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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/139 (1)

Diagnostics

PM 7/139 (1) Tospoviruses (Genus *Orthotospovirus*)

Specific scope

This Standard describes a diagnostic protocol for detection and identification of tospoviruses[1].

This protocol replaces the EPPO Standard PM 7/34 on *Tomato spotted wilt*, *Impatiens necrotic spot* and *Watermelon silver mottle* tospoviruses.

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

Approved in 2020-04.

1. Introduction

Members of the genus *Orthotospovirus* are the only plant-infecting viruses in the family *Tospoviridae* (ICTV online: https://talk.ictvonline.org/taxonomy/). The genus is named after its type member Tomato spotted wilt virus (TSWV; Brittlebank, 1919). Tospoviruses consist of enveloped quasi-spherical particles of 80–120 nm (Fig. 1). The genomes consist of three RNA segments, denoted large (L), medium (M) and small (S), complexed with the nucleocapsid (N) protein (De Haan *et al*., 1990). Species demarcation within the genus is based on the sequence of the N protein, in combination with plant host range, vector specificity and serological relationships of the N protein (Plyusnin *et al*., 2012).

At present the genus *Orthotospovirus* includes 18 officially recognized and 12 additionally described species (Hassani-Mehraban *et al*., 2019). The species, within the genus, group in at least five distinct clades and one single species (Fig. 2). Species within a clade have similar thrips vectors and have similar geographical distribution (Oliver & Whitfield, 2016). The following tospoviruses are recommended for regulation by EPPO: Chrysanthemum stem necrosis virus (CSNV), Impatiens necrotic spot virus (INSV), TSWV and Watermelon silver mottle virus (WSMoV).

Tospoviruses can infect a large number of plant species, including economically important vegetable crops such as bean, cucurbit, lettuce, pepper, potato, soybean, tomato and various ornamental species (EFSA, 2012). Individual species may have extended host ranges, such as TSWV, which has been reported to infect over 1300 different plant species (Parella *et al*., 2003; EFSA, 2012), whereas others have a more limited host range. Nearly all tospoviruses are known to be transmitted by thrips (order *Thysanoptera*, family *Thripidae*) in a persistent and propagative manner (Table 1; Rotenberg *et al*., 2015). Many of these thrips species also have wide host ranges and a worldwide distribution. To date, at least 15 different thrips species have been reported to vector tospoviruses. Tospoviruses are considered not to be seed transmitted (Pappu *et al*., 2009). A first report of seed transmission of a tospovirus (soybean vein necrosis virus) has recently been published by Groves *et al*. (2016), but this needs confirmation.

Tospoviruses are among the most damaging plant viruses worldwide (Pappu *et al*., 2009). Over recent decades, the increasing global trade of plants and plant products has resulted in the spread of both the tospoviruses and their vectors. Moreover, the fact that thrips are difficult to control has contributed to the spreading of known and unknown tospoviruses into new areas. The spread of *Frankliniella occidentalis* is considered the primary driver of the worldwide emergence of tospoviruses, although *Thrips palmi* is the major vector of several important tospoviruses in Asia (Rotenberg *et al*., 2015). Furthermore, the occurrence of reassortment (exchange of one or more genomic RNAs between virus isolates) and recombination within and between different species might contribute to a further emergence of tospovirus diseases. This is because they can differ with respect to viral properties relevant to

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

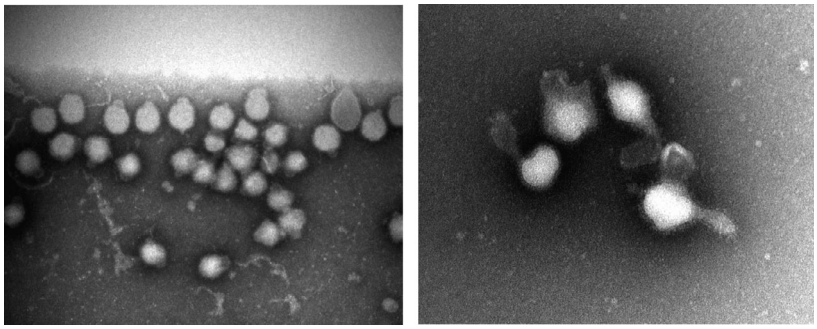

Fig. 1 Electron microscopy of tospovirus particles. Courtesy O. Schumpp, Agroscope (CH).

management, such as host range, symptomatology, vector transmissibility and the potential to breakdown host resistance (Tentchev *et al*., 2011; Gawande *et al*., 2015; Webster *et al*., 2015). As a result, tospoviruses are recognized as emerging diseases threatening food security worldwide (Oliver & Whitfield, 2016). For this reason, the protocol not only focuses on specific tospoviruses species but covers general detection and identification of tospoviruses. Early recognition of non-indigenous and potentially harmful viruses into new areas is a key factor in preventing the introduction and spreading of 'new' diseases.

A flow diagram describing the procedure for generic detection and identification of tospoviruses is given in Fig. 3.

Table 1. List of official and tentative species in the genus *Orthotospovirus* and their vectors if known*

Name†	Acronym	Vector	Reference‡
Alstroemeria necrotic streak virus	ANSV	*Frankliniella occidentalis*	Hassani-Mehraban *et al*. (2010)
Alstroemeria yellow spot virus	AYSV	*Thrips tabaci*	Hassani-Mehraban *et al*. (2019)
Bean necrotic mosaic virus†	BeNMV	Unknown	NA
Calla lily chlorotic spot virus†	CCSV	*Thrips palmi*	NA
Capsicum chlorosis virus†	CaCV	*Ceratothripoides claratris* *Frankliniella schultzei* *Thrips palmi*	NA
Chrysanthemum stem necrosis virus†	CSNV	*Frankliniella gemina* *Frankliniella occidentalis* *Frankliniella intonsa* *Frankliniella schultzei*	NA
Groundnut bud necrosis virus†	GBNV	*Frankliniella schultzei* *Scirtothrips dorsalis* *Thrips palmi*	NA
Groundnut chlorotic fan-spot virus¶	GCFSV	*Scirtothrips dorsalis*	Chu *et al*. (2001a)
Groundnut ringspot virus†	GRSV	*Frankliniella gemina* *Frankliniella intonsa* *Frankliniella occidentalis* *Frankliniella schultze*	NA
Groundnut yellow spot virus†	GYSV	*Scirtothrips dorsalis*	NA
Hippeastrum chlorotic ringspot virus	HCRV	Unknown	Dong *et al*. (2013)
Impatiens necrotic spot virus†	INSV	*Frankliniella fusca* *Frankliniella intonsa* *Frankliniella occidentalis* *Frankliniella schultzei*	NA
Iris yellow spot virus†	IYSV	*Frankliniella fusca* *Thrips tabaci*	NA
Lisianthus necrotic ringspot virus	LNRV	Unknown	Shimomoto *et al*. (2014)
Melon severe mosaic virus†	MSMV	Unknown	NA
Melon yellow spot virus†	MYSV	*Thrips palmi*	NA
Mulberry vein banding associated virus	MVBaV	Unknown	Meng *et al*. (2013)

(continued)

Table 1 *(continued)*

Name[†]	Acronym	Vector	Reference[‡]
Pepper chlorotic spot virus	PCSV	Unknown	Cheng *et al*. (2014)
Pepper necrotic spot virus	PNSV	Unknown	Torres *et al*. (2012)
Polygonum ring spot virus[†]	PolRSV	*Dictyothrips betae*	NA
Soybean vein necrosis virus[†]	SVNV	*Neohydatothrips variabilis*	NA
Tomato chlorotic spot virus[†]	TCSV	*Frankliniella intonosa* *Frankliniella occidentalis* *Frankliniella schultzei*	NA
Tomato necrotic ringspot virus	TNRV	*Ceratothripoides claratris* *Thrips palmi*	Seepiban *et al*. (2011)
Tomato necrotic spot virus	TNSV	Unknown	Yin *et al*. (2014)
Tomato spotted wilt virus[†]	TSWV	*Frankliniella bispinosa* *Frankliniella cephalica* *Frankliniella fusca* *Frankliniella gemina* *Frankliniella intonsa* *Frankliniella occidentalis* *Frankliniella schultzei* *Thrips setosus* *Thrips tabaci*	NA
Tomato yellow ring virus	TYRV	*Microcephalothrips abdominalis* *Thrips tabaci*	Hassani-Mehraban *et al*. (2005)
Tomato zonate spot virus	TZSV	*Frankliniella occidentalis*	Dong *et al*. (2008)
Watermelon bud necrosis virus[†]	WBNV	*Thrips palmi*	NA
Watermelon silver mottle virus[†]	WSMoV	*Thrips palmi*	NA
Zucchini lethal chlorosis virus[†]	ZLCV	*Frankliniella zucchini*	NA

*Table based on Oliver & Whitfield (2016), list updated and supplemented with references of species not included in the official ICTV classification.
[†]Virus species included in the ICTV classification.
[‡]Reference of first report or description for species not included in the ICTV classification.
NA: Not applicable.
[¶]Synonym *Peanut chlorotic fan-spot virus* (PCFV).

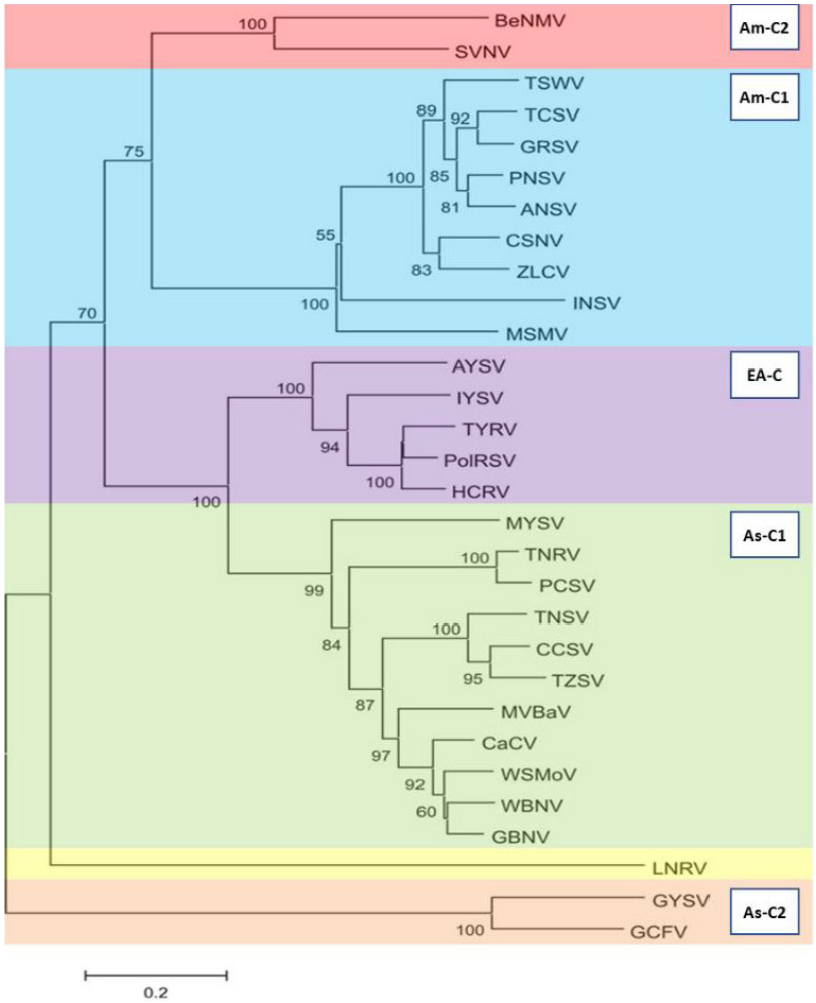

Fig. 2 Phylogenetic relationships of tospovirus species, including official and tentative members of the genus *Orthotospovirus*, based on the alignments of amino acid sequences of the nucleocapsid (N) protein. The tree was constructed using the neighbour-joining method implemented in MEGA7.0. Bootstrap values are shown as percentages derived from 1000 replicates. Those values less than 50% are not shown (according to Hassani-Mehraban *et al*., 2019). Am-C1 and C2: American Clade 1 and 2; As-C1 and C2: Asian Clade 1 and 2; EA-C: Eurasian Clade. [Colour figure can be viewed at wileyonlinelibrary.com]

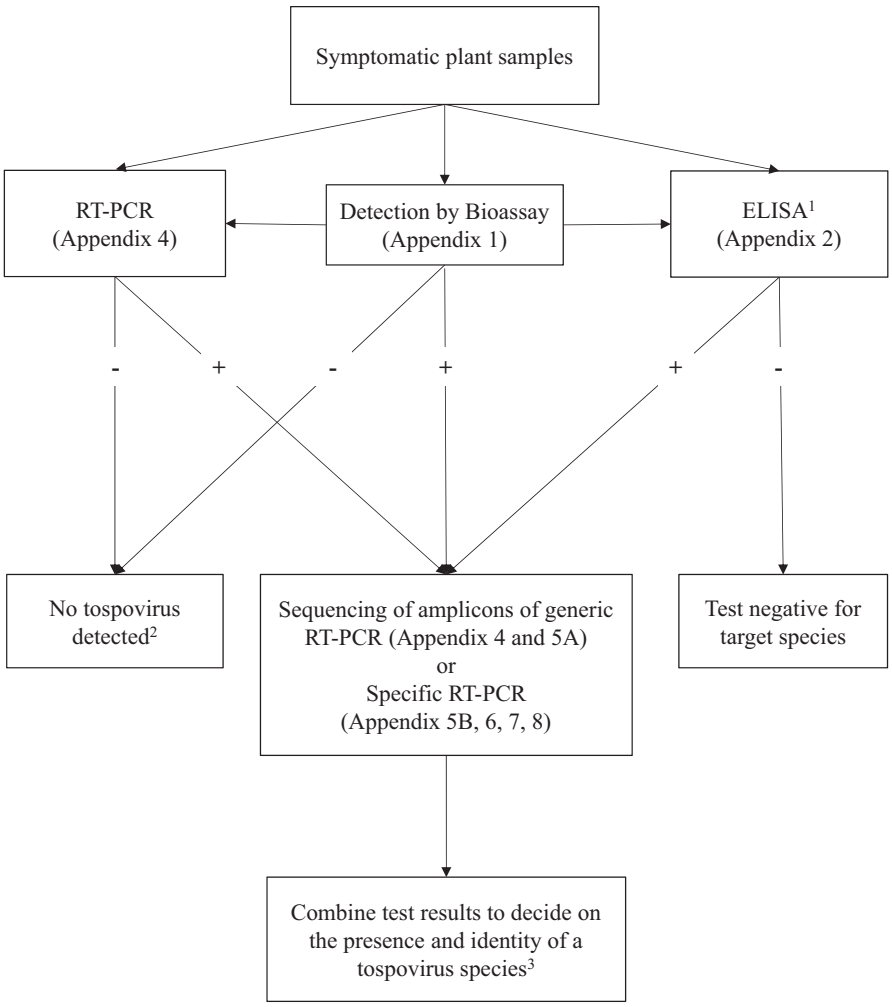

Fig. 3 Flow diagram for detection and identification of tospoviruses in symptomatic plant samples.
[1] ELISA can be performed when validated specific antisera are available see Table 4 in Appendix 2.
[2] Conclusion which can only be reached in the case of a validated test or in combination with results of other tests.
[3] This statement is made because of the lack of validation data and/or risks of cross reactions.

2. Identity

Name: *Orthotospovirus*.
Taxonomic position: *Bunyavirales*, *Tospoviridae*.
EPPO Code: 1TOSPG.

Name: Chrysanthemum stem necrosis virus.
Acronym: CSNV.
Taxonomic position: *Bunyavirales*, *Tospoviridae*, *Orthotospovirus*.
EPPO Code: CSNV00.
Phytosanitary categorization: EPPO A1 list no. 313, EU Annex II/A1.

Name: Impatiens necrotic spot virus.
Acronym: INSV.
Taxonomic position: *Bunyavirales*, *Tospoviridae*, *Orthotospovirus*.
EPPO Code: INSV00.
Phytosanitary categorization: EPPO A2 list no. 291, EU annexes I/B and II/A2.

Name: Tomato spotted wilt virus.
Acronym: TSWV.
Taxonomic position: *Bunyavirales*, *Tospoviridae*, *Orthotospovirus*.
EPPO Code: TSWV00.
Phytosanitary categorization: EPPO A2 list no. 290, EU annexes I/B and II/A2.

Name: Watermelon silver mottle virus.
Acronym: WSMoV.
Taxonomic position: *Bunyavirales*, *Tospoviridae*, *Orthotospovirus*.
EPPO Code: WMSMOV.
Phytosanitary categorization: EPPO A1 list no. 294, EU annexes I/B and II/A2.

Note Virus nomenclature in Diagnostic protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2018b, https://talk.ictvonline.org/taxonomy/). Accepted species names are italicized when used in their taxonomic context, whereas virus names are not, corresponding to ICTV instructions. The integration of the genus name within the name of the species is currently not consistently adopted by ICTV working groups and therefore species names in diagnostic protocols do not include the genus name. Names of viruses not included in the official ICTV classification are based on first reports.

3. Detection

3.1. Disease symptoms

Tospoviruses can induce a wide variety of symptoms on economically important ornamental plants, vegetables and industrial crops (e.g. EPPO, 1999a–c; EPPO, 2005). Given the wide host range and factors affecting symptom expression, it is not feasible to give comprehensive symptom descriptions for each tospovirus species.

Symptoms on plants include the following: chlorotic or necrotic local lesions, ringspots, concentric ringspots, line patterns, green island mosaic on leaves, stem discoloration, followed by systemic symptoms such as wilting, stunting, mottling, crinkling, bronzing, distortion (curling), rugosity, chlorosis and (top) necrosis, which may vary on the same host species.

Symptoms on fruits usually consist of irregular discolouration, e.g. yellow/orange flecks and occasionally rings on fruits of pepper and tomato, or necrotic lesions or rings. Yield might be reduced and marketing quality seriously affected.

Factors affecting symptom expression include the plant species and cultivar, the developmental stage at the time of inoculation, the nutritional and environmental conditions as well as the virus species and isolate (strain) (Mumford *et al*., 1996a).

3.2. Test sample requirements

Due to uneven distribution and the occurrence of infections limited to certain tospovirus-host combinations, e.g. INSV in specific pepper varieties (Roenhorst & Verhoeven, 1997), samples are preferably taken from symptomatic material. In systemically infected plants, young symptomatic leaves or fruits should be taken when present. In the case of necrotic symptoms, 'living' tissue at the edge of the necrotic tissue should be used. In addition, it is advisable to sample tissue from several parts of the plant to address the uneven distribution of the viruses.

3.3. Screening tests

For detection of tospoviruses the following tests are recommended.

3.3.1. Bioassay

Mechanical inoculation of herbaceous test plants can be used for the generic detection of tospoviruses. It does not lead to species identification, as the symptoms produced on the most suitable and recommended test plants are not discriminatory. However, test-plant inoculations can be used for virus detection and subsequent identification by other methods. The bioassay is described in Appendix 1.

3.3.2. Serological methods ELISA

Double-antibody sandwich-enzyme-linked immunosorbent assay [(DAS)-ELISA] or triple-antibody sandwich [(TAS)-ELISA] should be performed using antisera that have been assessed for their reliability for the detection of tospovirus species. Instructions to perform an ELISA test are described in the EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015a) and further information is provided in Appendix 2.

3.3.3. Molecular methods

Procedures for sample preparation and RNA extraction for molecular methods are described in Appendix 3. For generic detection of tospoviruses a conventional RT-PCR test is described, using either degenerate primers or combinations of primers (Table 2). The conventional RT-PCR from Hassani-Mehraban *et al*. (2016) is recommended for the generic detection of tospoviruses and is described in Appendix 4. This test consists of five reactions using different primer combinations that allow detection and further assignment of tospovirus isolates to any of the American, Asian and Eurasian clades.

Other tests for broad detection of tospoviruses have been described by Chen *et al*. (2012) and Mumford *et al*. (1996b) (Appendix 5A). Further molecular tests, including both conventional and real-time RT-PCRs that can be used for detection (and identification) of different tospovirus species, are listed in Table 2. This table provides information on the species that have been successfully detected and/or identified by each test. Please note that when (semi-) specific tests are used for detection, negative results do not exclude the presence of a tospovirus.

3.3.4. Other tests

Electron microscopy (EM) can be used for the detection of tospoviruses since they have a typical morphology, i.e. enveloped quasi spherical particles 80–120 nm in diameter (Fig. 1). Instructions to perform EM are described in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b).

Lateral flow tests (LFTs) are available as a preliminary screening tool for some tospoviruses, such as TSWV and INSV. They allow detection in symptomatic material and provide results within a few minutes. Tests should be performed according to the manufacturers' instructions. A positive result should be confirmed by an additional serological

Table 2. Molecular tests for detection and/or identification of tospoviruses* (only published data is included in this table)

Reference	Method	Primers	Am-C1[†,‡]	Am-C2[†,‡]	As-C1[†,‡]	As-C2[†,‡]	EA[†,‡]	LNRV[‡]	Target (gene)	Appendix
Boben *et al.* (2007)	Real-time RT-PCR	CSNV	CSNV						S RNA (N)	6
Boonham et al. (2002)[§]	Real-time RT-PCR	TSWV-CP	TSWV						S RNA (N)	8
Chen *et al.* (2012)	RT-PCR	gM410/gM870c	GRSV		CaCV		IYSV		M RNA (NSm)	–
		gL2740/gL3920c	INSV		CCCV		TYRV		L RNA (L)	
			TCSV		GBNV					
			TSWV		MYSV					
					WBNV					
					WSMoV					
Chen *et al.* (2013)	Real-time RT-PCR	INSV	INSV						S-RNA (NSs)	7
Chu *et al.* (2001b)	RT-PCR		GRSV		WSMoV	GCFSV[¶]			L RNA (L)	-
			INSV							
			TSWV							
Chu *et al.* (2001b)	RT-PCR	WSMoV-N			WSMoV				S RNA (N)	-
Hassani-Mehraban *et al.* (2016)	RT-PCR	AM1-F/R	ANSV	BeNMV	CaCV	GCFSV[¶]	IYSV	LNRV	S RNA (N)	4
		AM2-F/R1/R2	AYSV		CCSV		PolRSV			
		AS-EA-F/AS1-R	CSNV		GBNV		TYRV			
		AS-EA-F/AS2-R	GRSV		MYSV					
		LNRV	INSV		TNRV					
			MSMV		WSMoV					
			TCSV							
			TSWV							
Mortimer-Jones *et al.* (2009)	Real-time RT-PCR	TSWV 1 for	TSWV							-
		TSWV-1 rev								
		TSWV-1 probe								
Mumford *et al.* (1994)	RT-PCR	L1/L2	TSWV						L RNA (L)	5B
Mumford *et al.* (1996b)	RT-PCR	S1/S2 UNIV	GRSV						S RNA (N)	5A
			INSV							
			TCSV							
			TSWV							
Mumford *et al.* (1996b)	RT-PCR	S1/S2 INSV	INSV						S RNA (N)	5A

Table based on Hassani-Mehraban *et al.* (2016) reporting data from original papers, supplemented with additional tests described and/or referred to in this protocol.

*Virus species indicated by acronyms as in Table 1.

[†]Clades within the genus *Tospovirus*, i.e. American Clades 1 (Am-C1) and 2 (Am-C2), Asian Clades 1 (As-C1) and 2 (As-C2), Eurasian Clade (EA) and *Lisianthus necrotic ringspot virus* (LNRV).

[‡]Tospoviruses that can be positively detected with particular test(s), are indicated by their acronyms, unless indicated differently. Test results for other tospovirus species are not reported or unknown.

[§]Test may cross react with GRSV and TCSV (Boonham *et al.*, 2002).

[¶]Synonym of *Peanut chlorotic fan-spot virus* (PCFV).

and/or molecular test. LFTs are generally used for testing symptomatic samples at locations without laboratory facilities, e.g. in the field or in greenhouses.

4. Identification

Molecular tests are recommended for identification because identification by serological tests is often hampered by cross-reactions (Hassani-Mehraban *et al*., 2016). DAS-ELISA can be used in specific cases, provided that the test is validated.

4.1. Serological methods

For the tospoviruses recommended for regulation by EPPO and regulated by a number of EPPO members, i.e. CSNV, INSV, TSWV and WSMoV, commercial antibodies are available. Details on their performance are provided in Appendix 2. Note that the analytical specificity of a test is critical when using it for definite identification of an unknown tospovirus isolate.

4.2. Molecular methods

4.2.1. RT-PCR tests

Several conventional and real-time RT-PCR tests have been described for the identification of specific tospoviruses, in particular CSNV, INSV, TSWV and WSMoV. Table 2 provides an overview of the available tests. Recommended tests are described in the corresponding appendices. Hassani-Mehraban *et al*. (2016), see Appendix 4, additionally provides primer sets for amplification of the (near) complete Nucleocapsid (N) gene for 19 out of 30 tospoviruses. It should be noted, however, that these primer sets have been designed for confirmation of the identity of the used isolates and, therefore, are not validated for detection of (all isolates of) a particular species.

4.2.2. Sequencing

Sequence analysis of amplicons obtained by the generic test described by Hassani-Mehraban *et al*. (2016) can be used for identification (see Table 2 and Appendix 4). Definite identification should be based on the sequence of the complete N-gene according to the species demarcation criteria of ICTV. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016). In specific cases, sequencing of additional genes or regions might be required, especially when reassortment and/or recombination events can be expected.

4.2.3. Other tests

The Panel on Diagnostics in Virology and Phytoplasmology noted that high-throughput sequencing is a technology that may be used to obtain (near) complete genome sequences, and analysis of these sequences can be used for identification of a virus isolate.

5. Reference material

Tospovirus isolates for reference are available from:

- Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, 38124, Braunschweig, Germany (https://www.dsmz.de/catalogues/catalogue-plant-viruses-and-antisera.html.
- National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands.
- Wageningen University and Research, Plant Sciences Group, Droevendaalsesteeg 1, 6708 PB Wageningen.

Information is also available from EPPO-Q-bank (https://qbank.eppo.int/).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7. Performance characteristics

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further Information

Further information on these organisms can be obtained from:

JW Roenhorst, National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands.

N Mehle, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia.

Y Shneyder, All-Russian Plant Quarantine Centre, 140150 Russia, Moscow Region, Bykovo, Pogranichnaya str., 32, Russia.

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was prepared by an Expert Working Group composed of J.W. Roenhorst (lead author), National Plant Protection Organization, Wageningen (NL), K. de Jonghe, Flanders Research Institute for Agriculture, Fisheries and Food, Merelbeke (BE), N. Mehle, National Institute of Biology, Ljubljana (SI), O. Schumpp, Agroscope, Nyon (CH) and Y. Shneyder, All-Russian Plant Quarantine Center, Moscow Oblast (RU). The Panel on Diagnostics in Virology and Phytoplasmology reviewed this protocol.

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Appendix 1 – Bioassays

Test plants

Test plant species that can be used for generic detection of tospoviruses are *Datura stramonium*, *Nicotiana benthamiana* and *Nicotiana occidentalis*-P1. These test plants produce symptoms upon mechanical inoculation with the currently known tospovirus species (Table 3).

For inoculation, different buffers [e.g. 0.02 M phosphate buffer pH 7.4, 2% (w/v) polyvinylpyrrolidone (PVP; MW 10000)] as well as water have been used successfully. It is advisable to inoculate immediately after grinding the plant material because tospoviruses may lose infectivity quite rapidly. Furthermore, note that the leaf sap of some host plant species might inhibit virus transmission (Roenhorst *et al*., 2013).

Test plants should be inoculated at a young stage (3–6 leaves) by rubbing the inoculum onto carborundum-dusted leaves. After inoculation, plants should be rinsed with water and kept in a glasshouse at 18–25°C with supplementary illumination for a day length of at least 14 h. First symptoms generally develop within 7 days after inoculation. Further details on quality control are described by Roenhorst *et al*. (2013).

Table 3. Overview of test plants used for detection of tospoviruses*

Tospovirus name†	Acronym	*Datura stramonium*	*Nicotiana benthamiana*	*Nicotiana occidentalis*-P1
Alstroemeria necrotic streak virus	ANSV	nd‡	+/+	nd
Alstroemeria yellow spot virus	AYSV	+/−	+/+	+/−
Bean necrotic mosaic virus	BeNMV	nd	nd	nd
Calla lily chlorotic spot virus	CCSV	nd	nd	nd
Capsicum chlorosis virus	CaCV	nd	+/+	+/+
Chrysanthemum stem necrosis virus	CSNV	+/−	+/+	+/+
Groundnut bud necrosis virus†	GBNV	nd	(+)/+	(+)/+
Groundnut chlorotic fan-spot virus	GCFSV	nd	nd	nd
Groundnut ringspot virus†	GRSV	−/−	(+)/+	+/+
Groundnut yellow spot virus†	GYSV	nd	nd	nd
Hippeastrum chlorotic ringspot virus	HCRV	nd	nd	nd
Impatiens necrotic spot virus†	INSV	(+)/-	+/+	+/(+)
Iris yellow spot tospovirus†	IYSV	nd	+/+	+/−
Lisianthus necrotic ringspot virus	LNRV	nd	+/+	nd
Melon severe mosaic virus	MSMV	nd	nd	nd
Melon yellow spot virus	MYSV	nd	(+)/(+)	+/+
Mulberry vein banding associated virus	MVBaV	nd	nd	nd
Pepper chlorotic spot virus	PCSV	nd	nd	nd
Pepper necrotic spot virus	PNSV	nd	nd	nd

(continued)

Table 3 *(continued)*

Tospovirus name[†]	Acronym	*Datura stramonium*	*Nicotiana benthamiana*	*Nicotiana occidentalis*-P1
Polygonum ringspot virus[†]	PolRSV	nd	nd	nd
Soybean vein necrosis virus	SVNV	nd	nd	nd
Tomato chlorotic spot virus[†]	TCSV	(+)/+	+/+	+/+
Tomato necrotic ringspot virus	TNRV	nd	−/+	−/+
Tomato necrotic spot virus	TNSV	nd	+/+	nd
Tomato spotted wilt virus[†]	TSWV	+/+	+/+	+/+
Tomato yellow ring virus	TYRV	nd	+/+	nd
Tomato zonate spot virus	TZSV	nd	nd	nd
Watermelon bud necrosis virus[†]	WBNV	nd	nd	nd
Watermelon silver mottle virus[†]	WSMoV	nd	+/+	nd
Zucchini lethal chlorosis virus[†]	ZLCV	nd	nd	nd

Note that reactions of test plants are variable, depending on the virus isolate, test-plant accession and climatic conditions like temperature, light conditions (season), etc.

*Test plant reactions indicated are based on experience of the NPPO-NL and/or the Laboratory of Virology of Wageningen University, the Netherlands.

[†]Virus species included in the ICTV classification.

[‡]Test plant reactions: nd, not determined; symptoms local/systemic: +, symptoms for all or the majority of isolates tested; (+), symptoms for only part of the isolates tested; −, no symptoms.

Appendix 2 – Data on antisera for ELISA

Instructions to perform an ELISA test are provided in the EPPO Standards PM 7/125 *ELISA tests for viruses*.

The source of antibodies is critical. Several antisera are available for generic or semi-generic detection of tospoviruses and for detection and identification of species (Table 4). In general, it is recommended to follow the protocol provided by the supplier of the antiserum. Note that identification by serological tests is often hampered by cross-reactions of the antiserum (Hassani-Mehraban *et al*., 2016).

The following antisera are used for detection of tospoviruses. Performance characteristics are provided if available. For analytical specificity see Table 4.

'Generic' antisera

- Tosposcreen (Loewe Biochemica GmbH.): analytical sensitivity: CSNV (1:10,000), GRSV (1:10,000), INSV (1:100,000), TCSV (1:10,000), TSWV (1:10,000); selectivity: no back ground reactions observed in the following matrices: *Calibrachoa* spp., *Capsicum* spp., *Catharanthus* spp., *Chrysanthemum* spp., *Cyclamen* spp., *Impatiens* spp., *Lactuca* spp., *Nicotiana* spp., *Pelargonium* spp., *Petunia* spp., *Saintpaulia* spp., *Solanum* spp., (according to manufacturer)
- Tospovirus broad-spectrum (Bioreba)

'Specific' antisera

- Agdia: TSWV, WSMoV + GBNV (http://www.agdia.com/)
- Bioreba: INSV, TSWV (http://www.bioreba.ch)
- DSMZ*: CSNV AS-0529, INSV AS-0115-0117/1, INSV BF-0115, TSWV AS-0105-0106/3, TSWV BF-0105 (https://www.dsmz.de)
- Loewe Biochemica GmbH: INSV, TSWV (https://www.loewe-info.com)
- Neogen/Adgen: INSV, TSWV (http://www.agrifoodtest.nl)
- Prime Diagnostics: INSV, TSWV (https://www.wur.nl/en/show/Prime-Diagnostics-2.htm)

*For all antisera, no background reactions observed in *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *Nicotiana tabacum* (tobacco) and *Solanum lycopersicum* (tomato).

Table 4. Antisera for detection and/or identification of tospoviruses

	Generic		CSNV		INSV						TSWV							WSMoV
Antiserum (supplier) Species*	Tospovirus broad-spectrum[8] (Bioreba)	Tosposcreen[5] (Loewe)	CSNV[3,4] (DSMZ)	CSNV[4] BF[#] (DSMZ)	INSV[1,2] (Bioreba)	INSV[4,5] (DSMZ)	INSV[3,4] BF[#] (DSMZ)	INSV[3,5] (Loewe)	INSV[5] (Neogen)	INSV[6] (Prime Diagnostics)	TSWV[5,7] (Agdia)	TSWV[1,2] (Bioreba)	TSWV[4,5] (DSMZ)	TSWV[3,4] BF[#] (DSMZ)	TSWV[5] (Loewe)	TSWV[3,5] (Neogen)	TSWV[6,9] (Prime Diagnostics)	WSMoV + GBNV[1,7] (Agdia)
ANSV			(+)	(+)		–							(+)	(+)				
AYSV										–							–	
BeNMV																		
CCSV																		
CaCV			–	–		–							–	–				
CSNV		+	+	+		–		–				(+)[9]	(+)	(+)[$]	(+)[9]	(+)	(+)	
GBNV																		+
GCFSV																		
GRSV	+	+	–	–		–				–			–	–			(+)	–
GYSV																		
HCRV																		
INSV	+	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–
IYSV		–	–	–		–	–			–			–	–			–	–
LNRV																		
MSMV																		
MYSV										–							–	
MVBaV																		
PCSV																		
PNSV																		
PolRSV																		
SVNV																		
TCSV	+	+	(+)	(+)		–	–			–			(+)	(+)			(+)	–
TNRV																		
TNSV										–							–	
TSWV	+	+	(+)	(+)	–	–	–		–	–	+	+	+	+	+	+	+	–
TYRV			–	–		–	–			–			–	–			–	
TZSV																		
WBNV																		
WSMoV		–	–	–		–	–						–	–				+
ZLCV																		

Data provided by [1]E. Ince (Tarimormam, TR), [2]S. Linhartova (UKZUZ, CZ), [3]N. Mehle (NIB, SI), [4]W. Menzel (DSMZ, DE), [5]Y. Shneyder (VNIIKR, RU), [6]R. van der Vlugt (Prime Diagnostics, NL) and/or R. Kormelink (WUR, NL), and websites of [7]Agdia, [8]Bioreba. [9]Data from a Proficiency test organized by NIB SI.TR 2018-01

*For acronyms see Table 1: +, positive reaction; (+), positive reaction less strong than homologous reaction; –, no reaction; empty cell, no data.

[#]B-Fast ELISA.

[$]Only one of two CSNV isolates showed cross-reactivity.

Appendix 3 – Sample preparation and RNA extraction methods for PCR tests

This appendix describes sample preparation and RNA extraction methods for different hosts and types of plant material. These initial steps are critical for the result of a PCR test and are more dependent on the matrix than the following amplification steps, therefore they are described in this appendix.

In general, a wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals. The RNeasy Plant Mini Kit (Qiagen), or the Sbeadex® Maxi Plant Kit (LGC Genomic) can be used following the manufacturers' instructions or the instructions in this Appendix for the RNeasy plant mini kit. The Sbeadex® Maxi Plant Kit can be used in combination with a KingFisher KF96 system for high throughput. RNA extraction using CTAB (e.g. after Gambino *et al*., 2008) is robust and is considered by the Panel on Diagnostics in Virology and Phytoplasmology as an appropriate method which will not affect the performance of the molecular tests. Other extraction methods may be used if they are verified.

Grinding and RNA extraction by RNeasy plant mini kit*

(1) 0.2–0.3 g of plant material is ground in either:
   (a) liquid nitrogen using an iced mortar and pestle; the tissue powder is then decanted into an RNase-free, liquid-nitrogen-cooled, 2 mL microcentrifuge tube and 900 µL RLT buffer (Qiagen, included in the kit);
   (b) 900 µL RLT** buffer from the kit using a tissue homogeniser, e.g. FastPrep^{R}-24 with QuickPrep^{TM} 24 × 2 mL adapter (MP Biomedicals) in a 2 mL tube, with garnet matrix and ¼-inch. ceramic sphere (MP Biomedicals); centrifuge tubes before opening to prevent cross-contamination;
   (c) 1 mL PBS-extraction buffer (dissolve in 800 mL of distilled water: 8.0 g NaCl, 0.2 g KH_2PO_4, 1.14 g Na_2HPO_4, 0.2 g KCl; adjust volume to 1 L; add 20.0 g PVP (MW 40 000), 2.0 g BSA and 0.5 mL Tween 20; adjust pH to 7.4) by using homogenization bags and Homex 6 (Bioreba); continue with an aliquot of 100 µL and add 450 µL of RLT buffer (Qiagen, included in the kit);
   (d) 3.5 mL of GH + grinding buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5.2, 25 mM EDTA and 2.5% PVP (MW 10 000)); 1 mL of the homogenate is lysed by incubation in a thermomixer at 850 rpm for 10 min at 65°C and centrifuged for 2 min at 16 000 g; continue with 500 µL supernatant;
(2) Proceed with RNA extraction following the manufacturer's instructions.
(3) Total RNA is eluted twice with 50 µL (total of 100 µL) of RNase-free water pre-warmed to 65°C. Tenfold diluted RNA is suitable for testing when inhibition by the matrix is expected.
(4) Extracted RNA should preferably be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or −80°C for longer periods.

*Note that when using the RNeasy plant mini kit (Qiagen) there is no need to add 2-mercaptoethanol.

**Qiagen recommends in cases of tissue solidification the use of buffer RLC instead of buffer RLT.

Appendix 4 – Conventional RT-PCR (Hassani-Mehraban *et al*., 2016)

1. General information

1.1. This conventional RT-PCR is suitable for the detection of tospoviruses in leaf material of *Nicotiana benthamiana* and other plant host species.
1.2. The test is based on the publication of Hassani-Mehraban *et al*., 2016.
1.3. The target sequences are located in the nucleocapsid gene and its 5' upstream untranslated region of the S-RNA.
1.4. Oligonucleotides and average amplicon size (amplicon location and size depend on the virus species concerned).

Clade(s)	Primers	Amplicon size
Asian clade 1 and Eurasian clade	AS-EA-F: 5′-GGG GGA TCC AGA GCA ATC GAG G-3′ AS1-R: 5′-GCT TCA GTC CTC TTA AAT GTC C-3′ EA-R: 5′-TTG TTC AAT GAA GCA GCA CC-3′	370 bp (As-1) 790 bp (EA)
American clade 1*	AM1-F: 5′-GGG GGA TCC AGA GCA ATT GTG TC-3′ AM1-R: 5′-CTT TGC TTT TCA GCA CAG TGC A-3′	760 bp
Asian clade 2	AS-EA-F: 5′-GGG GGA TCC AGA GCA ATC GAG G-3′ AS2-R: 5′-CTT TGA AGA TGA CCT CAT CT-3′	520 bp
LNRV	LNRV-F: 5′-CCA GTA AAA GAC GAA ATC CC-3′ LNRV-R: 5′-GAT TCA AAT CGC CCA GCA GTC C-3′	430 bp
American clade 2	AM2-F: 5′-GGG GGA TCC AGA GCA ATC GG-3′ AM2-R1: 5′-GCA ACT CTA CCA GCT TG-3′ AM2-R2: 5′-GCA ACT TTA GCA GCT TG-3′	670 bp
Internal positive control	Nad-5F: 5′- GAT GCT TCT TGG GGC TTC TTG TT-3′ Nad-5R: 5′- CTC CAG TCA CCA ACA TTG GCAT AA-3′	181 bp

*Recently there are indications that the American clade 1 primers will miss some isolates of TSWV (NVWA, pers. comm. 2020).

1.5. The test has been successfully performed using a Bio-Rad C1000/S1000 Thermal Cycler with deepwell blocks.

2. Methods

2.1. Nucleic Acid Extraction and Purification
See Appendix 3, alternative procedures may also be suitable.

2.2. One-step Reverse Transcription PCR (one-step RT-PCR)
The RT-PCR test consists of five reactions using different primer combinations, and the internal positive control *nad5* is run in a separate tube. It might be possible to use this control in combination with the target reactions in a duplex format, but this has not been evaluated so far.

2.2.1. Master Mix for Asian clade 1 and Eurasian clade

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	15.5	NA
One-step RT-PCR buffer (*Qiagen OneStep RT-PCR Kit*)	5×	5.0	1x
dNTPs (*Qiagen*)	10 mM	1.0	0.4 mM
PCR Forward primer AS-EA-F	10 µM	0.5	0.2 µM
PCR Reverse primer AS1-R	10 µM	0.5	0.2 µM
PCR Reverse primer EA-R	10 µM	0.5	0.2 µM
OneStep RT-PCR enzyme mix (*Qiagen*)	NA	1.0	NA
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.2. Master Mix for American clade 1 or Asian clade 2 or LNRV

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	16.0	NA
One-step RT-PCR buffer (*Qiagen OneStep RT-PCR Kit*)	5×	5.0	1x
dNTPs (*Qiagen*)	10 mM	1.0	0.4 mM
PCR Forward primer AM1-F or AS2-F or LNRV-F (separate reactions)	10 µM	0.5	0.2 µM
PCR Reverse primer AM1-R or AS2-R or LNRV-R (separate reactions)	10 µM	0.5	0.2 µM
OneStep RT-PCR enzyme mix (*Qiagen*)	NA	1.0	NA
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.3. Master Mix for American clade 2

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	6.5	NA
One-step RT-PCR Reaction mix (*SuperScript III One-Step RT-PCR System, Life Technologies*)	2×	12.5	1×
dNTPs (*Qiagen*)	10 mM	1.0	0.4 mM
PCR Forward primer AM2	10 µM	1.0	0.2 µM
PCR Reverse primer AM2-R1	10 µM	1.0	0.2 µM
PCR Reverse primer AM2-R2	10 µM	1.0	0.2 µM
SuperScript III RT-Platinum Taq DNA Polymerase (*Life Technologies*)	NA	1.0	NA
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Note that the test for American Clade 2 species has been performed with Life Technologies reagents but it is expected that similar results can be obtained with Qiagen reagents as well.

2.2.4. Master Mix for internal positive control *nad5*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	16.0	NA
One-step RT-PCR buffer (*Qiagen OneStep RT-PCR Kit*)	5×	5.0	1×
dNTPs (*Qiagen*)	10 mM	1.0	0.4 mM
PCR Forward primer Nad-5F	10 µM	0.5	0.2 µM
PCR Reverse primer Nad-5R	10 µM	0.5	0.2 µM
OneStep RT-PCR enzyme mix (*Qiagen*)	NA	1.0	NA
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.5. RT-PCR cycling conditions

- *Asian Clade 1 and Eurasian Clade*: reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, elongation at 72°C for 60 s; terminal elongation at 72°C for 5 min; hold at 20°C.
- *American Clade 1*: see RT-PCR conditions above except for annealing at 50°C.
- *Asian Clade 2*: see RT-PCR conditions above except for annealing at 51°C.
- *LNRV*: see RT-PCR conditions above except for annealing at 58°C.
- *American Clade 2*: reverse transcription at 50°C for 30 min; denaturation at 94°C for 2 min; 35 cycles consisting of denaturation at 94°C for 60 s, annealing at 58°C for 30 s, elongation at 68°C for 60 s; terminal elongation at 68°C for 5 min; hold at 20°C.
- Internal positive control *nad5*: see RT-PCR conditions for Asian Clade 1 and Eurasian Clade.

3. Essential procedural information

3.1. Controls

- For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.
- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of one species belonging to the target clade or a matrix sample that contains one of the target species (e.g. naturally infected host tissue or host tissue spiked with a target species).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target species. This can include nucleic acid extracted from one species belonging to the target clade, total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPCs can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel *et al*., 2002). Note that for this test the IPC has not been evaluated in a duplex format.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 370 bp for Asian clade 1, 520 bp for Asian clade 2, 790 bp for Eurasian clade, 760 bp for American clade 1, 670 bp for American clade 2 and 430 bp for LNRV. Note that because of the generic nature of the primers amplicon sizes of individual virus species might deviate from the indicated sizes.
- IPC if used should produce amplicon of expected size (~181 bp for *nad5*).

When these conditions are met

- A test will be considered positive if amplicons of the appropriate size are produced.
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that for viruses and viroids bands of different sizes may correspond to strains of the target organism

and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

Note that amplicons obtained by the generic tospovirus tests can be sequenced for identification of the species (Hassani-Mehraban *et al*., 2016). The article additionally provides specific primers for amplification of the (near) complete nucleocapsid gene of most common tospovirus species. Sequencing and analysis of these amplicons allow a definite identification of the virus isolate.

4. Performance characteristics

The test of Hassani-Mehraban *et al*. (2016) has been primarily developed for confirmation of the presence of a tospovirus in plant material showing symptoms. Therefore, validation data are limited.

4.1. Analytical sensitivity data
Not determined.

4.2. Analytical specificity data
For the 19 species tested, sequencing of the obtained amplicons resulted in correct identification of the isolate, which has been confirmed by comparison with the identities obtained by sequencing of the (almost) complete N-gene.

4.3. Data on repeatability and reproducibility
Although the performance characteristics for repeatability and reproducibility have not been evaluated according to PM 7/98, the test has been repeated and reproduced on several occasions with consistent results.

Appendix 5 – Conventional RT-PCR tests

A. Conventional RT-PCR tests for GRSV, INSV, TCSV and TSWV (Mumford *et al*., 1996b)

1. General Information

1.1. The conventional RT-PCR is suitable for the detection and/or identification of GRSV, INSV, TCSV and TSWV in leaf material of ornamental and vegetable plants.

1.2. The test is adapted from the publication of Mumford *et al*. (1996b).

1.3. S1/S2 Univ primers were designed to target the N gene of: GRSV, INSV, TCSV and TSWV. The S1/S2 INSV primers target the Nucleocapsid (N) gene using the S-RNA sequences of INSV isolates NL-07 (NC_003624) and TSWV-I. The internal positive control primers IC1 5S-RNA and IC2 5S-RNA have been designed by Kolchinsky *et al*. (1991).

1.4. Oligonucleotides and average amplicon size:

Primer	Sequence	Amplicon size
Forward primer S1 Univ	5′-TGT A(A/G)T G(G/T)T CCA T(A/T)G CA-3′	871 bp
Reverse primer S2 Univ	5′-AGA GCA AT(C/T) GTG TCA-3′	
Forward primer S1 INSV	5′-AAA TCA ATA GTA GCA TTA-3′	602 bp
Reverse primer S2 INSV	5′-CTT CCT CAA GAA TAG GCA-3′	
Forward primer IC1	5S-RNA 5′-TTT AGT GCT	Differs in size
Reverse primer IC2	GGT ATG ATC GC-3′	according
	5S-RNA 5′-TGG GAA GTC	to the host
	CTC GTG TTG CA-3′	plant

1.5. The test has been successfully performed on a Veriti Thermal Cycler (Applied Biosystems)

2. Methods

2.1. Nucleic Acid Extraction and Purification
See Appendix 3; alternative procedures may also be suitable.

2.2. Reverse Transcription (RT; to produce cDNA from RNA)

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	2.1	N.A.
RT buffer (M-MLV RT 5X Reaction Buffer (*Promega*)	5×	2.0	1×
dNTPs (*Invitrogen*)	3.5 mM	3.0	1 mM
Reverse Primer (S2 univ or S2 INSV or IC2)	10 µM	1.0	1 µM
M-MLV Reverse transcriptase (*Promega*)	200 U/µL	0.5	100 U
RNase inhibitor (*Promega*)	120 U/µL	0.4	48 U
Subtotal		9.0	
RNA		1.0	
Total		10.0	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.2. RT cycling conditions
Reverse transcription at 42°C for 60 min

2.3. PCR

2.3.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water *	NA	3.5	NA
Dream Taq PCR Master Mix (*Thermo Scientific*)**	2×	12.5	1×
Forward primer (S1 univ or S1 INSV or IC1)	10 µM	2.0	0.8 µM
Reverse primer (S2 univ or S2 INSV or IC2)	10 µM	2.0	0.8 µM
Subtotal		20.0	
cDNA		5.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.
**Polymerase included in the Master Mix.

2.3.2. PCR cycling conditions

Denaturation at 94°C for 5 min; 29 cycles consisting of denaturation at 94°C for 60 s, annealing at 48°C for 60 s, elongation at 72°C for 60 s; 1 cycle at 94°C for 60 s, followed by terminal elongation at 55°C for 60 s and 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification of nucleic acid of the target organism. This can include a known amount of nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product, artificial RNA or DNA). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel *et al*., 2002). The internal control primers IC1 5S-RNA and IC2 5S-RNA have been designed on the transcribed units of the 5S rRNA gene family that is highly conserved in the plant kingdom (Kolchinsky *et al*., 1991). The presence of an amplicon indicates RNA extraction was successful. Run under standard conditions (annealing at 55°C).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 871 bp (for S1/S2 Univ) and 602 bp (for S1/S2 INSV).
- IPC if used should produce amplicon of expected size, e.g. ~181 bp for *nad5*, and different sizes for 5S-RNA according to host plant.

When these conditions are met

- A test will be considered positive if amplicons of 871 bp (for S1/S2 Univ) and 602 bp (for S1/S2 INSV) are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that for viruses and viroids bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance characteristics available

Although the performance characteristics for repeatability and reproducibility have not been evaluated according to PM 7/98, the test has been repeated and reproduced on several occasions with consistent results.

B. Conventional RT-PCR test for TSWV (Mumford *et al*., 1994)

'The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.'

1. General Information

1.1. The conventional RT-PCR is suitable for the detection and/or identification of TSWV in leaf material of ornamental and vegetable plants.
1.2. The test is adapted from the publication of Mumford *et al*. (1994).
1.3. The L1/L2 TSWV primers were designed to target the RNA-dependent RNA polymerase gene of the L-RNA of TSWV isolate NC_002052.
1.4. Oligonucleotides and average amplicon size:

Primer	Sequence	Amplicon size
Forward primer L1	5′-AAT TGC CTT GCA ACC AAT TC-3′	276 bp
Reverse primer L2	5′-ATC AGT CGA AAT GGT CGG CA-3′	

1.5. The test has been successfully performed on a Veriti Thermal Cycler (Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification
See Appendix 3; alternative procedures may also be suitable.
2.2. One step Reverse Transcription PCR
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	7.95	NA
GoTaq® Flexi Buffer, $MgCl_2$ free (*Promega*)	5×	5.0	1×
$MgCl_2$ (*Promega*)	25 mM	3.5	3.5 mM
dNTPs (*Invitrogen*)	10 mM	2.0	0.8 mM
Forward primer L1	10 µM	2.5	1 µM
Reverse primer L2	10 µM	2.5	1 µM
AMV reverse transcriptase (*Promega*)	10 U/µL	0.15	1.5 U
RNase inhibitor (*Promega*)	40 U/µL	0.15	6 U
GoTaq DNA polymerase (*Promega*)	5 U/µL	0.25	1.25 U
Subtotal		24.0	
RNA		1.0	
Total		25.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3. RT-PCR cycling conditions
Reverse transcription at 48°C for 45 min; denaturation at 94°C for 2 min; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, elongation at 72°C for 90 s; terminal elongation at 72°C for 10 min; hold at 20°C.

3. Essential procedural information

3.1. Controls
For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification of nucleic acid of the target organism. This can include a known amount of nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product, artificial RNA or DNA). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel *et al*., 2002), The internal control primers IC1 5S-RNA and IC2 5S-RNA have been designed on the transcribed units of the 5S rRNA gene family that is highly conserved in the plant kingdom (Kolchinsky *et al*., 1991). The presence of an amplicon indicates RNA extraction was successful. Run under standard conditions (annealing at 55°C).

3.2. Interpretation of results
Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of ~276 bp.
- IPC if used should produce amplicon of expected size, e.g. ~181 bp for *nad5*.

When these conditions are met

- A test will be considered positive if amplicons of ~276 bp are produced.

- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that for viruses and viroids bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance criteria available

The test has not been validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Limited data on the analytical specificity were obtained at the NPPO of the Netherlands.

4.1. Analytical sensitivity data
Not determined.

4.2. Analytical specificity data
No cross-reactions occurred with isolates of TCSV, GRSV, IYSV, TYRV and ANSV.

4.3. Selectivity
No background reactions observed for *Datura stramonium*, *Lactuca sativa*, *Nicotiana rustica* and *Solanum lycopersicum*.

4.4. Repeatability and reproducibility
Although the performance characteristics for repeatability and reproducibility have not been evaluated according to PM 7/98, the test has been repeated and reproduced on several occasions with consistent results.

Appendix 6 – Real-time RT-PCR test for CSNV (Boben *et al.*, 2007)

'The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.'

1. General Information

1.1. This one-step real-time RT-PCR protocol is suitable for the detection and identification of CSNV in leaf material of ornamental and vegetable plants

1.2. The test is based on primers and probe published by Boben *et al*. (2007).

1.3. The target sequence is located within the nucleocapsid (N) gene using the nucleotide sequence of GenBank accession no AF067068, the forward and reverse primers starting at position 215 and 284, respectively, and the probe covering positions 241-255.

1.4. Oligonucleotides:

Primer/probe	Sequence
CSNV-F CSNV-R	5'-TGA ATT TGA GGA AGA ACA GAA CCA-3'
Probe CSNV-MGB	5'-CTG ATC CAG GTT GTC ATT GCA-3' 5'-FAM - TTG CAT TCA ACT TCC-MGB-3'

1.5. The test has been successfully performed using the AgPath-ID (Ambion) reagents and a range of different real-time PCR systems including ABI (7900, 7900HT Fast, ViiA^{TM} 7)

2. Methods

2.1. Nucleic Acid Extraction and Purification
See Appendix 3; alternative procedures may also be suitable.

2.2. One step[2] real-time RT-PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	0.0	NA
Real-time RT PCR buffer (*AgPath-ID^{TM} One-step RT-PCR kit (Ambion)*)	2×	5.0	1×
Forward primer CSNV_F	10 µM	0.9	0.9 µM
Reverse primer CSNV_R	10 µM	0.9	0.9 µM
Probe CSNV-MGB	2.5 µM	0.8	0.2 µM
RT-PCR enzyme *(AgPath-ID^{TM} One-step RT-PCR kit (Ambion))*	25×	0.4	1×
Subtotal		8.0	
RNA		2.0	
Total		10.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.2. Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 10 min (45°C as used for TSWV is appropriate as well); denaturation at 95°C for 10 min; 45 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min.
Note that the same real-time RT-PCR conditions can be used for the real-time RT-PCR tests for

[2]A two-step procedure was also shown to be appropriate (see Boben *et al.,* 2007).

CSNV, INSV and TSWV, as described in Appendices 6, 7 and 8, respectively.

3. Essential procedural information

3.1. ControlsFor a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using conserved primers preferably amplifying RNA targets, such as *nad5* (Botermans *et al*., 2013).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note that when analysing the raw data, it is important to adjust the cycle threshold (C_t) of the amplification plot to a point within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. When looking at the log view, this corresponds to the linear part of the amplification plot. It has been experimentally determined that an automatic baseline and threshold at 0.2 are usually suitable when using AgPath-ID (Ambion) reagents and Applied Biosystems thermal cyclers (7900, 7900HT Fast, ViiA^{TM} 7).

4. Performance characteristics available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by the National Institute of Biology (NIB), Slovenia, using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (C_t).

4.1. Analytical sensitivity data

Not determined.

4.2. Analytical specificity data

Both inclusivity and exclusivity of the test scored 100%. Eight out of eight samples from CSNV-infected plants gave positive results, i.e. *Chrysanthemum* spp. (2), *Gerbera* sp. (1) and *Chrysanthemum* spp. co-infected by TSWV(2), *Nicotiana benthamiana* (1) and *Nicotaina tabacum* cv. White Burley (2). No cross-reactions occurred with 34 non-targets, i.e. 20 samples from TSWV-infected plants, i.e. *Chrysanthemum* spp. (14), *Nicotiana rustica* (1), *Senecio pericallis* (3) and *Solanum lycopersicum* (2), three samples of INSV-infected plants, i.e. *N. benthamiana* (1) and *S. pericallis* (2), one sample of a CMV-infected *Cucurbita maxima* plant.

In silico NCBI BLAST analyses confirmed the specificity of the CSNV amplicon. The alignment of all the publically available sequences of CSNV only revealed some mismatches within the primers and probe sequences. It is expected, however, that most of them will not significantly hamper amplification.

4.3. Selectivity

No reactions were obtained for non-CSNV-infected samples of *Capsicum annuum* (4), *Chrysanthemum* spp. (2), *Gerbera* sp. (1), *Solanum lycopersicum* (1) and *Verbena* spp. (2).

4.4. Data on repeatability and reproducibility

Although the performance characteristics for repeatability have not been evaluated according to PM 7/98, the test has been repeated on several occasions with consistent results. Two samples, one with estimated high and one with estimated medium CSNV amount, tested in five runs on two machines on different days, resulted in identical results.

Appendix 7 – Real-time RT-PCR test for INSV (modified by Chen *et al.*, 2013)

'The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.'

1. General Information

1.1. This one-step real-time RT-PCR protocol is suitable for the detection and identification of INSV in leaf material of ornamental and vegetable plants.

1.2. The test is based on the primers and probe published by Chen *et al*. (2013).

1.3. The target sequence is located within the NSs gene (non-structural protein). NSs gene sequences of seven INSV isolates from GenBank (Accession nos. NC 003624, GU112504, EU095193, GQ336989, DQ425096, X66972 and AB109100) and six other tospovirus species [*Capsicum chlorosis virus* (NC 008301), *Groundnut bud necrosis virus* (NC 003619), *Melon yellow spot virus* (NC 008300), TSWV (NC 002051), *Tomato zonate spot virus* (NC 010489), *Water-melon silver mottle virus* (NC 003843)] were used to design the INSV specific primers and probe.

1.4. Oligonucleotides:

Primer/probe	Sequence
INSV-F	5′-CCA GAC CAA CAA CAG GAC AGT A-3′
INSV-R	5'-TGA AGA ACC GGC TGT ATG TG-3'
Probe INSV-P	5'-FAM - TCA CTG GCA ATG TCT GCA ACT TC -TAMRA-3'

1.5. The test has been successfully performed using the AgPath-ID (Ambion) reagents and the real-time PCR system ABI 7900HT Fast.

2. Methods

2.1. Nucleic Acid Extraction and Purification

See Appendix 3; alternative procedures may also be suitable.

2.2. One step[3] real-time RT-PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	0.0	NA
Real-time RT PCR buffer (*AgPath-ID™ One-step RT-PCR kit (Ambion)*)	2×	5.0	1×
Forward Primer INSV_F	10 µM	0.9	0.9 µM
Reverse Primer INSV_R	10 µM	0.9	0.9 µM
Probe INSV-P	2.5 µM	0.8	0.2 µM
RT-PCR enzyme *[AgPath-ID™ One-step RT-PCR kit (Ambion)]*	25×	0.4	1×
Subtotal		8.0	
RNA		2.0	
Total		10.0	

*Molecular grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.2. Real-time RT-PCR conditions: reverse transcription at 48°C for 10 min (45°C as used for TSWV is appropriate as well), denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min

Note that the same real-time RT-PCR conditions can be used for the real-time RT-PCR tests for CSNV, INSV and TSWV, as described in Appendix 6, 7 and 8, respectively.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid

[3]Two-step procedure was shown to be appropriate also (see Chen *et al.,* 2013). It has been used not only to detect INSV in plant hosts, but also in western flower thrips.

extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using conserved primers preferably amplifying RNA targets, such as *nad5* (Botermans *et al*., 2013).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC if applicable) should be positive.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note that when analysing the raw data, it is important to adjust the cycle threshold (C_t) of the amplification plot to a point within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. When looking at the log view, this corresponds to the linear part of the amplification plot. It has been experimentally determined that an automatic baseline and threshold at 0.2 are usually suitable when using AgPath-ID (Ambion) reagents and Applied Biosystems thermal cyclers (7900, 7900HT Fast, ViiA™ 7).

4. Performance characteristics available

Validation data for the two-step procedure is available from the publication of Chen *et al*. (2013). In addition, the following validation data were generated by the National Institute of Biology (NIB), Slovenia.

4.1. Analytical sensitivity data

INSV was detected up to a 10^{-5} dilution in 10-fold serial dilutions of INSV-infected *Nicotiana benthamiana* leaf homogenates in homogenates of non-infected chrysanthemum leaves.

4.2. Analytical specificity data

Both inclusivity and exclusivity of the test scored 100%. Three out of three samples from INSV-infected plants gave positive results, i.e. *Impatiens* sp. (1) and *N. benthamiana* (2). No cross-reactions were observed for eight non-targets, i.e. non-infected *Chrysanthemum* sp. (1), TSWV-infected *Chrysanthemum* spp. (3), *Solanum lycopersicum* (1) and *Capsicum annuum* (1), respectively, and CSNV-infected *Chrysanthemum* sp. (1) and *N. benthamiana* (1).

4.3. Selectivity

Not determined.

4.4. Data on repeatability and reproducibility

Although the performance characteristics for repeatability have not been evaluated according to PM 7/98, the test has been repeated on several occasions with consistent results. Five chrysanthemum samples, one with estimated high, three with estimated medium INSV levels and one non-infected sample, tested in triplicate resulted in 100% identical results. In addition, three chrysanthemum samples, one with estimated high and one with estimated medium INSV amount, and one non-infected sample, tested two times by different operators on a different day, resulted in identical results.

Appendix 8 – Real-time RT-PCR for TSWV (Boonham *et al*., 2002)

'The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.'

1. General Information

1.1. The real-time PCR is suitable for the detection of TSWV in leaf material of ornamental and vegetable plants as well as in individual thrips.

1.2. The test is based on primers and probe published by Boonham *et al*. (2002).

1.3. The target sequence is located in the conserved regions of the nucleoprotein N (N-gene).

1.4. Oligonucleotides:

Primer/probe	Sequence
TSWV-CP-17F	5′-CTC TTG ATG ATG CAA AGT CTG TGA-3′
TSWV-CP-100R	
Probe TSWV-CP-73T	5′-TCT CAA AGC TAT CAA CTG AAG CAA TAA-3′
	5′-6-FAM-AGG TAA GCT ACC TCC CAG CAT TAT GGC AAG-TAMRA-3′

1.5. The test has been successfully performed using the AgPath-ID (Ambion) reagents and a range of different real-time PCR systems including ABI (7900, 7900HT Fast, ViiA™ 7) and Bio-Rad C1000 + cfx96 real-time PCR module.

2. Nucleic Acid Extraction and Purification

See Appendix 3; alternative procedures might work as well.

2.1. One step[4] real-time RT-PCR

2.1.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Real-time RT PCR buffer *[AgPath-ID™ One-step RT-PCR kit (Ambion)]*	2×	5.0	1×
PCR forward Primer TSWV-CP-17F	10 µM	0.9	0.9 µM
PCR reverse Primer TSWV-CP-100R	10 µM	0.9	0.9 µM
Probe TSWV-CP-73T	2.5 µM	0.8	0.2 µM
RT-PCR enzyme *[AgPath-ID™ One-step RT-PCR kit (Ambion)]*	25×	0.4	1×
Subtotal		8.0	
RNA		2.0	
Total		10.0	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.1.2. Real-time RT-PCR cycling conditionsReverse transcription at 45°C (48°C as used for CSNV and INSV is appropriate as well) for 10 min; denaturation at 95°C for 10 min; 45 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min.

Note that the same real-time RT-PCR conditions can be used for the real-time RT-PCR tests for CSNV, INSV and TSWV, as described in Appendices 6, 7 and 8, respectively.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using conserved primers preferably amplifying RNA targets, such as *nad5* (Botermans *et al*., 2013).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC if applicable) should be positive.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note that when analysing the raw data it is important to adjust the cycle threshold (C_t) of the amplification plot to a point within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. When looking at the log view, this corresponds to the linear part of the amplification plot. It has been experimentally determined that an automatic baseline and threshold are usually suitable when using AgPath-ID (Ambion) reagents and Applied Biosystems thermal cyclers (7900, 7900HT Fast, ViiA™ 7) and Bio-Rad (C1000, CFX96).

4. Performance characteristics available

Validation data were generated according to PM7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* with a slightly different protocol by the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Belgium.

4.1. Analytical sensitivity data

In serial dilutions of TSWV-infected material in *Chrysanthemum morifolium*, TSWV could be detected

[4]A two-step procedure was also shown to be appropriate (see Boonham *et al.,* 2002).

up to 10^7 and 10^3 dilutions for material with high and low virus concentration, respectively.

4.2. Analytical specificity data
Inclusivity scored 100%. In addition to the assessment of the specificity of Boonham *et al*. (2002), six samples of plants infected by different isolates gave positive results, i.e. *Capsicum annuum* (1), *Chrysanthemum* spp. (2), *Phalaenopsis* sp. (1) and *Solanum lycopersicum* (2). Moreover, testing over 250 different diagnostic and survey samples, mainly chrysanthemum and tomato, with both this real-time RT-PCR and ELISA produced consistent results (inclusivity).Exclusivity: No cross-reactions occurred with the tospoviruses CSNV, INSV, and WSMoV, neither with chrysanthemum virus B, cucumber mosaic virus, pepino mosaic virus, potato virus Y, tobacco mosaic virus, tomato black ring virus, tomato mosaic virus, tomato ringspot virus, tomato yellow leaf curl virus and chrysanthemum chlorotic mottle viroid and chrysanthemum stunt viroid (exclusivity). Cross-reactions may occur with GRSV and TCSV.

4.3. Selectivity
Not determined.

4.4. Data on repeatability and reproducibility
Although the performance characteristics for repeatability have not been evaluated according to PM 7/98, the test has been repeated on three and reproduced on two occasions with 100% identical results.