SHORT COMMUNICATION

NATURAL SPREAD AND MOLECULAR ANALYSIS OF POSPIVIROIDS INFECTING ORNAMENTALS IN ITALY

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SUMMARY

In many European countries different species of symptomless ornamental plants were found to be infected by pospiviroid*s*, including *Potato spindle tuber viroid* (PSTVd), a quarantine pathogen. In order to investigate the pospiviroid status of ornamental plants in Italy, a survey was carried out in 2009 and 2010. A total of 111 ornamental plants belonging to nine different solanaceous genera were analyzed. Forty-eight plants were found infected by three pospiviroids. PSTVd was identified in *Solanum jasminoides* and for the first time in *Cestrum* spp., *Citrus exocortis viroid* was found for the first time in *Lycianthes rantonnetii* and *Cestrum* spp. and *Tomato apical stunt viroid* in *S. jasminoides*. The latter represents the first record of this viroid in Italy. All the pospiviroid isolates detected were characterized molecularly.

Key words: *Potato spindle tuber viroid, Citrus exocortis viroid, Tomato apical stunt viroid,* survey, diagnosis.

Viroids, the smallest pathogens known, have a non coding genome composed of a small (246-401 nt) naked single strand RNA (Flores *et al.*, 2004). Viroids are classified into the families *Avsunviroidae* and *Pospiviroidae* based on genomic and biochemical properties. Most viroids belong to the family *Pospiviroidae,* which includes five genera (*Pospiviroid, Hostuviroid, Cocadviroid, Apscaviroid* and *Coleviroid*). The genus *Pospiviroid* comprises ten species, *Potato spindle tuber viroid* (PSTVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Mexican papita viroid* (MPVd), *Tomato planta macho viroid* (TPMVd), *Chrysanthemum stunt viroid* (CSVd), *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd), *Iresine viroid* 1 (IrVd-1), *Columnea latent viroid* (CLVd) (Flores *et al*., 2005) and *Pepper chat fruit viroid* (Verhoeven *et al*., 2009). The *Pospiviroid* genome structure consists of five structural domains: terminal left (TL), pathogenicity (P), central (C), variable (V) and terminal right (TR) (Keese and Symons, 1985). Each domain is characterised by a precise structure and is assumed to contribute to certain functions *in vivo* (e.g. pathogenicity, replication, host specificity, etc.). Members of the family *Pospiviroidae* replicate via the asymmetrical rolling circle mechanism mediated by the host DNA-dependant RNA polymerase II (Schindler and Mühlbach, 1992).

At least five pospiviroid species (PSTVd, MPVd, TASVd, TCDVd, TPMVd) infect horticultural solanaceous plants inducing similar symptoms (Singh *et al*., 2003) and causing severe economic damages. In particular, PSTVd can cause more than 64% losses to potato crops (Pfannenstiel and Slack, 1980), thus it has been included in the list of quarantine pathogens both in the EU and in many other countries.

In the past years, different pospiviroid species were found in several ornamental plants, the most susceptible being: *Solanum jasminoides* infected by CSVd, CEVd, PSTVd and TASVd (Verhoeven *et al*., 2006, 2008a, 2008b, 2008c, 2010a), *Petunia* spp. by CSVd, PSTVd and TCDVd (Verhoeven *et al*., 1998, 2007; Mertelik *et al*., 2009), *Brugmansia* spp. by PSTVd and TCDVd (Verhoeven *et al*., 2008a, 2010a) and *Streptosolen jamensonii* by PSTVd and TASVd (Verhoeven *et al*., 2008b, 2010b)

To investigate the pospiviroid status of ornamental plants in Italy a survey was carried out in 2009 and 2010 in the framework of two projects funded by the Italian Ministry of Agriculture (PSTVd-free and Stra.Te.Co). Specifically, a total of 111 plants belonging to nine solanaceous ornamental genera (*Brugmansia* spp., *Cestrum* spp., *Datura* spp., *Nierrembergia* spp., *Petunia* spp., *Solandra* spp., *Solanum jasminoides*, *S. rantonnetii* syn. *Lycianthes rantonetii*, *Streptosolen jamensonii* and *Surfinia* spp) were collected from several nurseries and gardens.

Total nucleic acids were extracted with a commercial kit (Real, Spain) in accordance with the instruction manual. Two µl of total RNA were used in RT-PCR assays using generic primers for pospiviroids (Verhoeven *et al*., 2004) and the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, UK),

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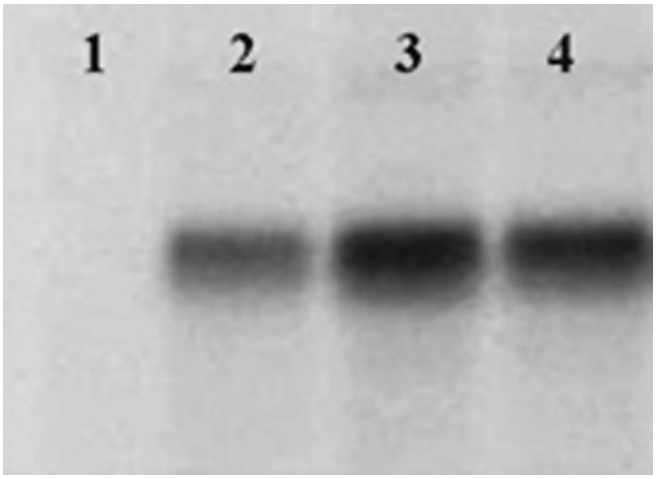

Fig. 1. Autoradiograph of Northern blot hybridization analysis of DIG-labeled full length PSTVd cRNA probe with nucleic acids analyzed by a bidirectional gel electrophoresis. Lane 1 = healthy control, lane 2 = naturally-infected *Cestrum auricantum*, lane 3 = naturally-infected *S. jasminoides*, lane 4 = PSTVd control.

following the instruction manual. Retro-transcription and amplification were performed on a thermal cycler programmed according to the protocol of Verhoeven *et al*. (2004). PCR products were sequenced and analysed for the initial identification of any viroid. Ultimate identification was based on sequence analysis of full-length amplicons generated by RT-PCR using specific primers designed by Di Serio (2007) for PSTVd and TASVd, primers for PSTVd from OEPP/EPPO diagnostic protocol (2004), primers designed by Önelge (1997) for CEVd and TASVd and by Levy and Hadidi (1992) for CEVd. Amplified products were purified, cloned in pGEM-T-easy plasmid vector (Promega, USA) and sequenced (Bio-Fab Research, Italy). Multiple alignments of PSTVd, CEVd, TASVd nucleotide sequences were done using the Clustal W multiple sequence alignment program. Secondary structures were predicted by the mFold program (Zucker, 1989). To rule out possible contaminations, PSTVd infections were also detected by Northern blot hybridization using a specific digoxigenin-labelled probe (Fig. 1).

Although none of the ornamental hosts showed symptoms, several samples tested positive for pospiviroid infection (Table 1). In fact, 48 plants were infected by the following viroids: PSTVd (*S. jasminoides* and *Cestrum* spp.), TASVd (*S. jasminoides*) and CEVd (*Cestrum* spp. and *L. rantonnetii*)

PSTVd was found in 25 out of 35 *S. jasminoides* and in 22 out of 29 *Cestrum* spp. (*Cestrum auricantum*, *C. rubrum*, *C. x cultum*, *C. endlicheri* and *C. nocturnum*) plants. PSTVd isolates showed two different patterns, those from *S. jasminoides* were highly homologous among themselves and with the isolates described by Verhoeven *et al*. (2008a) (99-100% identity), conserving the informative mutation sites described by Navarro *et al*. (2009). Isolates from *Cestrum* spp. showed a variability ranging from 94 to 100% among themselves and the multiple alignment revealed several substitution and insertions in different genomic domains (reference sequence accession No. NC002030), i.e. U/G_{49}, C_{130} and C_{317} for isolates from *C. nocturnum,* A_{315} for isolates from *C. rubrum* and *C. x cultum* and C_{320} for all isolates from *Cestrum* spp. They did not show the characteristic informational site identified in the Italian isolates but appeared to be more similar to the reference PSTVd sequence (Table 2).

TASVd was identified for the first time in Italy in four *S. jasminoides* plants, in two of which together with PSTVd. The sequences obtained from no less than two clones from each plant were very homogeneous among themselves (99% to 100% identity) but less (91 to 99% identity) with other sequences deposited in GenBank. All TASVd isolates characterized in this work presented in the TR three conserved substitutions previously unreported, 162 C>U, 168 U>C, 202 U>G.

CEVd was identified for the first time in *L. rantonnetii* and *C. auricantum*, in the latter mixed with PSTVd. CEVd sequences from these hosts, were very homogeneous (97 to 100% identity), showing few muta-

Table 1. Solanaceous ornamentals analyzed and pospiviroids detected.

Species	Viroid	No. infected plants/ No. analyzed plants	Accession number
Brugmansia sp.		0/8	
Cestrum sp.	CEVd, PSTVd	1/29; 22/29	HQ452399 to HQ452417
Datura sp.		0/6	
Nierrembergia sp.		0/5	
Petunia sp.		0/8	
Solandra sp.		0/5	
Solanum jasminoides	TASVd, PSTVd	4/35, 25/35	HQ667139 to HQ667141
Lycianthes rantonnetii	CEVd	1/5	HQ667138
Streptosolen jamensonii		0/5	
Surfinia sp.		0/5	
Total		48/111	

Table 2. Summary of the most variable positions obtained by multiple alignment of PSTVd sequences.

PSTVd molecular analysis						
Isolate[a]	49-50	64-65	125-126	238	308-311	320
Reference sequence	A-G	A--G	G-A	C	U-ACU	-
Tomato mild	.-.	.--.	.-.	.	.CCU.	-
Solanum jasminoides	.-.	.-U.	.--	-	.-C..	-
Lycianthes rantonnetii	.-.	.-U.	.--	-	.-C..	-
Streptosolen jamensonii	.-.	.-U.	.--	-	.-C..	-
Brugmansia spp	.-.	.-U.	.-.	.	.-C..	-
Tomato Italy	.-.	.-U.	.--	-	.-C..	-
Tomato the Netherlands	.-.	.-UU	.--	-	.-C..	-
Cestrum auricantum 1[b]	.U.	.-UU	.-.	.	.-C..	C
Cestrum auricantum 2	.U.	.-UU	.-.	.	.-C..	C
Cestrum cultum 2	.U.	.AGU	.-.	.	.AC..	C
Cestrum cultum 3	.U.	.AGU	.-.	.	.AC..	C
Cestrum endlicheri 2	.U.	.AGU	.-.	.	.AC..	C
Cestrum endlicheri3	.U.	.AGU	.-.	.	.AC..	C
Cestrum nocturnum 2	.G.	.A-U	.C.	.	.-CUA	C
Cestrum nocturnum 3	.G.	.-$_{A}$-$_{U}$U	.C.	.	.-CUA	C
Cestrum rubrum 1	.U.	.-UU	.-.	.	.AC..	C
Cestrum rubrum 2	.U.	.AGU	.-.	.	.AC..	C
Domain	P	P	V	V	P	TL

[a]Reference variant from potato (acc. No. NC002030) and the others sequences from ornamental species [*S. jasminoides* (EF192393), *S. rantonnetii* (EF459700), S. *jamensonii* (EF580923) and *Brugmansia* spp (FM998547)] and from tomato [mild variant (X76844), Dutch variant (FJ872823) and Italian variant (AY372398)].
[b]Below this line variants isolated in this work are reported
Dots indicate identity with the reference sequence and slashes denote gaps. Subscript position indicates the presence of polymorphisms. Informative position identified by Navarro *et al.* (2009) are highlighted in grey.

Table 3. Summary of the most interesting polymorphisms in the terminal left (TL) and in the pathogenetic (P) domains obtained by multiple alignment of CEVd isolates.

CEVd molecular analysis					
Isolate	Acc. No.	50	61-64	294	308-309
Reference sequence	NC001464	G	AAGA	C	CU
Severe strain	EU382204	.	....	.	..
Tomato	X53716	A	G.AT	.	UC
Verbena	EU877744-5	.	....	.	..
Impatiens	EU877742-3-6	.	....	.	..
Solanum jasminoides	AM774356-7	U_C	G.AC	A	UC
Cestrum auricantum	This work	C	G.AC	A	UC
Lycianthes rantonnetii	This work	U	G.AC	A	UC
Domain		TL	P	P	P

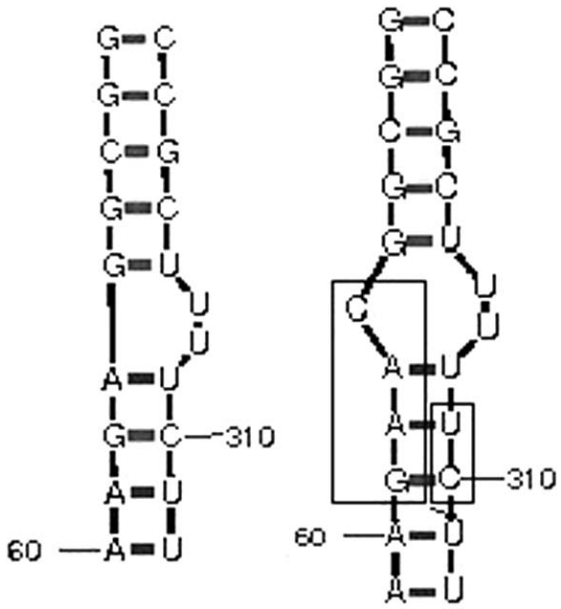

Fig. 2. Comparison of the predicted secondary structures of two fragments from two different CEVd isolates obtained using Mfold software (Zucker, 1989). Panel A: secondary structure of the CEVd pathogeneticity domain (+) strand of the reference sequence (acc. no. NC001464). Panel B: secondary structure of the CEVd pathogeneticity domain (+) strand of the isolate from *S. jasminoides*. The boxes highlight the positions of the two polymorphisms.

tions, all located in the V domain, similar to those of isolates from *S. jasminoides* previously characterized in Germany, the Netherlands and Austria (Verhoeven *et al.*, 2008c; Gottsberger and Suarez-Mahecha, 2010). Differences with isolates from *Solanum lycopersicum*, *Verbena* spp. and *Impatiens* spp. are mainly located in the P and TL domains (Table 3).

Two of these mutation sites involved more than one nucleotide so the relevance of these polymorphisms was investigated by folding the predicted secondary structure. As reported in Fig. 2, polymorphisms did not affect the hairpin structure. In fact, mutations at positions 61 and 62 were balanced by the complementary mutations at positions 309 and 310. The presence of insertion C_{64} did not affect the secondary structure either.

In conclusion, this study provides comprehensive evidence that some symptomless solanaceous ornamentals grown in Italy are infected by different pospiviroids that represent a potential source of infections for sensitive horticultural crops. Current legislative measures refer only to PSTVd on *S. jasminoides* and *Brugmansia* spp., but as reported here other species can host this pathogen as well as other pospiviroids. Because of the high genetic homology of pospiviroids, there is no specific diagnostic tool (except sequencing) for detecting specifically PSTVd, which is the only viroid covered by compulsory health standards. To complicate more the problem, we have now shown the presence of mixed pospiviroidal infections in the same plant.

In light of these findings, it seems desirable to check a larger number of ornamental plants for the presence of pospiviroids, using genus-specific methods able to identify all members of the genus through a single analytical procedure.

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