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International Plant Protection Convention
Protecting the world's plant resources from pests

ISPM 27
ANNEX 24

ENG

INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DIAGNOSTIC PROTOCOLS

DP 24: *Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Watermelon silver mottle virus*

Produced by the Secretariat of the
International Plant Protection Convention (IPPC)

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ISPM 27
Diagnostic protocols for regulated pests

DP 24: *Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Watermelon silver mottle virus*

Adopted 2017, published 2017

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1. Pest Information

The genus *Tospovirus* includes the plant-pathogenic, thrips-transmitted members of the family *Bunyaviridae*. Tospoviruses are transmitted exclusively by thrips belonging to the family Thripidae, subfamily Thripinae (Riley *et al.*, 2011). There are 11 definite members of the genus *Tospovirus*, of which *Tomato spotted wilt virus* (TSWV) is the type species, and at least 15 tentative members (King *et al.*, 2012). The latest information on classification of the genus *Tospovirus* may be obtained from the International Committee on Taxonomy of Viruses (see http://ictvonline.org). Tospoviruses have been classified according to serological differences but more recent classifications are based on molecular data (de Avila *et al.*, 1993). Viruses in the family *Bunyaviridae* have genomes composed of three negative or ambisense single-stranded RNAs that occur as ribonucleoprotein complexes (RNPs). Characteristic pleomorphic virus particles are formed by enclosure of RNPs in a host-derived membrane studded with surface projections composed of virally encoded glycoproteins. The viruses of this family are quasi-spherical, enveloped plant viruses 70–110 nm in diameter (Mumford *et al.*, 1996b; EPPO, 1999a).

Tospoviruses cause devastating crop losses because of their wide distribution, broad host range (approximately 1 000 plant species) and the circulative replicative relationship between the virus and its thrips vector. This diagnostic protocol covers the three most economically important tospoviruses: TSWV, *Impatiens necrotic spot virus* (INSV) and *Watermelon silver mottle virus* (WSMoV). Examples of economically important hosts for TSWV are *Arachis hypogaea* (peanut), *Capsicum annuum* (sweet pepper), *Carica papaya* (papaya), *Lactuca sativa* (lettuce), *Nicotiana tabacum* (tobacco), *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potato) (EPPO, 1999a). Ornamental hosts for TSWV include *Alstroemeria* spp., *Antirrhinum* spp., *Begonia* spp., *Celosia* spp., *Gerbera* spp., *Impatiens* spp., *Iris* spp. and *Zinnia* spp. (EPPO, 1999a). INSV also causes significant damage in vegetable crops as well as in ornamental plants, including *Ageratum* spp., *Begonia* spp., *Chrysanthemum* spp. and *Impatiens* spp. (EPPO, 1999b; Windham *et al.*, 1998, revised in 2015). WSMoV is a pathogen of cucurbits, the principal hosts being *Citrullus lanatus* (watermelon) and *Cucumis melo* (melon) (EPPO, 1999c). Spread or movement of all three of the viruses and their vectors on infected nursery stock is common, making detection and removal of infected material crucial.

TSWV is one of the most widespread plant viruses and occurs in countries of Africa, Asia, Central America and the Caribbean, Europe, North America, Oceania and South America (EPPO, 1999a). INSV has a more restricted geographic distribution than TSWV, being present within Africa, Asia, Australasia, Central America and the Caribbean, Europe and North America (EPPO, 1999b). WSMoV is currently restricted to Asia and possibly parts of South America (EPPO, 1999c). The limited distribution described for the latter two viruses may reflect the fact that they were distinguished only recently (EPPO, 1999b).

The three viruses are all transmitted and spread in nature by thrips (*Frankliniella* spp. and *Thrips* spp.), which acquire the virus during the larval stages and transmit it via the adults. The viruses are not reported to be seed- or pollen-transmitted or mechanically transmitted by contact between plants. However, experimentally, they may be transmitted mechanically or by grafting (EPPO, 1999a, b, c).

2. Taxonomic Information

Name: *Tomato spotted wilt virus* (TSWV)

Synonyms: *Pineapple yellow spot virus* (EPPO, 1999a)

Taxonomic position: *Bunyaviridae, Tospovirus*

Common names: None

Name: *Impatiens necrotic spot virus* (INSV)

Synonyms: None

Taxonomic position: *Bunyaviridae*, *Tospovirus*

Common names: None

Name: *Watermelon silver mottle virus* (WSMoV)

Synonyms: *Watermelon silver mottle tospovirus*; *Watermelon silvery mottle virus*; *Watermelon tospovirus*; TSWV-W (EPPO, 1999c)

Taxonomic position: *Bunyaviridae*, *Tospovirus*

Common names: None

3. Detection

All plant parts of infected hosts, except seeds and pollen, can potentially harbour the three viruses. Lists of hosts of TSWV, INSV and WSMoV hosts are provided in EPPO (1999b), (1999a) and (1999c), respectively.

Tospoviruses generally induce symptoms that include leaf necrosis, chlorosis, ring patterns, mottling, silvering, local lesions and stunting. Symptoms depend on the strain of the virus, the host plant, and the environmental conditions at the time of infection and plant growth. However, in combination with other information such as the presence of thrips, symptoms can be an indicator of the presence of a tospovirus. More detailed symptom descriptions for TSWV, INSV and WSMoV are given below and have been described also in Cho *et al.* (1987), Lisa *et al.* (1990), Yeh *et al*. (1992), Daughtrey (1996) and Chatzivassiliou *et al.* (2000).

TSWV symptoms on tomato include leaf bronzing, curling, necrotic spots, necrotic streaks and stunting of the plants. Fruit symptoms are usually either irregular yellow–orange flecks and occasionally rings on red and green fruits, or necrotic lesions or rings on other fruits. Ripe fruits of affected plants have paler red or yellow skin. Affected plants may have severe necrosis and sometimes die prematurely. On *C. annuum*, the first symptom is vein yellowing, which is usually followed by chlorosis, stunting and yellowing of the plant, chlorotic line patterns or mosaics with necrotic spots on leaves, and necrotic streaks on stems extending to terminal shoots. Yellow spots or necrotic streaks may be observed on ripe fruits (EPPO, 1999a). On *L. sativa*, the main symptom is the appearance of numerous necrotic lesions; other symptoms include leaf discoloration and one-sided growth. On *N. tabacum*, necrotic lesions, necrotic rings and chlorotic rings are observed on leaves.

INSV symptoms on New Guinea impatiens hybrids include stunting, leaf spots and black discoloration at the leaf bases. A range of symptoms occur on ornamental plant hosts such as *Alstroemeria* spp., *Gladiolus* spp. and *Lobelia* spp., and on vegetable crops such as *C. annuum*, *Cichorium endivia* (endive), *Cucumis sativus* (cucumber) and *L. sativa* (EPPO, 1999b).

WSMoV symptoms on *C. lanatus* include foliar mottling, crinkling, yellow spotting and narrowing of leaf laminae as well as the growth of small, malformed fruits with necrotic spots or silver mottling, a reduced fruit set, severe stunting, shortened internodes, upright growth of branches and tip necrosis. On *C. melo*, foliar mottling, stunting, upright growth of branches and tip blight are observed (Yeh *et al.*, 1992; EPPO, 1999c).

Appropriate sample selection is important for the detection of tospoviruses because they can be unevenly distributed in naturally infected hosts. Virus titre is likely to be low in hosts that have been infected recently by viruliferous thrips, depending on environmental conditions and on the host species or cultivar. Symptomatic leaves (or parts of symptomatic leaves, for example around necrotic lesions) should be used when available. It is recommended that newly expanded leaves should be selected rather than senescing material. Leaves should be stored at 4 °C for no more than seven days before processing, or at −80 °C if storage for an extended period is required.

Detection and identification of TSWV, INSV and WSMoV can be achieved using biological, serological or molecular tests following the flow diagram shown in Figure 1. Lateral flow tests may be used as a preliminary screening tool for virus detection in symptomatic material.

The tests described in Figure 1 are the minimum requirements to detect and identify the three viruses (e.g. during routine diagnosis of a pest widely established in a country), but further tests may be required where the national plant protection organization (NPPO) requires additional confidence in the identification (e.g. detection in an area where the virus is not known to be present). For example, sequencing of amplicons generated using molecular tests may be done. When a virus is suspected to be present in a new region or host it is recommended that both a serological test and a molecular test be used for detection.

The recommended techniques for the tests are described in the following sections. In all tests, positive and negative controls must be included.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

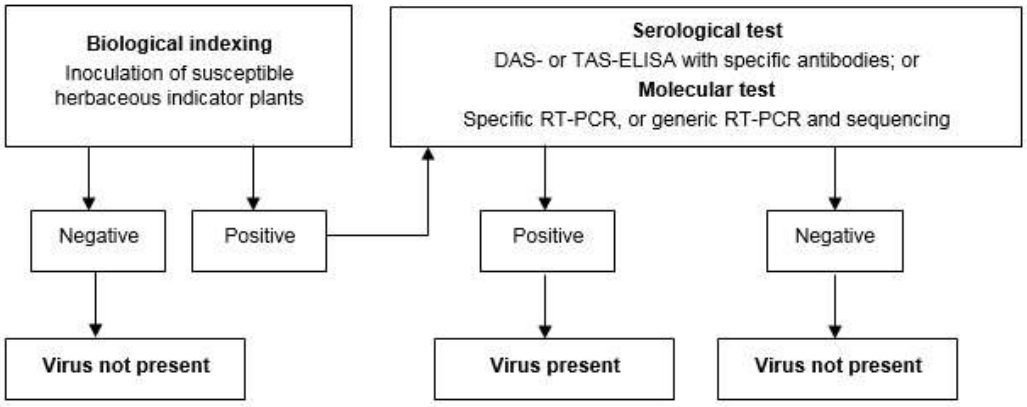

Figure 1. Minimum requirements for the detection and identification of *Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Watermelon silver mottle virus* (e.g. for the routine diagnosis of a pest widely established in a country).

DAS-ELISA, double-antibody sandwich enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; TAS-ELISA, triple-antibody sandwich enzyme-linked immunosorbent assay.

3.1 Biological detection

Herbaceous indicator species used to detect TSWV, INSV and WSMoV are given in Table 1. At least two species and at least two plants per species should be used, and positive and negative controls should be included in biological tests.

Indicator plants should be propagated from seed, planted in a well-drained soil mixture and maintained in an insect-proof facility at approximately 20–25 °C. Indicator plants should be kept in the dark for 24 h before inoculation to enhance susceptibility. Plant material to be tested should be macerated with chilled inoculation buffer (0.01 M phosphate buffer, pH 7.0, containing 1% sodium sulphite) using a chilled mortar and pestle; approximately 1 g tissue to 4 ml buffer. Tospoviruses are very labile, therefore buffers should be kept ice-cold and inoculum used as soon as possible after preparation. Sap

extract should be applied to the leaves of young plants with a small amount of Celite (Imerys Minerals California, Inc.[1] (mixed with sap) or carborundum powder (applied lightly to leaves). Using a gloved finger, the sap should be gently rubbed down the top surface of the lamina away from the plant stem. The inoculum should be allowed to sit on the leaves for a minimum of 1 min, then the leaves should be washed carefully to remove any residual abrasive powder. Following inoculation, the indicator plants should be maintained at either approximately 20 °C (for TSWV and INSV) or approximately 20–25 °C (for WSMoV). Symptoms usually develop within 7 to 28 days, depending on the indicator plant and the inoculum type and concentration.

Herbaceous indexing is considered to be a reliable and sensitive method of detection, but there are no quantitative data published on its specificity, sensitivity or reliability. It is not a rapid test (symptom development requires at least seven days after inoculation), it requires dedicated facilities (such as temperature-controlled greenhouse space) and the symptoms may be confused with those of other pests (in particular other tospoviruses). However, virus concentration is often greater in infected herbaceous indicator species than in the natural host plants. TSWV, INSV and WSMoV can be detected more reliably by other tests described in the protocol by testing inoculated herbaceous indicator plants.

Table 1. Selected herbaceous indicator species for *Tomato spotted wilt virus*, *Impatiens necrotic spot virus* and *Watermelon silver mottle virus*

Species[†,‡]	Family	Symptoms	Reference
Tomato spotted wilt virus			
Petunia hybrida cultivars Pink Beauty and Minstrel	Solanaceae	Local necrotic lesions on inoculated leaves, not systemic	Brunt *et al*. (1996); Kormelink (2005)
Nicotiana tabacum cultivars Samsun and White Burley; *Nicotiana glutinosa*; *Nicotiana clevelandii*; *Nicotiana rustica*	Solanaceae	Local necrotic lesions on inoculated leaves, systemic necrotic patterns and leaf deformation	Brunt *et al.* (1996); Kormelink (2005)
Nicotiana benthamiana	Solanaceae	Chlorotic to necrotic ring spots, local lesions on inoculated leaves, systemic chlorosis, mosaic stunting	Vaira *et al.* (1993); Louro (1996)
Cucumis sativus	Cucurbitaceae	Chlorotic spots with necrotic centres, not systemic	Brunt *et al.* (1996); Kormelink (2005)
Datura stramonium	Solanaceae	Chlorotic and necrotic spots and rings on inoculated leaves,	Vaira *et al.* (1993)

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Species[†,‡]	Family	Symptoms	Reference
		systemic mosaic and mottling	
Lycopersicon esculentum cv. Marmande	Solanaceae	Chlorotic to necrotic spots and rings on inoculated leaves, systemic mosaic, systemic chlorosis and necrotic spots	Vaira *et al.* (1993); Brunt *et al.* (1996)
Impatiens spp.	Balsaminaceae	Chlorotic to necrotic spots or rings on inoculated leaves, systemic chlorotic to necrotic spots	Daughtrey *et al*. (1997)
Impatiens necrotic spot virus			
Impatiens spp.	Balsaminaceae	Some necrotic spots or rings, systemic chlorotic or necrotic spots	Brunt *et al.* (1996)
Nicotiana tabacum cv. White Burley	Solanaceae	Local necrotic lesions on inoculated leaves (some isolates)	Vaira *et al.* (1993); Daughtrey *et al.* (1997)
Nicotiana benthamiana	Solanaceae	Chlorotic to necrotic ring spots or local lesions on inoculated leaves, systemic chlorosis and stunting	Vaira *et al.* (1993); Daughtrey *et al.* (1997)
Nicotiana clevelandii	Solanaceae	Local necrotic lesions on inoculated leaves, systemic mosaic	Vaira *et al.* (1993)
Datura stramonium	Solanaceae	Chlorotic spots or systemic mosaic	Vaira *et al.* (1993); Daughtrey *et al.* (1997)
Petunia hybrida	Solanaceae	Small necrotic spots on inoculated leaves, not systemic	Daughtrey *et al.* (1997)
Lycopersicon esculentum	Solanaceae	Variable between isolates, lesions on inoculated leaves only	Vaira *et al.* (1993); Daughtrey *et al.* (1997)
Watermelon silver mottle virus			
Nicotiana benthamiana	Solanaceae	Systemic mottling	Yeh *et al.* (1992)
Datura stramonium	Solanaceae	Local lesions on inoculated leaves, systemic mottling or necrotic spots	Yeh *et al*. (1992)

Species†,‡	Family	Symptoms	Reference
Petunia hybrida	Solanaceae	Local lesions on inoculated leaves, not systemic	Yeh *et al*. (1992)
Chenopodium amaranticolor; Chenopodium quinoa	Chenopodiaceae	Local lesions on inoculated leaves, not systemic	Yeh *et al*. (1992)
Cucumis sativus	Cucurbitaceae	Systemic chlorotic spots and mottling, rolling of leaf edges	Yeh *et al*. (1992)
Nicotiana rustica	Solanaceae	Local lesions, systemic necrotic spots and mottling	Yeh *et al*. (1992)

† The indicator species are in the order recommended for each virus.

‡ The names used in the table are the names mentioned in the references cited (e.g. *Lycopersicon esculentum* is used in the listed references while the accepted binomial name for tomato is *Solanum lycopersicum*).

3.2 Serological detection

3.2.1 Lateral flow tests

Lateral flow tests can be done on symptomatic material in the field and they provide results within a few minutes. However, there are no quantitative data available on the specificity, sensitivity or reliability of lateral flow tests, and false negatives and false positives may occur. Positive tests must be confirmed by additional serological or molecular tests.

Lateral flow tests are commercially available for TSWV and INSV and may be used to rapidly detect these viruses. No tests are currently available for WSMoV. The tests are designed for use with symptomatic material. Different formats are available from Agdia[2], Forsite Diagnostics[2] and Neogen[2], and the tests should be done according to these manufacturers' instructions. There is no positive or negative control; rather, there is an internal control to verify the test has performed as it should.

3.2.2 DAS-ELISA and TAS-ELISA

Double-antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) or triple-antibody sandwich (TAS)-ELISA should be performed using kits that have been assessed for their reliability and specificity. Some tests may cross-react with other tospoviruses. All tests should be done according to the manufacturer's instructions. ELISA is highly recommended for screening large numbers of samples.

Samples should be tested in duplicate using two wells on the microtitre plate, and with appropriate controls run alongside. Positive controls can be infected tissue or virus maintained in indicator plants (frozen at −80 °C or lyophilized). Negative controls should preferably be healthy plant material from the same species as that being tested as well as extraction buffer. A healthy negative control is important as certain plant extracts, for example *Fuchsia*, may give false positive results (Louro, 1996).

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The ELISA methodologies, including reagents, were validated in a European Union DIAGPRO test performance study (SMT 4-CT98-2252) (EPPO, 2004) with all laboratories accurately detecting TSWV and INSV (antisera source: Neogen-Adgen[1]) and WSMoV (antiserum source: DSMZ[1]). The respective antisera reacted only with the homologous virus species. Although test performance studies have been conducted, identification based on serological methods can be affected by cross-reactions (See Supplemental Data in Hassani-Mehraban *et al*., 2016).

3.2.3 Interpretation of ELISA results

The recommendations for the interpretation of ELISA results described below are based on the EPPO protocol PM 7/125 (1) (EPPO 2015).

The serological test will be considered valid only if:

- the positive controls included in the test produce the expected colour or colorimetric response
- and the negative controls included in the test produce a negative response and do not produce a response similar to the positive control.

The ELISA is considered positive if the average optical density (OD) value from each of the duplicate sample wells is ≥2× the OD value of the negative control of healthy plant extracts. When using polyclonal antibodies, it is essential that the negative controls are as similar as possible to the matrix tested in the same plate.

The ELISA is considered negative if the OD value from each of the duplicate sample wells is <0.1 or is <2× the OD value of the negative control of healthy plant extracts.

The test should be repeated when duplicate wells differ by more than 50% OD value.

3.3 Molecular detection

Molecular methods may be more expensive or time-consuming than serological methods, especially for large-scale testing. However, molecular methods are generally more sensitive than serological methods (see, for example, Chu *et al*. (2001)). The reverse transcription (RT)-polymerase chain reaction (PCR) method described in this diagnostic protocol enables the detection of TSWV, INSV or WSMoV using species-specific primers, or tospovirus species (including *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) as well as TSWV, INSV and WSMoV) using genus-specific primers. Liu *et al*. (2009) described primers for RT-PCR detection of INSV that target the nucleoprotein gene and generate an amplicon approximately 364 base pairs (bp) in size, but no data were provided on cycling parameters or specificity. The protocols described below give some indication of specificity.

Real-time RT-PCR methods have been published for TSWV but not for INSV or WSMoV. However, the specificity of the TSWV method published by Roberts *et al.* (2000) and Dietzgen *et al*. (2005) has not been reported, while the method of Boonham *et al*. (2002) cross-reacts with GRSV and TCSV. Detection of a tospovirus using real-time RT-PCR may result in an inability to confirm the identity of the virus using other methods because of the inherent sensitivity of real-time RT-PCR. If it is used as a confirmatory test then the issue of the lack of specificity of the real-time RT-PCR may not be a concern. The real-time RT-PCR method described by Boonham *et al*. (2002) has been used for monitoring the presence of viruliferous thrips, and can detect viruses even in individual thrips.

In addition, both Chen *et al.* (2012) and Hassani-Mehraban *et al.* (2016) described generic and specific primers for use in RT-PCR for the detection and/or identification of tospoviruses. This protocol provides the sequences of the generic primers that can be used for the detection of TSWV, INSV and/or WSMoV. Sequence analysis of the amplicons obtained by the tests described by Hassani-Mehraban *et al.* (2016) can be used for provisional identification of the species. The specific primers for TSWV, INSV and WSMoV described in the latter publication were used only to confirm the identity of isolates and have not been fully validated or optimized for routine use.

For molecular tests, plant extracts that are fresh or frozen (stored between −20 and −80 °C for periods of up to one year) can be used. Extraction of RNA should be done using the RNeasy Plant Mini Kit (Qiagen[1]), SV Total RNA Isolation System (Promega[1]) or any other appropriately validated protocol, according to the manufacturer's instructions.

3.3.1 Conventional RT-PCR

The generic primers of Mumford *et al.* (1996a) for tospoviruses are:

S1 UNIV-forward (F): 5′-TGT A (G/A) TG (T/G)TCCAT(T/A)GCA-3′

S2 UNIV-reverse (R): 5′-AGA GCA AT (T/C) GTG TCA-3′

The primers of Mumford *et al.* (1994) and (1996a) for TSWV (primers L1 and L2) and INSV (primers S1 and S2) are, respectively:

L1 TSWV-R: 5′-AAT TGC CTT GCA ACC AAT TC-3′

L2 TSWV-F: 5′-ATC AGT CGA AAT GGT CGG CA-3′

S1 INSV-F: 5′-AAA TCA ATA GTA GCA TTA-3′

S2 INSV-R: 5′-CTT CCT CAA GAA TAG GCA-3′

The primers of Chu *et al.* (2001) for WSMoV are:

WSMoV-NR: 5′-ACA GAA AGG TTA GCA CTG AA-3′

WSMoV-NF: 5′-ACA GAG GAC TCC ACT CCC GG-3′

The RT reaction is done in a microfuge tube containing 10 µl reaction mixture composed of: 0.2 µM reverse primer (S2 UNIV-R, L1 TSWV-R, S2 INSV-R or WSMoV-NR), 1 mM dNTPs, 2 µl of 5× M-MLV buffer, 100 U M-MLV reverse transcriptase, 0.5 U RNase inhibitor and 1 µl RNA sample. The cycling parameters are: 37 °C for 1 h.

Following RT, 40 µl of PCR reaction mixture is added to the tube. The mixture is composed of: 0.2 µM forward primer (S1 UNIV-F, L2 TSWV-F, S1 INSV-F or WSMoV-NF), 1.5 mM $MgCl_2$, 5 µl of 10× Taq polymerase buffer and 1.25 U Taq DNA polymerase. The reaction is performed under the following thermocycling parameters: 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 48 °C (S1 and 2 UNIV primers), 50 °C (WSMoV-NR/NF primers) or 55 °C (S1/S2 INSV and L1/L2 TSWV primers) and 1 min at 72 °C; followed by a final extension for 10 min at 72 °C. The PCR products are analysed by gel electrophoresis.

The S1/S2 INSV and L1/L2 TSWV primers produce a 602 bp and a 276 bp amplicon with INSV and TSWV, respectively. The WSMoV-NR/NF primers produce a 700 bp amplicon with WSMoV. The generic S1/S2 UNIV primers produce an 871 bp amplicon with TSWV, INSV and other tospoviruses, or a 933 bp amplicon with WSMoV.

Broad-spectrum degenerate primers of Chen *et al.* (2012) for *Tospovirus*:

gM410-F: 5′-AAC TGG AAA AAT GAT T(T/C) (A/T/C/G) (T/C) TTG TTG G-3′

gM870c-R: 5′-ATT AG(C/T) TTG CA(T/G) GCT TCA AT(A/T/G/C) AA(A/G)G C-3′

First strand complementary DNA (cDNA) synthesis is carried out at 50 °C for 30 min and terminated by heating at 94 °C for 2 min followed by PCR amplification carried out as follows: 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 10 min.

The degenerate primers gM410-F and gM870c-R were designed based on the NSm gene sequences of a range of tospoviruses including TSWV, INSV and WSMoV and they amplify a 0.5 kb fragment. All tospoviruses included in the study, except peanut chlorotic fan-spot virus (PCFV), were detected including the viruses targeted in this protocol. No amplification was observed with healthy controls or with non-tospoviruses included in the study (Chen *et al.*, 2012).

Primers for generic detection of American clade 1 tospoviruses (including TSWV and INSV; Hassani-Mehraban *et al.*, 2016):

AM1-F: 5′-GGG GGA TCC AGA GCA ATT GTG TC-3′

AM1-R: 5′- CTT TGC TTT TCA GCA CAG TGC A-3′

Primers for generic detection of Asian clade 1 tospoviruses (including WSMoV; Hassani-Mehraban *et al.*, 2016):

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′

Following RNA extraction, 1 µl RNA extract is added to the following reaction mixture: 16.0 µl water, 5 µl One-step RT-PCR buffer (Qiagen[1]), 1 µl dNTPs (10 mM each), 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl One-step RT-PCR enzyme mix (Qiagen[1]).

Reverse transcription is done at 50 ˚C for 30 min; followed by denaturation at 95 ˚C for 15 min; then 35 cycles consisting of denaturation at 94 ˚C for 30 s, annealing at 50 ˚C (American clade 1 primers) or 52 ˚C (Asian clade 1 primers) for 30 s, elongation at 72 ˚C for 60 s; terminal elongation at 72 ˚C for 5 min; then maintained at 20 ˚C.

American clade 1 and Asian clade 1 primers will produce amplicons of approximately 760 and 370 bp, respectively.

In the DIAGPRO test performance study laboratories detected TSWV, INSV and WSMoV accurately, but there were insufficient molecular data to compare detection with the serological tests. The specificity of the molecular tests has been evaluated by Mumford *et al.* (1996a) and Chu *et al*. (2001). Mumford *et al.* (1996a) showed that the primers S1 INSV-F and S2 INSV-R were specific under the conditions of the study for INSV and did not cross-react with TSWV, TCSV or GRSV. Hassani-Mehraban *et al*. (2016) listed at least 29 tospovirus species, not all of which are officially recognized by the International Committee on Taxonomy of Viruses (http://www.ictvonline.org/virusTaxonomy.asp) and not all of them were tested for cross-reactions by Mumford *et al.* (1996a). The broad-spectrum degenerate primers described by Chen *et al.* (2012) were able to detect isolates of TSWV, INSV, WSMoV and other tospoviruses. Species identification was possible by restriction fragment length polymorphism (RFLP) analysis or sequence analysis of the amplicon. The American clade 1 and Asian clade 1 primers described by Hassani-Mehraban *et al.* (2016) also have been shown to detect isolates of TSWV, INSV and WSMoV, respectively. Provisional species identification was possible by sequence analysis of the amplicons.

3.3.2 Real-time RT-PCR

The real-time RT-PCR described by Boonham *et al*. (2002) was used to detect all isolates of TSWV included in the analysis. Positive results were observed also with the tospoviruses TCSV and GRSV, but no reactions were observed with INSV, WSMoV, *Iris yellow spot virus* (IYSV) or *Chrysanthemum stem necrosis virus* (CSNV). The total volume of the reaction was 25 µl, and reactions were carried out in 96-well reaction plates using the TaqMan EZ RT-PCR Kit (PE Biosystems[1]), but with the addition of 25 U M-MLV reverse transcriptase (Mumford *et al.*, 2000).

Cetyl trimethylammonium bromide (CTAB) extraction was carried out as described by Boonham *et al*. (2002). Leaf tissue (100–200 mg) was ground to a fine powder in liquid nitrogen using a mortar and pestle then placed in a sterile microcentrifuge tube. The ground tissue was mixed with 1 ml homogenizing buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 1% Na_2SO_3, 2% polyvinylpyrrolidone (PVP)-40). After incubation at 65 °C for 10 min, two chloroform:isoamyl alcohol (24:1) extractions were carried out. RNA was precipitated out of the aqueous layer by combination with an equal volume of 4 M LiCl, incubation overnight at 4 °C, and centrifugation for 30 min. The pellet was resuspended in 200 µl Tris-EDTA (TE) buffer containing 1% sodium dodecyl sulfate (SDS). To this was added 100 µl of 5 M NaCl and 300 µl ice-

cold isopropanol, then the suspension was incubated at −20 °C for 30 min. Following a 10 min centrifugation the pellet was washed with 70% ethanol, re-pelleted and dried.

After CTAB extraction the final pellet was resuspended in 50 µl diethylpyrocarbonate (DEPC)-treated water, and 1 µl RNA was used to prepare the final volume of 25 µl for the reaction (Mumford *et al*., 2000). Plates were cycled at 48 °C for 30 min, 95 °C for 10 min, and 40 cycles of 60 °C for 1 min and 95 °C for 15 s. Using suitable positive and negative controls each laboratory or user should validate the cycle threshold (Ct) values that represent a positive result. When positive results are obtained, TSWV-specific primers may be used to confirm identity as can RFLP analysis or sequence analysis of amplicons obtained by conventional RT-PCR.

Primers:

TSWV-CP-17-F: 5′-CTC TTG ATG ATG CAA AGT CTG TGA-3′

TSWV-CP-100-R: 5′-TCT CAA AGC TAT CAA CTG AAG CAA TAA-3′

Probe:

TSWV-CP-73T: FAM-5′-AGG TAA GCT ACC TCC CAG CAT TAT GGC AAG-3′TAMRA

3.3.3 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For RT-PCR a positive nucleic acid control, an internal control, a negative amplification control (no template control) and a negative extraction control are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction) and, with RT-PCR, the amplification. Pre-prepared (stored) virus-derived nucleic acid, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used.

Internal control. For conventional and real-time PCR, plant internal controls (e.g. a housekeeping gene (HKG) such as mitochondrial *nad5* (*NADH dehydrogenase 5*), or the ribosomal RNA gene) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors. The internal control primers should preferably be used in a duplex reaction with the target virus primers. However, because this may be difficult to achieve without reducing the sensitivity of the test, it is recommended, where practical, to run a duplex reaction of the virus primers with the HKG primers and also a simplex reaction with only the virus primers. Alternatively two separate simplex reactions (one for the plant marker and one for the target virus) may be performed. An RT-PCR using internal control primers (primers designed to detect a sequence conserved in plants such as the 5S ribosomal RNA gene (Kolchinsky *et al.* (1991)) may be used to confirm that RNA of sufficient quality for amplification has been extracted.

The *NADH dehydrogenase 5* gene fragment has been shown to be a reliable indicator of the performance of the extraction procedure and RT step for conventional RT-PCR (Menzel *et al*., 2002). The *nad5* primers are: sense, 5′-GAT GCT TCT TGG GGC TTC TTG TT-3′; and antisense, 5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′. The primers have been tested against many plant species, including *S. tuberosum* and other *Solanum* species (*S. bonariense*, *S. dulcamara*, *S. jasminoides*, *S. nigrum*, *S. pseudocapsicum*, *S. rantonnetii*, *S. sisymbriifolium*), *Acnistus arborescens*, *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Iochroma cyanea*, *Nicotiana* spp. and *Physalis* spp. (Seigner *et al*., 2008).

When an internal control is not mentioned in the description of a PCR method, the laboratory should choose an internal control and validate it.

Negative amplification control (no template control). This control is necessary for conventional and real-time RT-PCR to rule out false positives due to contamination during preparation of the reaction

mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Positive extraction control. This control is used to ensure that nucleic acid from the target virus is of sufficient quantity and quality for RT-PCR. Viral nucleic acid is extracted from known infected host tissue or healthy plant tissue that has been spiked with the virus. This helps validate the extraction procedure, ensuring that if the target virus is present in the plants being tested detection should occur.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the RNA extraction. If bulking of samples is done then the quantity of positive control should be adjusted accordingly (e.g. if ten lots of 20 mg sample are bulked for RNA extraction, then the positive control should consist of 2 mg infected leaf + 198 mg healthy plant tissue). If this is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved.

For RT-PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. The positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. It is possible that the control and the PCR amplicon may have the same sequence even in the absence of contamination, particularly if the target region is conserved. Alternatively, synthetic positive controls can be made with a known but unusual sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. If suitable uninfected host tissue is not available clean extraction buffer may be used. It is recommended that multiple controls be included when large numbers of positive samples are expected.

3.3.4 Interpretation of PCR results

For both conventional PCR and real-time PCR, the pathogen-specific PCR will be considered valid only if:

- the positive control produces a product of the correct size for the virus
- the negative extraction control and the negative amplification control do not produce a product of the correct size for the virus.

If the *nad*5 internal control primers are used, the negative extraction control, the positive extraction control (if used) and each of the test samples must produce a 181 bp amplicon (*nad*5). Failure of the samples to amplify with the internal control primers suggests, for example, that the RNA extraction has failed, the nucleic acid has not been included in the reaction mixture, the RT step has failed, compounds inhibitory to PCR are present in the RNA extract, or the RNA or DNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size.

Using real-time RT-PCR Roberts *et al*. (2000) showed that TSWV can be detected reliably in as little as 500 fg total RNA, and the method is approximately ten-fold more sensitive than detection by agarose gel analysis of amplicons with ethidium bromide staining. The real-time RT-PCR assay described by Dietzgen *et al*. (2005) was able to detect TSWV in a bulked sample of 1 infected leaf in 1 000 uninfected leaves, while ELISA could detect only 1 in 200 or 1 in 800, depending on the host.

4. Identification

As described in section 3.1, herbaceous indicators may be used for virus identification but at least two plant species and at least two plants per species should be used. In addition, positive and negative controls should be included in the test.

ELISA-based methods may be used for identification. As described in section 3.2.2, in an EU DIAGPRO test performance study all participating laboratories were able to accurately detect TSWV, INSV and WSMoV using the appropriate antiserum. Confirmation using a second method is recommended due to potential cross-reactions as described by Hassani-Mehraban *et al*. (2016).

As described in section 3.3.1, under the conditions of the validation studies, the primer pairs used for RT-PCR each produce an amplicon of a distinct size that can be used to identify the virus present in a sample. The amplicons may be sequenced to confirm identification, especially in situations where the virus is detected for the first time.

Real-time RT-PCR is not being recommended for identification because the specificity of the methods described by Roberts *et al*. (2000) and by Dietzgen *et al*. (2005) is unknown, while the method of Boonham *et al*. (2002) cross-reacts with GRSV and TCSV.

When positive and negative controls give the expected results, sequence analysis of the PCR product is usually not necessary except to specifically identify tospoviruses amplified using generic primers. Sequencing should also be done when an NPPO requires additional confidence in the result; for example, detection of a pest in an area where it is not known to occur. The International Committee on Taxonomy of Viruses states that when the nucleocapsid (N) protein sequence shows less than 90% amino acid identity, a different tospovirus species is indicated (Plyusnin *et al*., 2012).

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the virus is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- the original sample (labelled appropriately), kept frozen at −80 °C or lyophilized and kept at room temperature (note that lyophilization will affect viability)
- RNA extractions and RT-PCR amplification products, if relevant, kept at −80 °C.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Plant Pest and Disease Programme, Fera Science Limited, Sand Hutton, York, Y041 1LZ, United Kingdom (http://fera.co.uk/plantClinic/index.cfm; tel.: +44 1904 462000; fax: +44 1904 462111).

Department of Entomology, University of Wisconsin, 237 Russell Labs, 1630 Linden Drive, Madison, WI 53706, United States of America (Thomas German; e-mail: tlgerman@wisc.edu; tel.: +1 608 262 2956; fax: +1 608 262 3322).

A request for a revision to a diagnostic protocol may be submitted by NPPOs, regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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