NACIONALNI INŠTITUT ZA BIOLOGIJO
NATIONAL INSTITUTE OF BIOLOGY

European Union Reference Laboratory for Pests of Plants on Viruses, Viroids and Phytoplasmas

Co-funded by the European Union

EURL-Virology

(European Union Reference Laboratory for Pests of Plants on Viruses, Viroids and Phytoplasmas)

TESTING OF CHRYSANTHEMUM STEM NECROSIS VIRUS AND OTHER AMERICAN CLADE 1 TOSPOVIRUSES BY RT-PCR

VALIDATION REPORT

VERSION 30 APRIL 2024

The validation was performed by the National Institute of Biology, Department of Biotechnology and Systems Biology, Microbiology Unit, Laboratory for detection of viruses, viroids and phytoplasmas, Večna pot 121, Ljubljana, Slovenia, a partner of the European Union Reference Laboratory for pests of plants on viruses, viroids and phytoplasmas.

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Suggested Citation:

Mehle, N., Bajde, I., Brodarič, J., & Vučurović A., 2024. Validation report on the testing of Chrysanthemum stem necrosis virus and other American clade 1 tospoviruses by RT-PCR (version April 30, 2024). EURL-Virology; National Institute of Biology, Ljubljana, Slovenia.

Contents

1 Scope of validation

Detection of Chrysanthemum stem necrosis virus (CSNV) and other tospoviruses of American clade 1.

Method	RT-PCR: modified protocol from Appendix 4 of EPPO PM7/139(1) Tospoviruses
Harmful organism	Chrysanthemum stem necrosis virus and other tospoviruses of American clade 1
Sample type	Leaves of host plants (e.g., Chrysanthemum, tomato)

2 Description of the method

2.1 RNA extraction

Freeze dried leaf tissue (representing 100 mg fresh leaf weight) was ground in 450 µL of buffer RLT from the RNeasy Plant Mini Kit (Qiagen). RNA was then extracted from the entire homogenate according to the manufacturer's instructions. Total RNA was eluted twice with 50 µL (total of 100 µL) of RNase-free water pre-warmed to 65 °C. To monitor the RNA extraction procedure, real-time RT-PCR amplifying plant nad5 transcript (*nad5;* Botermans et al., 2013) was included in the analysis.

2.2 RT-PCR

Conventional RT-PCR was performed using the primers described in Appendix 4 of EPPO PM7/139(1) Tospoviruses, for American clade 1: AM1-F and AM1-R. These primers target sequences of the nucleocapsid gene and its 5'-upstream untranslated region of S-RNA.

Specificity: American clade 1 of viruses from the genus *Orthotospovirus*, which includes Alstroemeria necrotic streak virus (ANSV), Chrysanthemum stem necrosis virus (CSNV), Groundnut ringspot virus (GRSV), Impatiens necrotic spot virus (INSV), Melon severe mosaic virus (MSMV), Pepper necrotic spot virus (PNSV), Tomato chlorotic spot virus (TCSV), Tomato spotted wilt virus (TSWV), Zucchini lethal chlorosis virus (ZYLCV).

Expected amplicon size: 760 bp.

For sample analysis, we first used the master mix and the conditions of RT-PCR as described in Appendix 4 of EPPO PM7/139(1). We repeatedly did not obtain a PCR product with this protocol for CSNV samples or very weak bands, while we obtained clear bands for TSWV and INSV samples. Similar problems in detecting CSNV with the protocol from Appendix 4 of EPPO PM7/139(1) were also reported by several other NRLs in the EU. Therefore, several conditions were tested using the same primers. The best conditions (see specification below) for the tested sample batch was further validated.

Protocol that was validated:

- Mastermix with OneTaq^{R} One-Step RT-PCR Kit (NEB): 25 µL OneTaq One-Step Reaction Mix, 2 µL OneTaq One-Step Enzyme Mix, 2 µL 10 µM Forward Primer (AM1-F), 2 µL 10 µM Reverse Primer (AM1-R), 17 µL nuclease-free water, 2 µL RNA.

- Reaction conditions: 48 °C for 30 min, 94 °C for 1 min; 40 cycles of 94 °C for 15 sec, 50 °C for 30 sec, 68 °C for 1 min, followed by 68 °C for 5 min.
- Equipment used: ProFlex PR system (Applied Biosystems)

2.3 Sequencing of PCR products

Sanger sequencing of amplicons from the RT-PCR AM1-F/AM1-R was done to identify the tospovirus isolates. Guidelines described in Appendix 7 of EPPO Standard PM7/129(2) DNA barcoding as an identification tool for a number of regulated pests, have been followed. PCR amplicons were purified using MinElute PCR Purification Kits (Qiagen) according to the manufacturer's instructions, or PCR amplicon purification was ordered from Macrogen Europe (The Netherlands). The forward and reverse sequencing reactions for the purified PCR products were performed by Eurofins Genomics (GATC; Germany) or by Macrogen Europe (The Netherlands), using the Sanger method. DNA sequences were aligned using Geneious Prime software. The consensus sequences were compared with sequences from the GenBank database, using the BLAST algorithms (http://www.ncbi.nlm.nih.gov/blast).

Note: Sequencing of the PCR products is not part of the scope of validation

3 Validation procedure

Validation was performed according to EPPO PM7/98(5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.

3.1 Analytical specificity

To determine the analytical specificity, the isolates listed in Table 1 were tested by RT-PCR.

These include:

Target isolates:

- 27 isolates of tospovirus species from American Clade 1 (7 different virus species: ANSV, CSNV, GRSV, INSV, MSMV, TCSV, TSWV)

Non-target isolates:

- Two isolates of two different tospovirus species from Asian Clade 1, i.e., Capsicum chlorosis virus (CaCV) and Watermelon silver mottle virus (WSMoV)
- Three isolates of two different tospovirus species from Eurasian Clade, i.e., Iris yellow spot virus (IYSV) and Tomato yellow ring virus (TYRV)
- Chrysanthemum plant infected with pospiviroid Chrysanthemum stunt viroid (CSVd)

The RT-PCR products obtained with some isolates were sequenced for identification.

3.2 Analytical sensitivity

To determine the analytical sensitivity of the conventional RT-PCR test, the following experiments were carried out:

- Analysis of serial dilutions of RNA of CSNV PV-0529* in RNA of chrysanthemum leaves, which was infected with CSVd (PV-0735)
- Analysis of serial dilutions of RNA of CSNV PV-0529* in water
- Analysis of serial dilutions of RNA of CSNV NIB V 038 in water
- Analysis of serial dilutions of RNA of INSV PV-0280 in water
- Analysis of serial dilutions of RNA of TSWV PV-0204 in water

* The RNA of CSNV PV-0529 was extracted from the same freeze-dried plant material, but on different days.

3.3 Selectivity

Not evaluated.

3.4 Repeatability

Not evaluated.

3.5 Reproducibility

DSMZ (Germany) prepared several aliquots of freeze-dried homogenates of leaf tissue. These were aliquots of:

- CSNV PV-0529: undiluted and diluted in CSNV negative chrysanthemum (ratio: 1:1)
- CSNV PV-1219: undiluted and diluted in CSNV negative chrysanthemum (ratio: 1:3)
- INSV PV-0281
- TSWV PV-0182

Three aliquots of each preparation were included in the reproducibility assessment. Each aliquot was analyzed on different days. RNA extraction, RT-PCR and Sanger sequencing were performed for each sample from each aliquot.

Table 1. Isolates used for validation.

Virus*	Isolate	Collection	Origin	Collection year	Original host plant	Type of sample received	Experimental host plant
Genus *Orthotospovirus, American Clade 1*							
ANSV	PV-1027	DSMZ, Germany	Colombia	before 2011	*Alstroemeria* sp.	fresh	*Nicotiana rustica*
CSNV	PV-0529	DSMZ, Germany	Unknown	before 1998	*Chrysanthemum* sp.	fresh	*N. benthamiana*
CSNV	PV-1219	DSMZ, Germany	Slovenia	October 2001	*Chrysanthemum* sp.	lyophilized	*N. benthamiana*
CSNV	NIB V 038	NIB, Slovenia	Slovenia	2001	*Chrysanthemum* sp.	frozen	*N. benthamiana*
GRSV	PV-0205	DSMZ, Germany	South Africa	before 1988	*Arachis hypogaea*	fresh	*N. benthamiana*
INSV	PV-0281	DSMZ, Germany	Germany	before 1999	*Anemone coronaria*	fresh	*N. benthamiana*
INSV	PV-0280	DSMZ, Germany	USA	1990	*Hippeastrum* sp	lyophilized	*N. benthamiana*
INSV	PV-0485	DSMZ, Germany	unknown	1998	*Gloxinia* sp.	lyophilized	*N. benthamiana*
INSV	PV-1123	DSMZ, Germany	Germany	2014	*Lobelia speciosa*	lyophilized	*N. clevelandii*
INSV	PV-1189	DSMZ, Germany	Germany	2016	*Ocimum basilicum*	lyophilized	*N. benthamiana*
MSMV	VE440	IPSP CNR, Italy	Mexico	2007	melon	lyophilized	*N. benthamiana*
TCSV	PV-0390	DSMZ, Germany	Brazil	before 1994	pepper	fresh	*N. rustica*
TCSV	PV-0391	DSMZ, Germany	Brazil	1994	pepper	lyophilized	*N. rustica*
TSWV	PV-0182	DSMZ, Germany	Bulgaria	before 1988	*N. tabacum*	fresh	*N. rustica, N. benthamiana*
TSWV	PV-1175	DSMZ, Germany	Hungary	before 2015	pepper	fresh	*N. tabacum* cv. Xanthi, *N. rustica*
TSWV	PV-0393	DSMZ, Germany	Bulgaria	before 1993	*N. tabacum*	fresh	*N. tabacum* cv. Samsun
TSWV	PV-0204	DSMZ, Germany	Germany	before 1988	*Impatiens hawkeri* New-Guinea	fresh	*N. rustica*
TSWV	/	WSU, USA	USA		tomato	frozen	*N. benthamiana*
TSWV	108-19	UB-FA, Serbia	Serbia	2019	tomato cv Wrestler	frozen	*N. tabacum, Solanum lycopersicum*
TSWV	109-19	UB-FA, Serbia	Serbia	2019	tomato cv Wrestler	frozen	*N. tabacum, S. lycopersicum*
TSWV	France 77	INRA, France	France	2008	chilli pepper (RB strain)	fresh	*C. annuum* cv. Yolo wonder
TSWV	/	CREA, Italy	Italy, Lazio	2011	pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Bulgaria	2012	tomato	lyophilized	*S. lycopersicum*
TSWV	/	CREA, Italy	Italy, Calabria	2014	hot pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Italy, Lazio	2015	pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Italy, Lazio	2016	lisianthus	lyophilized	*Lysianthus*

TSWV	21007721	NPPO-NL, NVWA	Netherlands	2001	*Ligularia*	fresh	*N. benthamiana*
Genus *Orthotospovirus, Asian Clade 1*							
CaCV	PV-0864	DSMZ, Germany	Thailand	2005	tomato	lyophilized	*N. benthamiana*
WSMoV	PV-0283	DSMZ, Germany	Taiwan	1991	tomato	lyophilized	*N. benthamiana*
Genus *Orthotospovirus, Eurasian Clade*							
IYSV	PV-0528	DSMZ, Germany	unknown	1998	leek	lyophilized	*N. benthamiana*
TYRV	PV-0526	DSMZ, Germany	Iran	1998	tomato	lyophilized	*N. benthamiana*
TYRV	PV-0532	DSMZ, Germany	Iran	1998	tomato	lyophilized	*N. benthamiana*
Genus *Pospiviroid*							
CSVd	PV-0735	DSMZ, Germany	Germany	Dec 2001 or before	chrysanthemum cv Dream Time	lyophilized	*Chrysanthemum* sp.
Healthy tomato leaves							
/	/	DSMZ, Germany	unknown	unknown	tomato	lyophilized	*S. lycopersicum*

*for virus species name see EPPO PM7/139(1)

4 Results of validation

4.1 Evaluation of RNA extraction

The performance of RNA extraction was verified for each sample by real-time RT-PCR using a set of oligonucleotide primers and a probe for *nad5*. RNA extraction was considered successful if nad5 was detected with a Cq value below 33.6. If this was not achieved, the RNA extraction was repeated.

4.2 Analytical specificity

The inclusivity assessed with 27 isolates of tospovirus species from American Clade 1 is 100% (Table 2). The RT-PCR products obtained for ANSV (PV-1027), CSNV (PV-0529 and PV-1219), GRSV (PV-0205), INSV (PV-0281), MSMV (VE440), TCSV (PV-0390), TSWV (PV-0182) were sequenced, and the sequence analysis confirmed the assigned identity of the virus.

Exclusivity: Four out of five isolates of non-target tospoviruses and CSVd did not generate a RT-PCR product (Table 2). Analysis of WSMoV revealed a weak amplicon close to the expected size, which was sequenced, and sequence analysis showed that this was due to non-specific amplification of the host (*N. benthamiana*) tissue.

Table 2. Results of testing target and non-target virus isolates with RT-PCR

Virus*	Isolate	RNA dilution tested	RT-PCR	RT-PCR
Genus *Orthotospovirus, American Clade 1*				
ANSV	PV-1027	undiluted	pos*	pos
CSNV	PV-0529	10-fold dilution	pos*	pos
CSNV	PV-1219	undiluted	pos*	pos
CSNV	NIB V 038	undiluted	pos	pos
GRSV	PV-0205	undiluted	pos*	pos
INSV	PV-0281	undiluted	pos*	pos
INSV	PV-0280	undiluted	pos	pos
INSV	PV-0485	undiluted	pos	pos
INSV	PV-1123	undiluted	pos	pos
INSV	PV-1189	undiluted	pos	pos
MSMV	VE440	10-fold dilution	pos*	pos
TCSV	PV-0390	undiluted	pos*	pos
TCSV	PV-0391	undiluted	pos	pos
TSWV	PV-0182	undiluted	pos*	pos
TSWV	PV-1175	100-fold dilution	pos	pos
TSWV	PV-0393	10-fold dilution	pos	pos
TSWV	PV-0204	undiluted	pos	pos
TSWV	WSU, USA	undiluted	pos	pos
TSWV	108-19	undiluted	pos	pos
TSWV	109-19	undiluted	pos	pos
TSWV	France 77	undiluted	pos	pos
TSWV	CREA, Italy 2011	undiluted	pos	pos
TSWV	CREA, Italy 2012	undiluted	pos	pos
TSWV	CREA, Italy 2014	undiluted	pos	pos
TSWV	CREA, Italy 2015	undiluted	pos	pos
TSWV	CREA, Italy 2016	undiluted	pos	pos
TSWV	21007721	undiluted	pos	pos
Genus *Orthotospovirus, Asian Clade 1*				
CaCV	PV-0864	undiluted	neg	neg
WSMoV	PV-0283	undiluted	sus**	sus
Genus *Orthotospovirus, Eurasian Clade*				
IYSV	PV-0528	undiluted	neg	neg
TYRV	PV-0526	undiluted	neg	neg
TYRV	PV-0532	undiluted	neg	neg
Genus *Pospiviroid*				
CSVd	PV-0735	undiluted	neg	neg
Healthy tomato leaves				
tomato	DSMZ, Germany	undiluted	neg	neg

Results of two parallel tests are shown;
The RT-PCR products marked with asterisk (*) were sequenced and the sequence analysis confirmed the identity of the expected virus;
The suspect RT-PCR result (**) was sequenced and found to be due to amplification of a host (N.benthamiana) tissue.

4.3 Analytical sensitivity

Based on a dilution series prepared from RNA of CSNV, INSV and TSWV isolates in water, the analytical sensitivity was 10^{-1} for CSNV, 10^{-3} for INSV and 10^{-4} for TSWV (Table 3). It should be noted that the band for the 10^{-1} dilution of CSNV was very weak, and when the dilution of CSNV was prepared in RNA from chrysanthemum, we could not even see this weak band (data not shown). The result is probably related to the stochastic effect that can occur when we are close to the detection limit. However, a matrix effect cannot be excluded. Furthermore, we could see repeated bands when we tested RNA of CSNV mixed with chrysanthemum leaf material at a ratio of 1:3 (CSNV PV-1219) and at a ratio of 1:1 (CSNV PV-0529) (see reproducibility section). From this it can be concluded that the analytical sensitivity for CSNV is between 10^{0} and 10^{-1}.

Table 3. Results of testing the virus dilutions in water with RT-PCR

Sample		RT-PCR replicate 1	RT-PCR replicate 2
CSNV PV-0529	undiluted	pos	pos
	10^{-1}	pos	pos
	10^{-2}	neg	neg
	10^{-3}	neg	neg
	10^{-4}	neg	neg
CSNV NIB V 038	undiluted	pos	pos
	10^{-1}	pos	pos
	10^{-2}	neg	neg
	10^{-3}	neg	neg
	10^{-4}	neg	neg
INSV PV-0280	undiluted	pos	pos
	10^{-1}	pos	pos
	10^{-2}	pos	pos
	10^{-3}	pos	pos
	10^{-4}	neg	neg
TSWV PV-0204	undiluted	pos	pos
	10^{-1}	pos	pos
	10^{-2}	pos	pos
	10^{-3}	pos	pos
	10^{-4}	pos	pos
	10^{-5}	neg	neg
	10^{-6}	neg	neg
	10^{-7}	neg	neg

Results of two parallel tests are shown.

4.4 Selectivity

Not evaluated.

4.5 Repeatability

Not evaluated.

4.6 Reproducibility

The whole procedure (RNA extraction, RT-PCR and sequencing of PCR products) proved to be 100% reproducible when this parameter was evaluated on selected isolates and their dilutions (Table 4).

Table 4: Multiple testing of freeze-dried homogenates of leaf tissue infected with CSNV, INSV and TSWV by RT-PCR.

Test ID	29/23	31/23	32/23	**Positive replicates (%)**
CSNV PV-0529	pos CSNV	pos CSNV	pos CSNV	**100 %**
CSNV PV-0529 + negative chrysanthemum (ratio: 1:1)	pos CSNV	pos CSNV	pos CSNV	**100 %**
CSNV PV-1219	pos CSNV	pos CSNV	pos CSNV	**100 %**
CSNV PV-1219 + negative chrysanthemum (ratio: 1:3)	pos CSNV	pos CSNV	pos CSNV	**100 %**
INSV PV-0281	pos INSV	pos INSV	pos INSV	**100 %**
TSWV PV-0182	pos TSWV	pos TSWV	pos TSWV	**100 %**

5 Conclusions

Criteria		Results
Analytical sensitivity	Tested concentrations	• Dilutions of CSNV RNA in RNA of chrysanthemum • Dilutions of CSNV, INSV and TSWV RNAs in water
	LOD	For the dilutions in RNA of chrysanthemum: CSNV: 10^{0} For the dilutions in water: CSNV: 10^{-1} INSV: 10^{-3} TSWV: 10^{-4}
Analytical specificity	Number of tested samples	No of targets tested: 27 (tospoviruses of American clade 1) No of non-targets tested: 6 (5 isolates of other tospoviruses and one CSVd)
	Inclusivity	100%
	Exclusivity	83% (one false positive result; sequencing of amplicon confirmed that this was non-specific amplification of the host (*N. benthaminana*) tissue)
Selectivity	Effect of matrix	Not evaluated
Repeatability	nr. of replicates, percentage of identical results	Not evaluated
Reproducibility	nr. of replicates, percentage of identical results	No. of target isolates tested: 4 (for two isolates, two dilutions were evaluated) No. of different days: 3 Percentage of identical results (positive replicates): 100%

6 References

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