A DIAGNOSTIC METHOD FOR THE SIMULTANEOUS DETECTION AND IDENTIFICATION OF POSPIVIROIDS

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SUMMARY

Potato spindle tuber viroid (PSTVd) was the first species of the genus *Pospiviroid* detected in naturally infected symptomless ornamental plants (*Solanum jasminoides*) in 2006. Since then, other pospiviroid species were found in several naturally infected solanaceous ornamentals. The combination of latent infection, the widespread occurrence and the possibility of transmission to edible solanaceous crops (tomato and potato) has made the development of accurate and reliable molecular diagnostic detection methods a high priority. The aim of this study was the development of a genus-specific RT-PCR protocol, using universal pospiviroid primers, which would allow also a species-specific identification through restriction fragment length polymorphism (RFLP) analysis. The primers were designed to amplify the majority of the pospiviroid genome (*ca.* 80%) and the identification of each species using appropriate restriction endonuclease enzymes (*Alu*I and *Sau*96I). The method was evaluated for specificity using healthy and/or non-target viroid-infected plants and for sensitivity using serial dilutions of total RNA extracted from plants infected by different pospiviroid species. The reliability of the method was successfully ascertained during a survey when numerous plants of the families Solanaceae and Asteraceae were assayed for single and mixed infection of pospiviroid species.

Key words: RT-PCR, RFLP, *Pospiviroid*, diagnosis, genus-specific detection, species-specific detection

INTRODUCTION

Viroids, the smallest pathogens known, are composed of a highly complementary naked single-stranded circular RNA ranging in size from 246 to 401 nucleotides and do not encode any protein. Despite this minimal molecular composition, viroids contain sufficient information to infect host plants, to manipulate their gene expression and induce specific diseases (Hadidi *et al*., 2003).

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Viroids are classified into two families, *Pospiviroidae* [type species *Potato spindle tuber viroid* (PSTVd)] and *Avsunviroidae*, [type species *Avocado sunblotch viroid* (ASBVd)] (Flores *et al*., 2003; Owens *et al*., 2011), that replicate and accumulate in the nucleus and the chloroplast, respectively. The viroid classification scheme is also supported by other criteria, including the presence of hammerhead ribozymes in members of the family *Avsunviroidae* and of a central conserved region (CCR) in members of the family *Pospiviroidae*.

The family *Pospiviroidae* includes the genus *Pospiviroid* composed of ten phylogenetically related species: PSTVd, *Chrysanthemum stunt viroid* (CSVd), *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd), *Iresine viroid* 1 (IrVd-1), *Mexican papita viroid* (MPVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Tomato planta macho viroid* (TPMVd) and *Pepper chat fruit viroid* (PCFVd) (Verhoeven *et al*., 2009). These species show a high genomic identity, in some cases higher than 80% (Table 1). Moreover, most pospiviroids are almost indistinguishable in biological features from one other.

Most pospiviroids can cause severe diseases and damages to most species in the family Solanaceae, potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) in particular, in which they induce epinasty, stunting, leaf distortion or discoloration and necrosis (usually vein necrosis) (Singh, 1973; Galindo *et al*., 1982; Owens, 1990; Martinez-Soriano *et al*., 1996; Singh *et al*., 1999, 2003). Notwithstanding the above, PSTVd is currently the only Solanaceae-infecting pospiviroid with an explicitly regulated status in Europe (Council Directive 2000/29/EC), USA and Canada (NAPPO, 2007).

In 2006, PSTVd was found for the first time in latently infected solanaceous ornamentals. i.e. *Solanum jasminoides* and *Brugmansia* spp. (Verhoeven *et al*., 2008). Many other pospiviroid species were subsequently recovered from different symptomless such plants, that represent a sanitary hazard, as they constitute a source of inoculum for other susceptible hosts (Navarro *et al*., 2009). Simultaneous infections by PSTVd and TASVd, CEVd and TASVd, PSTVd and CEVd have recently been detected in *S. jasminoides* and *Cestrum auranticum* (Luigi *et al*., 2011; Torchetti *et al*., 2011).

This situation and the recent discovery that CSVd, another quarantinable pospiviroid, is able to infect

Table 1. Percentage of nucleotide identity among reference sequences for each pospiviroid species.

	CSVd	CEVd	CLVd	IrVd	MPVd	PCFVd	PSTVd	TASVd	TCDVd	TPMVd
CSVd	100%									
CEVd	75%	100%								
CLVd	73%	68%	100%							
IrVd	68%	70%	62%	100%						
MPVd	75%	75%	75%	71%	100%					
PCFVd	69%	71%	67%	74%	73%	100%				
PSTVd	73%	74%	76%	71%	**86%**	71%	100%			
TASVd	**81%**	**83%**	76%	67%	79%	74%	75%	100%		
TCDVd	75%	74%	75%	70%	**84%**	71%	**91%**	75%	100%	
TPMVd	75%	73%	75%	72%	**92%**	73%	**84%**	79%	**82%**	100%

Sequences retrieved from the NCBI database were: CSVd [NC_002015], CEVd [NC_001464.1], CLVd [NC_003538.1], IrVd 1[NC_003613.1], MPVd [NC_003637.1], PCFVd [NC_011590.1], PSTVd [NC_002030.1], TASVd [NC_001553.1], TCDVd [NC_000885.1] and TPMVd [NC_001558.1]. In bold are the highest percentages of identity.

symptomlessly *Argyranthemum frutescens* in Italy (Torchetti *et al*., 2012a) and France (Marais *et al*., 2011), have increased the concern for the incumbent phytosanitary risks (EFSA, 2011, 2012), thus calling for the adoption of regulatory measures based on the use of viroid-free propagation material and, when possible, on the interception of infected plants. Effectiveness of these defense strategies requires a rapid, efficient and reliable method for pospiviroids generic detection and specific identification.

In the past, different diagnostic methods have been developed either for the genus-specific or species-specific detection of members of the genus *Pospiviroid*: (i) northern blot hybridization (Owens and Diener, 1981; Singh, 1999); (ii) a single polyprobe for the simultaneous identification of eight viroids (Torchetti *et al*., 2012b); (iii) RT-PCR and Real time RT-PCR protocols using primers for pospiviroid generic detection (Bostan *et al*., 2004; Verhoeven *et al*., 2004; Botermans *et al*., 2013); (iv) specific primer pairs for species-specific detection (RT-PCR and real-time RT-PCR) (Yang *et al*., 1992; Hooftman *et al*., 1996; Spieker, 1996; Shamloul *et al*., 1997; Mumford *et al*., 2000; Boonham *et al*. 2004; Roenhorst *et al*., 2005)

This paper reports the development of a genus- and species-specific diagnostic method for pospiviroids. The method involves RT-PCR amplification of viroidal RNA using universal pospiviroid primers for genus detection, after which the amplified DNA is analyzed by restriction fragment length polymorphism (RFLP) for identification at the species level.

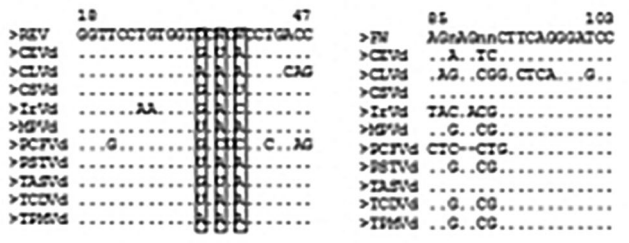

Fig. 1. Alignments of all pospiviroids in the two selected regions for primer design. Left, the reverse primer and in boxes the degenerate nucleotides are highlighted. Right, the forward primer, the letter n highlights the variable positions among different homologous primers.

MATERIALS AND METHODS

Source of materials. The CRA-Plant Pathology Research Centre (CRA-PAV, Rome) germplasm collection provided *S. jasminoides* plants infected by CEVd, TASVd and PSTVd in single and mixed infection, *Chrysanthemum* sp. plants infected by CSVd and healthy controls of *S. jasminoides*, tomato and potato. Positive controls infected by CEVd, CLVd, IrVd, MPVd, PCFVd, TASVd, TCDVd and TPMVd were kindly supplied by Dr. J. Th. J.Verhoeven (Dutch Plant Protection Service and Laboratory of Virology, Wageningen University) and maintained on tomato plants in a greenhouse.

To test the efficiency and accuracy of the method, 200 plants were collected from different nurseries and examined (Table 2). Their sanitary status has previously been ascertained using specific diagnostic methods (Di Serio, 2007; Önelge, 1997; Levy and Hadidi, 1992; Tomassoli *et al*., 2004; Verhoeven *et al*., 2010).

RNA extraction from pospiviroid-infected and uninfected plants. Total RNA was extracted from each control and test sample using the Spectrum™ Plant Total RNA Kit (Sigma, Germany) according to the manufacturer's instructions. RNA was finally eluted with 100 µl of RNase-free water.

Primer design and RT-PCR for detecting members of the genus *Pospiviroid*. Reference sequences of all pospiviroids were retrieved from the NCBI database (Table 1) and used to perform a multiple alignment for designing an universal genus-specific primer set (Table 3) by selecting the most conserved region among all pospiviroids (Fig. 1).

For efficiency testing, selected primers were first used in a two-steps, two-tubes RT-PCR (Menzel *et al*., 2002). Specifically, 2 µl of extracted total RNA were added to 18 µl of a random reverse transcriptase reaction mixture

Table 2. Plant species assayed for possible detection of naturally-infected pospiviroids.

	Species	Viroid detected	No. infected plants/ No. analyzed plants
Ornamental solanaceous plants	*Brugmansia* sp.		0/8
	Cestrum sp.	CEVd, PSTVd	1/29; 22/29
	Celosia	IrVd	4/4
	Datura sp.		0/6
	Nierembergia sp.		0/5
	Petunia sp.		0/8
	Solandra sp.		0/5
	Solanum jasminoides	CEVd, TASVd, PSTVd	46/50, 4/50, 25/50
	Solanum rantonnetii	CEVd	1/5
	Streptosolen jamensonii		0/5
	Surfinia sp.		0/5
Asteraceae plants	*Chrysanthemum spp.*		0/10
	Gazzania spp.		0/2
	Euryopa pectinatus		0/6
	Bideus spp		0/1
	Argyranthemum frutescens	CSVd	6/11
Solanaceous plants	*Solanum lycopersicum*		0/10
	Solanum tuberosum		0/10
	Capsicum spp		0/10
	Solanum melongea		0/10
	Total		83/200

Table 3. Primers designed in this study with nucleotide position of each primer indicated with reference to PSTVd sequence [NC_002030.1]. Degenerate nucleotides are highlighted in gray.

Name	Sequence	Position (nt)
POP-REV	5'-GGTCAGGWGWGHACCACAGGAACC-3'	15-39
POP-FW	5'-AGRAGYSCTTCAGGGATCC-3'	83-102
POP1-FW	5'-AGAAGTCCTTCAGGGATCC-3'	83-102
POP2-FW	5'-AGGAGCGCTTCAGGGATCC-3'	83-102
POP3-FW	5'-AAGAGCGGTCTCAGGAGCC-3'	83-102
POP4-FW	5'-ATCTCCTGTTCAGGGATCC-3'	83-102

containing: 50 mM Tris-HCl pH 7.4, 75 mM KCl, 5 mM MgCl2, 1 mM dNTPs, 0.1 ng random primer, 3 U M-MLV reverse transcriptase (Life Technologies, USA). Reverse transcription was performed at 42°C for 45 min. Subsequently, 5 µl of random cDNA were added to 45 µl of an amplification reaction composed of: 1× GoTaq buffer colorless (Promega, USA), 0.5 µM of the complementary primer POP-REV, 0.5 µM of each homologous primer or 1 µM of the combination POP1-FW and POP3-FW, 0.5 mM dNTPs, 5 U GoTaq polymerase (Promega, USA). The reaction was performed at 95°C for 5 min followed by 35 cycles of amplification (95°C for 45 sec; 60°C for 45 sec; 72°C for 1 min) and 7 min at 72°C for final extinction. All amplified products were visualized in 1.2% agarose gel stained with ethidium bromide.

A one-tube one-step RT-PCR protocol (Faggioli *et al*., 2005) was subsequently used for obtaining amplicons of different pospiviroid species as this method is faster than that of the two tubes-two steps RT-PCR and possible cross contamination is avoided. Specifically, 2 µl of total RNA were added to 48 µl of a reaction mixture containing: GoTaq Buffer 5× (Promega, USA); 200 µM each dNTPs; 10 mM DTT; 0.4 µM antisense primer (POP-REV), 0.3 µM of the POP1-FW primer and 0.1 µM of the POP3-FW primer; 2.5 U of avian myeloblastosis virus (AMV)-RT (Promega, USA); 20 U of RNase Out (Life Technologies, USA); 1.25 U GoTaq polymerase (Promega, USA). Synthesis of cDNA was done at 46°C for 30 min, followed by denaturation at 95°C for 30 sec. Amplification was carried out for 35 cycles under the following condition: denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 60 sec, followed by a final extension for 7 min at 72°C. The robustness of the one step-one tube amplification protocol was tested also on 9 ten-fold serial dilutions of total RNA extracts of the most economically important pospiviroids (CEVd, CSVd, PSTVd and TASVd).

Endonuclease digestion (RFLP analysis). Amplicons obtained from each pospiviroid control were sequenced and submitted to a bioinformatic tool (Restriction Mapper version 3.0) to generate a list of the restriction enzymes able to cut PCR products. Enzymes with more than 5 cuts and those with a similar pattern profile were discarded. Five restriction enzymes were chosen: *Alu*I, *Ava*II, *Hae*III, *Mbo*II, *Sau*96I (Fermentas, Lithuania). Ten µl of the amplified products were digested for 60 min at 37°C using

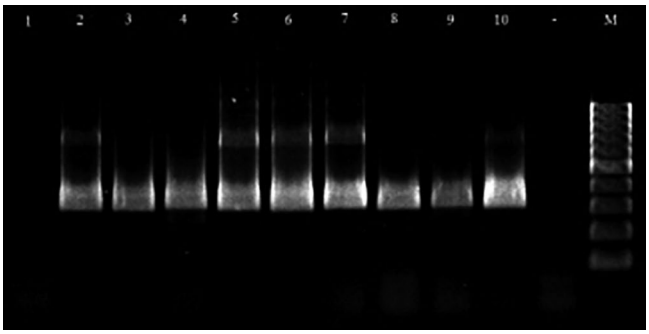

Fig. 2. Agarose gel electrophoresis analysis of amplified products obtained by one step one tube RT-PCR. Lane 1, healthy tomato; lanes 2 to 10, TCDVd, TASVd, CEVd, TPMVd, MPVd, CLVd, CSVd, IrVd and PSTVd; lane 11, water control; lane 12, DNA size markers (Gene Ruler^{TM} 100 bpDNA Ladder, Fermentas).

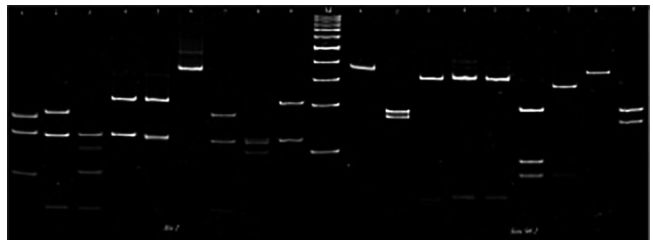

Fig. 3. Polyacrylamide gel electrophoresis analysis of the endonuclease digestion. On the left of the DNA size marker (lane M, 100 bp) *AluI* restriction fragment; on the right, *Sau96I* restriction fragment. Lane 1, TCDVd; lane 2, TASVd; lane 3, CEVd; lane 4, TPMVd; lane 5, MPVd; lane 6, CLVd; lane 7, CSVd; lane 8, IrVd; lane 9, PSTVd.

1× FastDigest Green Buffer (Fermentas, Lithuania), 1 µl of each restriction enzyme and nuclease-free water to bring the final volume to 30 µl. Ten µl of the digested products were resolved on vertical 5% (w/v) polyacrylamide gels in TBE buffer (89 mM Tris/borate, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA fragments were visualized in ethidium bromide. Fragments obtained by the endonuclease reaction were also resolved by a Bioanalyzer 2100 apparatus (Agilent Technologies, USA) to confirm the quality of the reaction. Electropherograms were obtained using the DNA 1000 Kit following the manufacturer's instructions. The resolution of the Bioanalyzer varied according to the length of the fragments analyzed. The apparatus has a limited resolution of about 5 bp with DNA fragments less than 100 bp, and 5% with fragments ranging from 100 to 500 bp. The repeatability of the reaction was tested *in vivo*, using, when possible, different variants of pospiviroid species and *in silico*, checking the percentage of the conservation of each restriction site among the different isolates retrieved from NCBI database.

RESULTS

Genus-specific RT-PCR. *Development.* The primers were designed in two conserved structural domains in the genome of pospiviroids: the terminal left and the pathogenesis domains. Whereas the complementary primer (POP-REV) was designed in a region of high identity for all pospiviroids using only three degenerate nucleotides (Table 3), the homologous primer was designed in a region with lower sequence identity. For this reason different forward primers were designed: POP-FW, taking into consideration sequence diversity among pospiviroids, had three degenerated nucleotides; POP1-FW, based on sequence identity with target viroids, had an expected higher specificity for CEVd, CSVd and TASVd, while POP2-FW had an expected higher specificity for PSTVd or TCDVd. These primers were tested using positive controls and only the POP-REV/POP1-FW set detected all pospiviroid species, except for CLVd and PCFVd. Subsequently, another homologous primer was designed for the same region specific for CLVd (POP3-FW) and was added to the mixture. Using the set POP-REV and POP1-FW/POP-3-FW the amplification of all pospiviroids, using two steps-two tubes RT-PCR was obtained (Fig. 2), with the exception of PCFVd. A new forward primer (POP4-FW), specific for PCFVd, was added, though unsuccessfully.

As expected, the size of each amplified product, synthesized using a mixture of three different primers (POP-REV, POP1 FW and POP3 FW) was 300 bp for each pospiviroid species. In agreement with sequencing analysis, nine pospiviroids were recognized by the primer set.

Implementation. The amplification protocol was implemented using one-step one-tube RT-PCR (all the reported results were obtained with this amplification method). In order to establish the best conditions for amplification, different concentrations of primers were used: POP-REV was tested at 0.2 and 0.4 µM; POP1-FW and POP3-FW at 0.1, 0.2, 0.3 and 0.4 µM. The best mixture was obtained using POP-REV, POP1-FW and POP3-FW at a concentration of 0.4, 0.3 and 0.2 µM respectively.

Endonuclease digestion (RFLP analysis) for species identification. For species-specific characterization, a restriction enzymes analysis was performed. Initially, an *in silico* analysis of the restriction profiles was done. Five different restriction enzymes were selected and applied, at least two of which (*Alu*I and *Sau*96I) proved essential for differentiateing all pospiviroid species and their possible variants. Both enzymes were used to cut the amplified products obtained with the genus-specific primer set and, as reported in Fig. 3, all the obtained profiles were as expected. The accuracy of the reaction was tested using also Bioanalyzer 2100 and all the endonuclease restriction profiles were confirmed (Fig. 4). Endonuclease reactions were performed for different durations: 15, 30 and 60 min. The best endonuclease digestion fragment profile was obtained after 60 min of incubation at 37°C.

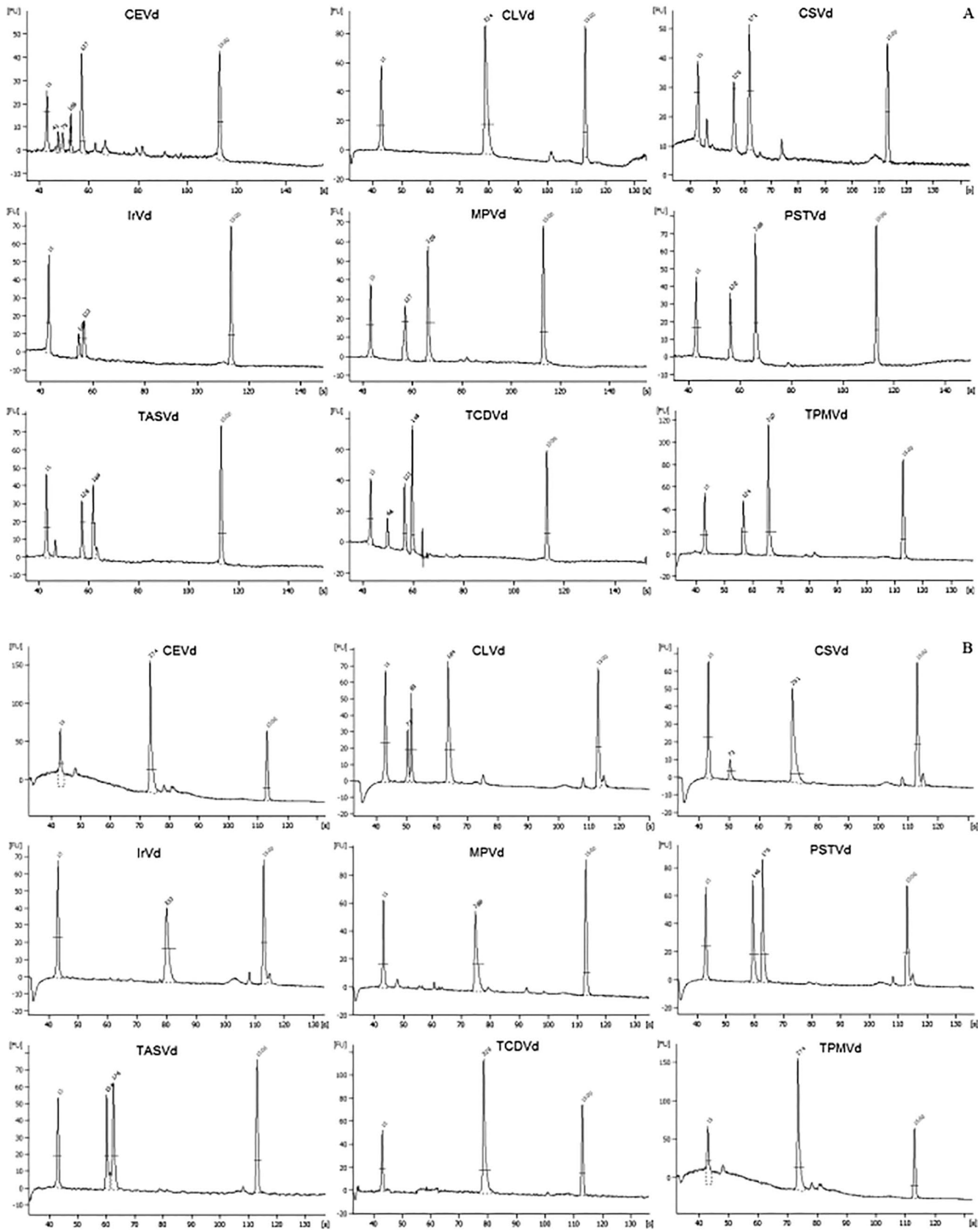

Fig. 4. Electropherograms of the endonuclease digestions performed with Bioanalyzer 2100 using the DNA 1000 kit. Time (in seconds) is reported on the *x*-axis, whereas the fluorescence value is found on the *y*-axis. The size in bp calculated using two size internal marker (15 and 1500 bp respectively the first and the last pick in each electropherograms) are reported over the peaks. Panel A, endonuclease restriction analysis performed using *AluI*; panel B, endonuclease restriction analysis performed using *Sau96I.*

Table 4. Endonuclease restriction pathways obtained by comparing pospiviroid isolates retrieved from NCBI and relative percentage (%) of distribution among isolates of the same species.

Pospiviroid	Amplification product (bp)	*Alu*I	%	*Sau96*I	%
CEVd	335	122-71-59-47-37 122-105-71-37	75 25	290-45	100
CLVd	333	No cut 247-89 151-97-89	25 36 39	187-80-66 No cut	24 76
CSVd	322	172-114-36	100	284-38	100
IrVd	334	121-115-98	100	No cut	100
MPVd	325	205-120	100	279-46	100
PCFVd	315	253-62	100	118-82-61-54	100
PSTVd	325	204-121	100	180-145	100
TASVd	327	168-122-37	100	173-154	100
TCDVd	322	147-134-78	100	No cut 279-44	10 90
TPMVd	326	205-121	100	279-47	100

Performance of the diagnostic protocol. The protocol was tested for its sensitivity, specificity and accuracy. The analytical sensitivity was assessed using serial ten-fold dilutions of total RNA extracted from different infected controls: PSTVd, CSVd, TASVd and CEVd. PSTVd was detected to the sixth dilution, TASVd and CSVd till the fifth, and CEVd till the third. The analytical specificity was evaluated using different healthy solanaceous (tomato, potato and ornamentals) and asteraceous species (chrysanthemum and daisy) as well as non-target viroids (i.e. *Hop stunt viroid* and *Peach latent mosaic viroid*). Amplified products were not obtained when healthy plant RNAs and non-target viroids were used (data not shown). The accuracy was estimated by analyzing 200 different plants species (summarized in Table 2), using both the generic and specific protocols for each pospiviroid species. The results obtained were in perfect agreement. The genus-specific protocol detected all infected samples that were positive using species-specific protocols. When species-specific RFLP was performed, the results confirmed the presence of the causal agent of the infection also with samples that had mixed natural infection (Fig. 5). The accuracy of the protocol was estimated also by *in silico* analysis, comparing different isolates of each pospiviroid species for evaluating the respective restriction site(s). Most (7 of 10) of the pospiviroid species showed the conservation of restriction sites of the two enzymes used practically in 100% of the isolates present in database. In three cases, (CEVd, CLVd and TCDVd) the *in silico* analysis showed different RFLP profiles among the isolates, in different percentage (Table 4), but, in spite of these differences, the 10 pospiviroid species were equally distinguishable.

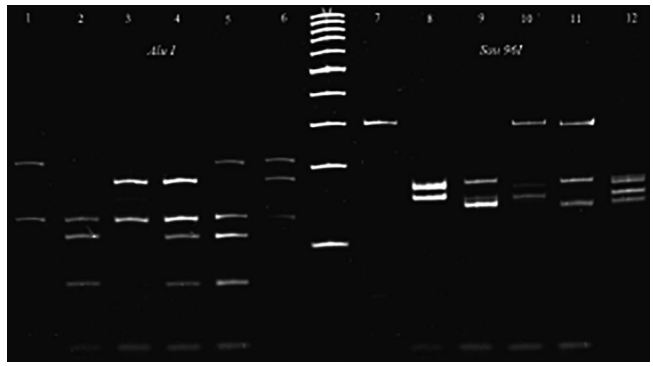

Fig. 5. Polycrylamide gel electrophoresis analysis of the endonuclease digestions of single and mixed infections. From lane 1 to 6 *Alu I* restriction fragment; from lane 7 to 12 *Sau96I* restriction fragment, lane M, 100 bp. Lanes 1 and 9, PSTVd; lanes 2 and 7; CEVd, lanes 3 and 8, TASVd; lanes 4 and 10, CEVd+TASVd; lanes 5 and 11, CEVd+PSTVd; lanes 6 and 12, PSTVd+TASVd.

DISCUSSION

The spread of pospiviroids, observed in several European countries in recent years, has made it essential to develop fast, efficient and sensitive detection methods to detect and identify them. Members of the genus *Pospiviroid* share genomic regions of very high identity that are interspersed with highly variable stretches. In this paper, the characteristics of the pospiviroid genomes have been exploited to produce a new diagnostic tool that rapidly detect and identify all pospiviroids.

A new primer set, composed of three different oligonucleotides was designed for detecting in a single reaction, all pospiviroids and, at the same time, to amplify the majority of their genomes. In the past, RT-PCR methods for the generic detection of pospiviroids in ornamental plants have been developed which amplify products with less than 200 bp in size, that do not allow subsequent species-specific identification (Bostan *et al*., 2004). Also, a double RT-PCR amplification reaction was required for the detection of almost all pospiviroids (Verhoeven *et al*., 2004). The primer set developed in this study amplifies, in a single PCR reaction, about 80% of each genome, producing a DNA fragment of about 300 bp. The primer set, however, did not detect PCFVd, although this viroid has a sequence with an identity level that should have made it recognizable. However, since only a single PCFVd isolate was tested in this study, the analysis of more isolates is desirable to confirm or not the reliability the method developed which, by and large, has shown a high specificity and sensitivity. It was tested on 200 field samples producing results identical to those obtained using pospiviroid species-specific reactions and detected the most widespread and economically important pospiviroid species (PSTVd, CEVd, CSVd and TASVd) present at a very low titers and in mixed infection.

The amplification of 300 bp DNA product for each pospiviroid species made it possible to subsequently

identify each of them by RFLP analysis. It seems worth pointing out that: (i) RFLP analysis avoids the need of sequencing amplified products, a practice more expensive and time-consuming than restriction analyses, i.e. the only available method for determining the pospiviroid species after amplification with universal primers; (ii) performing pospiviroid specie-specific analysis is critical and important for, according to phytosanitary regulations, only PSTVd and CSVd are quarantine pathogens in the EU, USA and Canada. Nonetheless, all pospiviroids may be considered as potentially quarantinable pathogens because of the harm that they may cause to infected plants.

The identification of each pospiviroid species was achieved by comparing patterns obtained with two restriction enzymes, but did not discriminate MPVd from TPMVd. These two viroids, however, share high sequence identity (> 90%) (Table 1) so that, following the reassessment of their molecular and biological properties, Verhoeven *et al*. (2011) suggested that they should be considered as possible variants of the same species. Furthermore, a RFLP analysis carried out combining two different restriction analyses appears to be a good tool to overcome the problem of the high mutational rate of viroidal genome. In fact, in the course of our studies, the simultaneous comparison of the two fragment patterns revealed immediately the presence of mutations. During RFLP analysis, two different profiles were identified for CEVd isolates from different hosts (tomato and *S. jasminoides*) and countries (the Netherlands and Italy). Specifically, the difference was due to A/G_{58} mutation occurred in the viroid genome from Italy. This different restriction profile was common to 25% of the isolates present in the NCBI database (Table 5). On the basis of the analysis of all pospiviroids isolates present in the NCBI database, other two species (CLVd and TCDVd) showed diverse possible RFLP patterns due to point mutations. This finding, however, did not hamper the newly developed method in identification and characterization of the *Pospiviroid* species.

In conclusion, the newly described method of detection and identification of pospiviroid species is accurate, sensitive and it may save time, money and reduce manipulation and contamination risks. This diagnostic tool could be easily used for preventing the introduction of propagation material infected by these viroids, especially in the light of possible regulatory changes that may require monitoring for all pospiviroid species in propagation materials moving within the European Union.

REFERENCES

Boonham N., Perez L.G., Mendez M.S., Peralta E.L., Blockley A., Walsh K., Barker I., Mumford R.A., 2004. Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. *Journal of Virological Methods* **116**: 139-146.

Bostan H., Nie X., Singh R.P., 2004. An RT-PCR primer pair for the detection of pospiviroids and its application in surveying ornamental plants for viroids. *Journal of Virological Methods* **116**: 189-193.

Botermans M., van de Vossenberg B.T.L.H., Verhoeven J.Th.J., Roenhorst J.W., Hooftman M., Dekter R., Meekes E.T.M., 2013. Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. *Journal of Virological Methods*, **187**: 43-50.

Di Serio F., 2007. Identification and characterization of *Potato spindle tuber viroid* infecting *Solanum jasminoides* and *S. rantonnetii* in Italy. *Journal of Plant Pathology* **89**: 297-300.

EFSA, 2011. Scientific opinion on the assessment of the risk of solanaceous pospiviroids for the EU territory and the identification and evaluation of risk management options. *EFSA Journal*, **2330**: 133.

EFSA, 2012. Scientific opinion on the risk to plant health posed by Chrysanthemum stunt viroid for the EU territory, with identification and evaluation of risk reduction options. *EFSA Journal* **3027**: 87.

Faggioli F., Ferretti L., Albanese G., Sciarroni R., Pasquini G., Lumia V., Barba M., 2005. Distribution of olive tree viruses in Italy as revealed by one-step RT-PCR. *Journal of Plant Pathology* **87**: 49-55.

Flores R., Randles J.W., Owens R.A., 2003. Classification. In: Hadidi A., Flores R., Randles J.W., Semancik J. (eds). Viroids, pp. 71-75. CSIRO Publishing, Collingwood, Victoria, Australia.

Galindo J., Smith D.R., Diener T.O., 1982. Etiology of planta macho, a viroid disease of tomato. *Phytopatology* **72**: 49-54.

Hadidi A., Flores R., Randles J.W., Semancik J.S., 2003. Viroids. CSIRO Publishing, Collingwood, Victoria, Australia.

Hooftman R., Arts M.J., Shamloul A.M., Van Zaayen A., Hadidi A., 1996. Detection of *Chrysanthemum stunt viroid* by reverse transcription-polymerase chain reaction and by tissue blot hybridization. *Acta Horticulturae* **432**: 88-94.

Levy R., Hadidi A., 1992. Direct nucleotide sequencing of PCR-amplified DNAs of the closely related *Citrus viroids* IIa and IIb (cachexia). *Proceedings 13th Conference of the International Organization of Citrus Virology (IOCV), Riverside, CA, USA*: 180-186.

Luigi M., Luison D., Tomassoli L., Faggioli F., 2011. Natural spread and molecular analysis of pospiviroids infecting ornamentals in Italy. *Journal of Plant Pathology* **93**: 491-495.

Marais A., Faure C., Deogratias J.M., Candresse T., 2011. First report of *Chrysanthemum stunt viroid* in various cultivars of *Argyranthemum frutescens* in France. *Plant Disease* **95**: 1196-1197.

Martinez-Soriano J. P., Galindo-Alonso J., Maroon, C.J.M., Yucel I., Smith D. R., Diener T. O., 1996. *Mexican papita viroid*: putative ancestor of crop viroids. *Proceedings of the National Academy of Sciences USA* **93**: 9397-9401.

Menzel W., Jelkmann W., Maiss E., 2002. Detection of four apple viruses by multiplexing RT-PCR assay with coamplification of plant mRNA as internal control. *Journal of Virological Methods*, **99**: 81-92.

Mumford R.A., Walsh K., Boonham N., 2000. A comparison of molecular methods for the routine detection of viroids. *Bulletin OEPP/EPPO Bulletin* **30**: 431-436.

NAPPO, 2007. Requirements for importation of potatoes into a NAPPO member country. Originally drafted in 2003, reviewed in 2007. NAPPO Regional Standard for Phytosanitary Measures. North American Plant Protection Organization. Ottawa, Canada. Regional Standard for Phytosanitary Measures, No. 3.

Navarro B., Silletti M.R., Trisciuzzi V.N., Di Serio F., 2009. Identification and characterization of *Potato spindle tuber viroid* infecting tomato in Italy. *Journal of Plant Pathology* **91**: 723-726.

Önelge N., 1997. Direct nucleotide sequencing of *Citrus exocortis viroid* (CEVd). *Turkish Journal of Agriculture and Forestry* **21**: 419-422.

Owens R.A., 1990. Mutational analysis of viroid pathogenicity: *Tomato apical stunt viroid*. *Molecular Plant-Microbe Interactions* **3**: 374-380.

Owens R.A., Flores R., Di Serio F., Li S.F., Pallás V., Randles J.W., 2011. Viroids. In: King A.M.Q., Adams M.J., Carstens E.B., Lefkowitz E.J. (eds). Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, pp. 1221-1234. Elsevier Academic Press, London, UK.

Owens R.A., Diener T.O., 1981. Sensitive and rapid diagnosis of *Potato spindle tuber viroid* disease by nucleic acid hybridization. *Science* **213**: 670-672.

Roenhorst J.W., Jansen C.C.C., Kox L.F.F., 2005. Application of real-time RT-PCR for large-scale testing of potato for Potato spindle tuber pospiviroid. *Bulletin OEPP/EPPO Bulletin* **35**: 133-140.

Shamloul A.M., Hadidi A., Zhu S.F., Singh R.P., Sagredo B., 1997. Sensitive detection of *Potato spindle tuber viroid* using RT-PCR and identification of a viroid variant naturally infecting pepino plants. *Canadian Journal of Plant Pathology/ Revue Canadienne de Phytopathologie* **19**: 89-96.

Singh R.P., 1973. Experimental host range of the potato spindle tuber 'virus'. *American Potato Journal* **50**: 111-123.

Singh R.P., 1999. Development of the molecular methods for potato virus and viroid detection and prevention. *Genome* **42**: 592-604.

Singh R.P., Xianzhou N., Singh M., 1999. *Tomato chlorotic dwarf viroid*: An evolutionary link in the origin of pospi-viroids. *Journal of General Virology* **80**: 2823-2828.

Singh R.P., Ready K.F.M., Nie X., 2003. Viroids of solanaceous species. In: Hadidi A., Flores R., Randles J.W., Semancik J. (eds). Viroids, pp. 125-133. CSIRO, Publishing Collingwood, Victoria, Australia.

Spieker R.L., 1996. The molecular structure of *Iresine viroid*, a new viroid species from *Iresine herbstii* ('beefsteak plant'). *Journal of General Virology* **77**: 2631-2635.

Tomassoli L., Faggioli F., Zaccaria A., Caccia R., Albani M., Barba M., 2004. Molecular diagnosis of *Chrysanthemum stunt viroid* for routine indexing. *Phytopathologia Mediterranea* **43**: 285-288.

Torchetti E.M., Navarro B., Di Serio F., 2011. First record of *Citrus exocortis viroid* infecting *Solanum jasminoides* in Italy. *Journal of Plant Pathology* **93**: S4.

Torchetti E.M., Navarro B., Trisciuzzi V.N., Nuccitelli L., Silletti M.R., Di Serio F., 2012a. First report of *Chrysanthemum stunt viroid* in *Argyranthemum frutescens* in Italy. *Journal of Plant Pathology* **94**: 451-454.

Torchetti E.M., Navarro B., Di Serio F., 2012b. A single polyprobe for detecting simultaneously eight pospiviroids infecting ornamentals and vegetables. *Journal of Virological Methods* **186**: 141-146.

Verhoeven J.Th.J., Jansen C.C.C., Willemen T.M., Kox L.F.F., Owens R.A., Roenhorst J.W., 2004. Natural infections of tomato by *Citrus exocortis viroid, Columnea latent viroid, Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology* **110**: 823-831.

Verhoeven J.Th.J., Jansen C.C.C., Roenhorst J.W., 2008. First report of pospiviroids infecting ornamentals in the Netherlands: *Citrus exocortis viroid* in *Verbena* sp., *Potato spindle tuber viroid* in *Brugmansia suaveolens* and *Solanum jasminoides*, and *Tomato apical stunt viroid* in *Cestrum* sp. *Plant Pathology* **57** : 399.

Verhoeven J.Th.J., Jansen C.C.C., Roenhorst J.W., Flores R., de la Pena M., 2009. *Pepper chat fruit viroid*: biological and molecular properties of a proposed new species of the genus *Pospiviroid*. *Virus Research* **144**: 209-214.

Verhoeven J.Th.J., Jansen C.C.C., Botermans M., Roenhorst J.W., 2010. First Report *of Iresine viroid* 1 in *Celosia plumosa* in the Netherlands. *Plant Disease* **94**: 920.

Verhoeven J.Th.J., Roenhorst J.W., Owens R.A., 2011. *Mexican papita viroid* and *Tomato planta macho viroid* belong to a single species in the genus *Pospiviroid*. *Archives of Virology* **156:** 1433-1437.

Yang X., Hadidi A., Garnsey S.M., 1992. Enzymatic cDNA amplification of citrus exocortis and cachexia viroids from infected citrus hosts. *Phytopathology* **82**: 279-285.

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