the nucleic acids targets were serially diluted so as to obtain at least six orders of dilutions. Each extract and the corresponding dilutions were tested using two amplification protocols: a qualitative RT-PCR (Loreti et al. 1999) and the qRT-PCR developed in this work.

With regards to the sensitivity and the starting amount of RNA molecules, the most efficient extraction method was no.1 (phenol) followed by extraction method no.2 (lmwRNA <200 nt) and then extraction method no.3 (Tri-Reagent) (Table 3).

Table 2 Effect of primer and probe concentrations on the C_t values obtained from the same target

Mixture	[primers]	[probe]	C_t
A	0,3 μM	0,15 μM	25,98
B	0,6 μM	0,3 μM	24,76
C	1 μM	0,5 μM	22,31
D	1,2 μM	0,6 μM	37,53

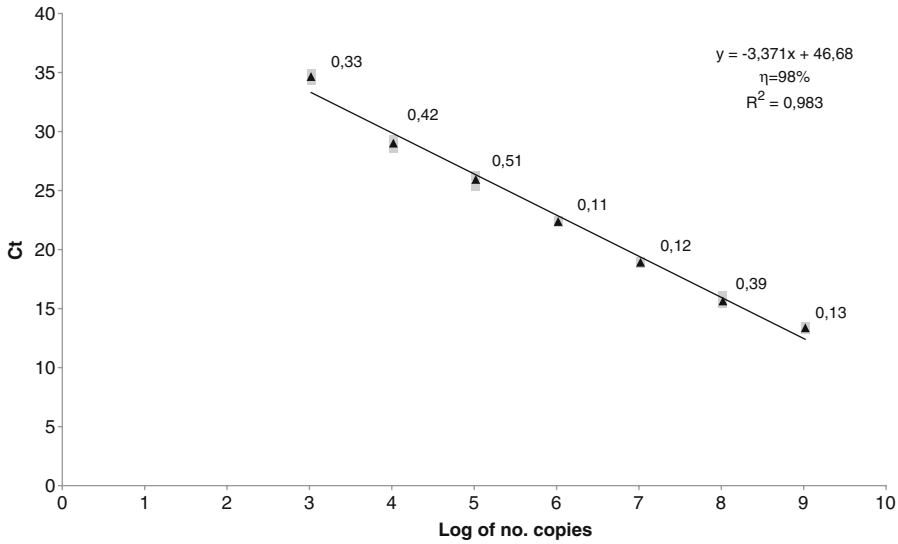

Fig. 1 Standard curve obtained for the PLMVd RNA quantification. The horizontal axis reports the logarithm of the number of copies of the in vitro transcript, while the vertical axis shows the Ct values obtained in the reactions. *Filled square* represent the Ct values obtained in each replicate and *filled triangle* the average Ct of the three replicates. The R^2 is used to assess the accuracy of the results obtained in triplicate experiments (more close to 1 means better fit), the exact standard deviation value was reported near each point of the graph

The comparison between the two amplification protocols highlighted the higher sensitivity of qRT-PCR with respect to the qualitative RT-PCR for every extraction method used. In fact, the qRT-PCR was able to detect PLMVd with all extraction methods, whereas the RT-PCR failed to detect PLMVd with extraction method no.5. The higher analytical sensitivity of the qRT-PCR protocol was also emphasised by the different behaviour of the two amplification methods in the framework of serial RNA dilution only (Table 3). For example, for extraction method no.1, the RT-PCR was only able to detect up to the second serial dilution, whereas the qRT-PCR was able to detect up to the sixth serial dilution.

Moreover, in order to test the repeatability of the protocol, the two most efficient extraction methods (no.1 and no.2) were also tested for their behaviour in absolute quantification. Specifically, three infected samples were extracted using the two methods, brought to the same TRNA target concentration (*ca* 10 ng/μl) and loaded onto the same plate. The obtained results, summarised in Table 4, evidenced, as expected, a higher repeatability of the kit-based protocol (extraction method no. 2)

Sample testing for protocol sensitivity and specificity

The qRT-PCR protocol was also tested for its specificity and sensitivity in detecting target, healthy and non target samples. The TNA from all samples were obtained using extraction method no.1. The specificity was tested using healthy GF 305, peach, apricot, plum and pear controls and tissues infected by non-target viroids as HSVd, PBCVd, ASSVd, ADFVd and PSTVd. None of the non target or healthy samples analysed has ever reacted positively in qRT-PCR. On the contrary, all 20 peach samples coming from commercial orchards and infected with different PLMVd variants (Luigi et al. 2009) resulted in positive reactions. Moreover, the qRT-PCR protocol was also able to detect three GF305 samples infected by the PLMVd 'calico'

Table 3 Comparison between the two amplification protocols on RNA extracted with the five methods

Extraction method	N° of starting RNA molecules	Analytical Sensitivity RT-PCR	Analytical Sensitivity qRT-PCR
Extraction 1 (phenol)	$2{,}83\ 10^7$	10^{-2}	10^{-6}
Extraction 2 (TRNA <200nt)	$2{,}85\ 10^7$	10^0	10^{-4}
Extraction 3 (Tri-Reagent)	$2{,}79\ 10^6$	10^0	10^{-2}
Extraction 4 (TRNA >200nt)	$8{,}33\ 10^5$	10^0	10^0
Extraction 5 (tissue print)	$1{,}38\ 10^5$	Not signal	10^0

Analytical sensitivity was evaluated comparing the last dilution in which the amplification signal was detected.

Table 4 Comparison between extraction method no. 1 and no. 2

Sample	Extraction method	Starting quantity of RNA (ng/μl)	Ct	N° of RNA molecules detected
1	Number 1	9,98	17,15	$5,75\ 10^8$
2	Number 1	9,97	18,32	$2,58\ 10^8$
3	Number 1	10,10	17,29	$5,22\ 10^8$
1	Number 2	10,02	15,05	$2,41\ 10^9$
2	Number 2	9,97	15,02	$2,46\ 10^9$
3	Number 2	9,97	14,90	$2,67\ 10^9$

isolate (Malfitano et al. 2003), coming from the CRA-PAV germplasm collection (originally kindly provided by Prof. Ragozzino).

Discussion

This work demonstrates the feasibility and reliability of a qRT-PCR protocol for the identification and quantification of PLMVd. The reliability, sensitivity and specificity of this protocol were optimised by designing specific primers and probes for use at certain concentrations. This was done with the help of a one-step Master Mix to standardise the implementation of the qRT-PCR reaction and through the comparison of different extraction methods.

In order to perform an absolute pathogen quantification and to evaluate the efficiency of the reaction, a standard curve was constructed using a series of ten-fold dilutions of target RNA obtained by in vitro transcription of a PLMVd clone. The 'goodness' of the method was demonstrated by the fact that optimal values of statistical coefficients were obtained. The line of best fit through the means of three replicates of seven points gave a coefficient of determination (R^2) very close to 1 (0,98) showing a good fit of the standard curve to the data points plotted: 98% of the variation in y is explained by variations in x only. Theoretically, the optimal value for the *y*-intercept should be around the 40 mark and the slope for an ideal standard curve should be −3.32. In our standardised conditions, the value of the *y*-intercept was 46 and the slope −3.371. Under these conditions, the analytical sensitivity of the method was very high: in fact, the qRT-PCR was capable of detecting up to 1 fg of PLMVd RNA. Its efficiency, calculated using the formula $\eta = 10\left(\frac{1}{slope}\right) - 1$, is 98%, meaning a quasi-doubling of the amplified product at each cycle, as should be in the case in an ideal reaction.

The protocol was used with five different extraction methods amongst those most frequently used for the detection of RNA plant pathogens. All five extraction methods used were appropriate, despite significant differences for the PLMVd diagnosis. The two non kit-based methods (extraction methods no.1 and no.3) both generated very good results in quantitative and qualitative amplification reactions. The greater efficiency revealed by extraction method no.1 could probably be due to the high amount of the starting tissue and to the use of liquid nitrogen in the first step that better protects the RNA from lysis. The comparison of the two kit-based extraction methods (no.2 and no.4) showed that extraction method no.2 generated better results in terms of amount of RNA extract and analytical sensitivity. This is probably due to the different nature of the compared commercial kits. In fact, the majority of commercial kits for total RNA extraction (TRNA) from plant tissues consist of a selective binding silica-based resin and a specialised high-salt buffer system able to retrieve RNA that is longer than 200 nt. Instead, the mirPremier™ microRNA Isolation Kit (Sigma) is specific for purifying and enriching miRNA and other small RNA (<200nt) from 'problematic' tissues. The results obtained in this work underline that highly structured viroid genomes could behave differently from other RNA molecules of the same length (337–350 nt in the case of PLMVd), but comparable to smaller molecules (<200 nt). Extraction method no.5 is a quick and easy extraction protocol that could be used in qRT-PCR starting from fresh tissue. Although it has not given satisfactory results for quantification purposes, its use could be very helpful in the presence of a large number of samples.

Although extraction method no.1 (phenol extraction) proved to be the most sensitive (Table 3), the excellent results also obtained with extraction method no.2 and its higher repeatability (Table 4) would suggest its use to standardise the procedure and to avoid the use of organic compounds.

The qRT-PCR protocol was tested for its sensitivity, specificity and reproducibility using target (different PLMVd variants), healthy (different host species) and non-target (different viroids) field sam-

ples. The fact that neither healthy GF 305, peach, apricot, plum and pear, nor non-target samples gave positive results pointed to the high specificity of the method. The sensitivity and reproducibility was tested on peach and GF 305 PLMVd-infected samples from commercial orchards or from a greenhouse, respectively. Sample testing included 20 different isolates of PLMVd, composed by different genome variants (Luigi et al. 2009) and three 'calico' isolates of PLMVd that contain, an insertion of 12 nucleotides (Malfitano et al. 2003). All PLMVd-infected samples tested gave a clear positive signal, stressing the sensitivity and reproducibility of the qRT-PCR protocol.

In conclusion, the qRT-PCR protocol for the identification and quantification of PLMVd has the potential to be used in disease management and PLMVd control, both of which have direct effects in sanitary and certification programmes.

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References

Albanese, G., Giunchedi, L., La Rosa, R., & Poggi Pollini, C. (1992). Peach latent mosaic viroid in Italy. *Acta Horticolturae, 309*, 331–338.

Ambros, S., Desvignes, J. C., Llàcer, G., & Flores, R. (1995). Peach latent mosaic viroid and pear blister canker viroid: detection by molecular hybridization and relationship with specific maladies affecting peach and pear trees. *Acta Horticulturae, 386*, 512–521.

Boonham, N., Gonzàlez, L., Lilia Peralta, E., Blockley, A., Walsh, K., Barker, I., et al. (2004). Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. *Journal of Virolological Methods, 116*, 139–146.

Boubourakas, I. N., Fukuta, S., & Kyriakopoulou, P. E. (2009). Sensitive and rapid detection of peach latent mosaic viroid by the reverse transcription loop-mediated isothermal amplification. *Journal of Virological Methods, 160*, 63–68.

Bussière, F., Lehoux, J., Thompson, D. A., Skrzeczkowski, J., & Perreault, J. P. (1999). Subcellular localization and rolling circle replication of peach latent mosaic viroid: hallmarks of group A viroids. *Journal of Virology, 73*, 6353–6360.

Desvignes, J. C. (1976). The virus diseases detected in greenhouse and in field by the peach seedling GF 305 indicator. *Acta Horticulturae, 67*, 315–323.

Desvignes, J. C. (1986). Peach latent mosaic viroid and its relation to peach mosaic and peach yellow mosaic virus diseases. *Acta Horticulturae, 193*, 51–57.

Faggioli, F., Ragozzino, E., & Barba, M. (2001). Simultaneous detection of stone or pome fruit viroids by single tube RT-PCR. *Acta Horticulturae, 550*, 59–63.

Faggioli, F., Ragozzino, E., Barba, M., Fideghelli, C., Della Strada, G., & Pavia, R. (2003). Influenza del viroide del mosaico latente del pesco in cultivar di pesco e nettarine. *Frutticoltura, 65*, 65–69.

Flores, R., & Llacer, G. (1988). Isolation of a rod-like RNA associated with peach latent mosaic disease. *Acta Horticolturae, 235*, 325–332.

Flores, R., Hernandez, C., Desvignes, J. C., & Llacer, G. (1990). Some properties of the viroid inducing peach latent mosaic disease. *Research in Virology, 141*, 109–118.

Flores, R., Randles, J. W., Bar-Joseph, M., & Diener, T. O. (1998). A proposed scheme for viroid classification and nomenclature. *Archives of Virology, 143*, 623–629.

Hernández, C., & Flores, R. (1992). Plus and minus RNAs of peach latent mosaic viroid self-cleave in vitro via hammerhead structures. *Proceedings of the National Academy United States of America, 89*, 3711–3715.

Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Science United States of America, 88*, 7276–7280.

Kyriakopoulou, P. E., Giunchedi, L., & Hadidi, A. (2001). Peach latent mosaic viroid and pome fruit viroids in naturally infected cultivated pear Pyrus communis and wild pear P. amygdaliformis: implications on possible origin of these viroids in the Mediterranean region. *Journal of Plant Pathology, 83*, 51–62.

Loreti, S., Faggioli, F., & Barba, M. (1995). A rapid extraction method to detect peach latent mosaic viroid by molecular hybridization. *Acta Horticulturae, 386*, 560–564.

Loreti, S., Faggioli, F., Cardoni, M., Mordenti, G., Babini, A. R., Poggi Pollini, C., et al. (1999). Comparison of different diagnostic methods for the detection of peach latent mosaic viroid. *OEPP Bulletin/EPPO Bullulletin, 29*, 433–438.

Luigi, M., Faggioli, F., Barba, M., & Giunchedi, L. (2009). Identification and characterization of Peach latent mosaic viroid and Hop stunt viroid in different peach cultivars showing dapple fruit, fruit yellow mosaic and cracked sutures symptoms. 21st "International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops"- Julius-Kühn-Archiv, 427, 373–377.

Malfitano, M., Di Serio, F., Covelli, L., Ragozzino, A., Hernández, C., & Flores, R. (2003). Peach latent mosaic viroid variants inducing peach calico (extreme chlorosis) contain a characteristic insertion that is responsible for this symptomatology. *Virology, 313*, 492–501.

Mandic, C., Al Rwahnih, M., Myrta, A., Gomez, G., & Pallas, V. (2008). Incidence and genetic diversity of peach latent mosaic viroid and hop stunt viroid in stone fruits in Serbia. *European Journal of Plant Pathology, 120*, 167–176.

Olmos, A., Bertolini, E., Gil, M., & Cambra, M. (2005). Real-time assay for quantitative detection of non-persistently transmitted Plum pox virus RNA targets in single aphids. *Journal of Virological Methods, 128*, 151–155.

Ozaki, H., Yamamucki, Y., Sato, Y., Tomita, Y., Kawai, Y., Miyamoto, Y., et al. (1999). Peach latent mosaic viroid from stone fruits in Japan. *Annals of the Phytopathological Society of Japan, 65*, 3–8.

Ragozzino, E., Faggioli, F., & Barba, M. (2004). Development of an one-tube RT-PCR protocol for the detection of seven viroids in four genera: Apscaviroid, Hostuviroid, Pelamoviroid and Pospiviroid. *Journal of Virological Methods, 121*, 25–29.

Rizza, S., Nobile, G., Tessitori, M., Catara, A., & Conte, E. (2009). Real time RT-PCR assay for quantitative detection of Citrus viroid III in plant tissues. *Plant Pathology, 58*, 181–185.

Shamloul, A. M., & Hadidi, A. (1999). Sensitive detection of potato spindle tuber and temperate fruit viroids by reverse transcription-polymerase chain reaction–probe capture hybridization. *Journal of Virological Methods, 80*, 145–155.

Shamloul, A. M., Minafra, A., Hadidi, A., Giunchedi, L., Waterworth, H. E., & Allam, E. K. (1995). Peach latent mosaic viroid: nucleotide sequence of an Italian isolate, sensitive detection using RT-PCR and geographic distribution. *Acta Horticolturae, 386*, 522–530.

Shamloul, A. M., Faggioli, F., Keith, J. M., & Hadidi, A. (2002). A novel multiplex RT-PCR probe capture hybridization (RT-PCR–ELISA) for simultaneous detection of six viroids in four genera: Apscaviroid, Hostuviroid, Pelamoviroid and Pospiviroid. *Journal of Virological Methods, 105*, 115–121.

Tessitori, M., Rizza, S., Reina, A., & Catara, V. (2005). Real-time RT-PCR based on SYBR-Green I for the detection of citrus exocortis and citrus cachexia diseases 16th Conference of the International Organization of Citrus Virologists, 456–459.

Tsagris, E. M., Martínez de Alba, A. E., Gozmanova, M., & Kalantidis, K. (2008). Viroids. *Cell Microbiology, 10*, 2168–2179.