Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/34

Organization Européenne et Méditerranéenne pour la Protection des Plantes
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Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use, etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all diagnostic protocols:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards 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 or that proper positive and negative controls are included.

References

EPPO/CABI (1996) *Quarantine Pests for Europe*, 2nd edn. CAB International, Wallingford (GB).

EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

FAO (1997) *International Plant Protection Convention* (new revised text). FAO, Rome (IT).

IPPC (1993) *Principles of plant quarantine as related to international trade*. ISPM no. 1. IPPC Secretariat, FAO, Rome (IT).

IPPC (2002) *Glossary of phytosanitary terms*. ISPM no. 5. IPPC Secretariat, FAO, Rome (IT).

OEPP/EPPO (2003) EPPO Standards PM 1/2 (12): EPPO A1 and A2 lists of quarantine pests. *EPPO Standards PM1 General phytosanitary measures*, 5–17. OEPP/EPPO, Paris.

Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest : a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Diagnostic Protocols for Regulated Pests provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Nineteen EPPO standards on diagnostic protocols have already been approved and published. Each standard is numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69
PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77
PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253
PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259
PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265
PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275
PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279
PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292
PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298
PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310
PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256
PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264
PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270
PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280
PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288
PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

Several of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'intercomparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostic protocols for regulated pests[1]
Protocoles de diagnostic pour les organismes réglementés

Tomato spotted wilt tospovirus, *Impatiens necrotic spot tospovirus* and *Watermelon silver mottle tospovirus*

Specific scope

This standard describes a diagnostic protocol for *Tomato spotted wilt tospovirus*, *Impatiens necrotic spot tospovirus* and *Watermelon silver mottle tospovirus*.

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2003-09.

Introduction

The *Tospovirus Tomato spotted wilt virus* (TSWV) was first described in Australia (Brittlebank, 1919) and its viral etiology presented (Samuel *et al*., 1930). TSWV is now classified as the type member of its genus (family *Bunyaviridae*), which to date includes 13 species (van de Wetering, 1999), including the *Tospoviruses Impatiens necrotic spot virus* (INSV) and *Watermelon silver mottle virus* (WSMoV). *Tospovirus* isolates were originally identified based on serological differences and a serogroup classification system was established (de Avila *et al*., 1990) in which TSWV is the sole member of serogroup I, INSV is the sole member of serogroup III, and WSMoV is a member of serogroup IV. *Tospovirus* spp. are further classified based on molecular data (de Avila *et al*., 1993). They are all transmitted and spread in nature by thrips (Thysanoptera: *Thripidae*) which acquire virus during larval stages and transmit virus via the adults. However, less is known of the vector-virus relationship for WSMoV than for TSWV and INSV (OEPP/EPPO, 1999). These viruses have quasi-spherical enveloped particles 70–110 nm in diameter (OEPP/EPPO, 1999).

Identity

Name: *Tomato spotted wilt tospovirus*
Acronym: TSWV
Taxonomic position: Bunyaviridae, *Tospovirus*
Bayer computer code: TSWV00
Phytosanitary categorization: EPPO A2 list: No. 290, EU Annexes: I/B and II/A2

Name: *Impatiens necrotic spot tospovirus*
Acronym: INSV
Taxonomic position: Bunyaviridae, *Tospovirus*
Bayer computer code: INSV00
Phytosanitary categorization: EPPO A2 list: No. 291, EU Annexes: not specifically listed, but not distinguished at the time TSWV was included in Annexes I/B and II/A2.

Name: *Watermelon silver mottle tospovirus*
Acronym: WSMOV
Taxonomic position: Bunyaviridae, *Tospovirus*
Phytosanitary categorization: WSMoV EPPO A1 list: No. 294, EU Annexes: not specifically listed, but not distinguished at the time TSWV was included in Annexes I/B and II/A2)

Biology

Tomato spotted wilt tospovirus (TSWV)

TSWV is one of the most widespread and economically important plant viruses (Goldbach & Peters, 1994). It occurs in countries within the EPPO region, Asia, Africa, N. America, C. America and the Caribbean, S. America and Oceania (OEPP/EPPO, 1999). Of the TSWV insect vectors cited, the most important is *Frankliniella occidentalis* Pergande, which transmits TSWV in a persistent propagative fashion (Gera *et al*., 2000). Other vector(s) of TSWV are also important in some countries both within and outside Europe (Mumford *et al*., 1996a; OEPP/EPPO, 1999; Chatzivassiliou *et al*., 2000). TSWV is considered not to be seed-transmitted.

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Impatiens necrotic spot tospovirus (INSV)

INSV occurs where *F. occidentalis* is present as a vector (Naidu *et al*., 2001) and causes damage and losses akin to those of TSWV, largely on ornamental hosts, but also some vegetable crops (Vicchi *et al*., 1999). INSV has not often been reported on outdoor crops, but the detection of INSV in tomato in Italy represents a further step in what seems to be a progressive adaptation of INSV to outdoor vegetable crops (Finetti & Gallitelli, 2000). INSV has a more restricted geographic distribution than TSWV within the EPPO and EU region, Asia, N. America, Central America and Caribbean (OEPP/EPPO, 1999). INSV is not reported to be seed-transmitted.

Watermelon silver mottle tospovirus (WSMoV)

WSMoV naturally infects *Cucurbitaceae*, and experimentally infects other hosts including tomato (Yeh *et al*., 1992). It is spread naturally with its vector *Thrips palmi* (OEPP/EPPO, 1999). The virus currently has a restricted geographic distribution occurring in India, Japan and Taiwan. It causes significant losses in watermelon (Yeh & Chang, 1995). WSMoV is not present in the EU, but eradicated outbreaks of its vector *T. palmi* (Fang-Hua Chu *et al*., 2001) have been reported in the Netherlands (EPPO/CABI 1997) and the UK (OEPP/EPPO, 2001). WSMoV is not reported to be seed-transmitted.

Vectors

Vectors cited for the Tospoviruses in this protocol (OEPP/EPPO, 1999a–c) are as follows

TSWV – besides *F. occidentalis* (already mentioned), *Thrips tabaci* Lindeman, *Thrips setosus* Moulton, *Frankliniella fusca* Hinds, *Frankliniella intonsa* Trybom, *Frankliniella schultzei* Trybom and *Scirtothrips dorsalis* Hood.

INSV – *F. occidentalis*, *Frankliniella fusca*.

WSMoV – *T. palmi*.

Principal hosts

TSWV

TSWV infects at least 900 plant species, with the number of natural host species recorded steadily increasing (Peters, 1998). It occurs in ornamental, vegetable and weed hosts (OEPP/EPPO, 1999). The principal vegetable and industrial host crops in the EPPO region (EPPO/CABI, 1997; OEPP/EPPO, 1999b) are tomato, tobacco, lettuce, faba bean, capsicum, chicory, potato, aubergine and artichoke. The principal ornamental plants (OEPP/EPPO, 1999) are: *Alstroemeria*, *Anemone*, *Antirrhinum*, *Araceae*, *Aster*, *Begonia*, *Bouvardia*, *Calceolaria*, *Callistephus*, *Celosia*, *Cestrum*, *Columnea*, *Cyclamen*, *Dahlia*, *Dendranthema* × *grandiflorum*, *Eustoma*, *Fatsia japonica*, *Gazania*, *Gerbera*, *Gladiolus*, *Hydrangea*, *Impatiens*, *Iris*, *Kalanchoe*, *Leucanthemum*, *Limonium*, *Pelargonium*, *Ranunculus*, *Saintpaulia*, *Senecio cruentus*, *Sinningia*, *Tagetes*, *Verbena*, *Vinca* and *Zinnia*. 104 plant species were found to be infected with TSWV in the Netherlands (Verhoeven & Roenhorst, 1998). TSWV and INSV often occur together (OEPP/EPPO, 1999a).

INSV

INSV currently has a much more restricted host range than TSWV, but the number of natural host species recorded is steadily increasing (Roggero *et al*., 1999). It is much more frequently found on ornamental crops than on vegetables (OEPP/EPPO, 1999a) which include: *Impatiens*, *Aconitum*, *Alstroemeria*, *Anemone*, *Antirrhinum*, *Begonia*, *Bouvardia*, *Callistephus*, *Columnea*, *Cyclamen persicum*, *Dahlia*, *Dendranthema* × *grandiflorum*, *Eustoma grandiflorum*, *Exacum affine*, *Fatsia japonica*, *Gerbera*, *Gladiolus*, *Limonium*, *Lobelia*, *Pittosporum*, *Primula*, *Ranunculus*, *Senecio cruentus*, *Sinningia speciosa*, and *Zantedeschia aethiopica*. Vegetable hosts (OEPP/EPPO, 1999; Finetti *et al*., 2000) include: *Capsicum annuum*, *Cichorium endivia*, *Cucumis sativus*, *Lactuca sativa*, *Ocimum basilicum*, *Valerianella olitoria* and *Lycopersicon esculentum*. 41 plant species were identified to be infected with INSV in the Netherlands (Verhoeven & Roenhorst, 1998).

WSMoV

The principal hosts of WSMoV are watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) (OEPP/EPPO, 1999c). These crops are widely grown in Mediterranean countries and in northern countries in glasshouses. WSMoV presents a clear risk to these crops (OEPP/EPPO, 1999c).

Detection

Symptoms

TSWV can induce a wide variety of symptoms on economically important ornamental plants, vegetables and industrial crops (EPPO/CABI, 1997) including: necrotic or chlorotic local lesions, ring spots, ring spots in concentric rings, green island mosaic, stem discoloration, line patterns, wilting, stunting, mottling, bronzing, distortion, chlorosis, necrosis which may vary on the same host species (Web Figs 1, 2 and 3). Variables affecting symptom expression include the cultivar, age, and nutritional and environmental conditions of the plant, and differences between different isolates of TSWV on the same hosts (OEPP/EPPO, 1999; Mumford *et al*., 1996a). The range of symptoms for INSV is similar to that of TSWV and these viruses can occur together (OEPP/EPPO, 1999). Some symptoms are given below for TSWV (OEPP/EPPO, 1999), INSV and principal WSMoV hosts. Given the wide host range, and variables affecting symptom expression discussed, it is not feasible to give a comprehensive symptom list in this protocol for all TSWV and INSV host plants. Further symptom descriptions are given by Daughtrey (1996), Chatzivassiliou *et al*. (2000), Lisa *et al*. (1990), Yeh *et al*. (1992).

TSWV

On tomato, leaf symptoms include bronzing, curling, necrotic spots, necrotic streaks, stunting. Fruit symptoms are usually either irregular

yellow/orange flecks and occasionally rings on red fruits, or necrotic lesions or rings. Ripe fruits show paler red or yellow areas on the skin. Sometimes affected plants are killed by severe necrosis

On capsicum, symptoms include stunting and yellowing of the plant, chlorotic line patterns or mosaic with necrotic spots on leaves, necrotic streaks on stems extending to terminal shoots and yellow target spots or necrotic streaks may be observed on ripe fruits.

On lettuce, symptoms include leaf discoloration and one-sided growth. On tobacco, symptoms include necrotic lesions, necrotic rings, chlorotic rings. On aubergine, symptoms include necrotic lesions on leaves. On faba bean, symptoms include necrotic lesions on leaves.

INSV

On New Guinea *Impatiens* hybrids, symptoms include stunting, leaf spots and black discoloration at leaf bases.

WSMoV

On watermelon, symptoms include foliar mottling, crinkling, yellow spotting and narrowed leaf laminae, small malformed fruits with necrotic spots or silver mottling, reduced fruit set and upright growth of branches and tip necrosis. On melon, symptoms include foliar mottling, stunting, upright growth of branches and tip blight. Systemic symptoms are induced in experimental hosts including tomato and cucumber (Yeh *et al*., 1992).

Sampling

Indicator Plants

The sampling strategy recommended for TAS-ELISA is also recommended for selection of material for inoculation of indicator plants. The use of indicator plants is an essential aid to reliable tospovirus diagnosis due to uneven virus distribution in plant hosts. It is thus recommended that indicator plants as recommended in this protocol (Appendix 2) as diagnostic hosts for TSWV, INSV and WSMoV are inoculated from the original host plant with symptoms or suspect, at an early stage, before deterioration of the sample occurs.

Lateral Flow Tests

Whole or parts of leaves can be used with the extraction bottle. It is recommended that fresh, young expanded leaf material is used and not senescent material. The tests are designed to detect TSWV or INSV in symptomatic material (see pathogen key card provided with the lateral flow device test kits). When sampling a plant it is advisable to take tissue from several locations on the plant, in case of uneven distribution.

ELISA

TSWV, INSV and WSMoV tend to be unevenly distributed in natural hosts. Symptomatic plant material (leaves, stems) should be sampled where possible, but not senescent material. Expanded young leaves tend to have more detectable virus than older plant parts.

RT-PCR/MPCR

The sampling strategy recommended for ELISA is also recommended for selection of plant material for nucleic acid extraction.

Identification

Sample preparation

See Appendix I. For RT-PCR and MRT-PCR, it is recommended to include internal control primers to confirm RNA extraction (Appendix 3). The CTAB RNA extraction method recommended by Boonham *et al*. (2001) is best suited to the broad range of tospovirus hosts detailed in this protocol. Alternatives given in this protocol (Appendix 1), on the basis of good RNA yield and ease of use, comprise the methods of (Logemann *et al*., 1987) and a commercial kit for RNA extraction.

Screening tests

Detailed protocols as well as material for testing and indicator plants are described in Appendices 1–6. A decision scheme is presented in Fig. 4.

Indicator plants

Mechanical inoculation of appropriate indicator plants selected from those listed in Appendix 3 is carried out according to the method described in Appendix 1. Symptoms develop within 7 days on indicator plants inoculated with TSWV or INSV grown at 20 °C, and with WSMoV at 20–25 °C.

Serological Tests

TAS-ELISA and Lateral Flow Devices (LFD) are rapid methods for screening tospovirus samples. The TAS-ELISA methods employed in this protocol allow the specific detection of TSWV, INSV or WSMoV. For a serological comparison of tospovirus isolates using other commercially available antisera, refer to Adam *et al*. (1996). LFDs allow rapid detection (within minutes) of TSWV or INSV. Positive LFD results should be confirmed by ELISA or PCR-based methods. The sensitivity of detection of TAS-ELISA and LFD serological tests is not as great as that of PCR-based methods, so RT-PCR/MPCR should also be used where tospoviruses are suspected to be present but are not detected by serological tests in the original host (at low concentration in plant hosts, or at an early stage of infection). Positive controls (infected plant material, preferably hosts of the same species as the test plants where available) and negative controls (healthy plant material and buffer) should be included if possible. The use of healthy controls is important as certain plant extracts, e.g. *Fuchsia* may give false positive results (Louro, 1996). A buffer-only control should also be included. The ELISA value of the sample should be twice or more than that of the negative control.

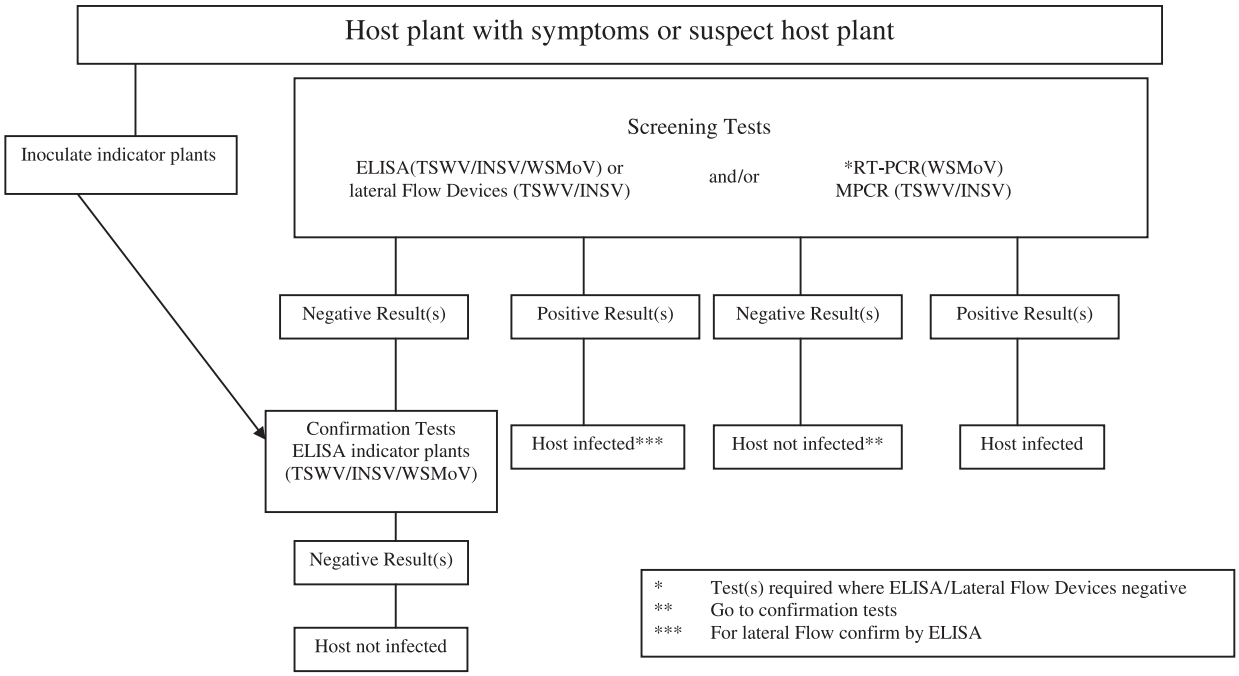

Fig. 4 Decision scheme for the detection and identification of Tospoviruses (*Tomato spotted wilt virus*, *Impatiens necrotic spot virus*, *Watermelon silver mottle virus*).

RT-PCR/MRT-PCR

RT-PCR/MRT-PCR methods for the detection of TSWV, INSV or WSMoV are carried out as described in Appendix 1. PCR-based methods are more sensitive than TAS-ELISA and are suitable for detection of tospoviruses present at low concentration in plant hosts. Methods given in this protocol enable both specific RT-PCR/MRT-PCR identification of TSWV, INSV or WSMoV or MRT-PCR using universal degenerate primers which provides a broad screening test for tospovirus species in serogroups I (TSWV), II (GRSV,TCSV), III (INSV) and (IV) WSMoV.

Confirmation

Where ELISA tests (TAS-ELISA-Appendix 1) for TSWV, INSV or WSMoV are positive, specific virus presence is confirmed. Where RT-PCR/MRT-PCR tests are positive, specific virus presence is confirmed. Confirmation of WSMoV where a positive test is achieved currently also requires sequencing to confirm a first record in Europe. Confirmation of TSWV, INSV and WSMoV species where serological screening test(s) (Appendix 1), and RT-PCR/MRT-PCR tests of original plant host material are negative, is achieved by ELISA testing of indicator plants using TAS-ELISA as described in this protocol. The use of indicator plants is an essential aid to reliable tospovirus diagnosis due to uneven virus distribution in plant hosts.

Possible confusion with similar species

None.

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 4, should have been followed. Where positive results are achieved using ELISA or molecular tests described in this protocol, the sample is positive. Positive LFD results should be confirmed by ELISA or PCR-based methods. Where positive results are achieved using ELISA tests for indicator plants, the sample is positive (but infection of indicator plants from the original host material is not always successful). Where negative results are obtained using serological tests for samples from the original host, appropriate PCR-based tests should be applied to the original host. This procedure aids positive identification where virus is at low titre in the original host.

Where original host material is screened by RT-PCR/MRT-PCR methods, there is a possibility of false negative results due to virus distribution in the original host. The material should be inoculated to indicator plants, and virus presence or absence confirmed by ELISA testing of the indicator plants.

Report on the diagnosis

The report on the execution of the protocol should include:

- results obtained by the recommended procedures
- information and documentation on the origin of the infected material
- a description of the disease symptoms (with photographs if possible)
- an indication of the magnitude of the infection

- comments as appropriate on the certainty or uncertainty of the identification.

Further information

Further information on this organism can be obtained from: J. Morris, Central Science Laboratory, Virology team PLH6, Sand Hutton, York, Y041 1LZ, UK. E-mail: jane.morris@csl.gov.uk

Acknowledgements

This protocol was originally drafted by: J. Morris, Central Science Laboratory, Virology team PLH6 Sand Hutton, York, Y041 1LZ, UK.

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Appendix 1 Materials

Materials for tests

TAS-ELISA

10× Phosphate buffered saline solution (PBS): NaCl 80 g (136 mM); KH_2PO_4 2 g (1.5 mM); $Na_2HPO_4 \cdot 12H_2O$ 29 g

(9 mM); KCl 2 g (2.7 mM); distilled water to 1 L. This concentrate gives pH 7.4 at × 1.

Extraction buffer (PBS-T): used for tissue maceration, and microtitre plate washes (Clark & Adams, 1977). 10× PBS 100 mL; 10% Tween 20 5 mL (0.05%); distilled water 895 mL. Mix well.

Carbonate coating buffer pH 9.6: Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; distilled water to 1 L. Check pH. Store at 4 °C.

Antibody buffer: PBS-T 100 mL; 5% dried milk powder or 0.2% bovine serum albumin. Prepare on day of usage.

Substrate buffer: diethanolamine 95 mL; distilled water 800 mL. Mix and adjust to pH 9.8 with concentrated HCl. Make up to 1 L with distilled water. Add 0.02% $MgCl_2$. Store at 4 °C. Add substrate (disodium p-nitrophenyl phosphate) to microtitre plates at 1 mg/mL dissolved in substrate buffer.

TSWV antisera: trapping antiserum (rabbit polyclonal) – use at 1 : 1000 (ADGEN-1071-01); detecting antiserum (TSWV monoclonal) – use at 1 : 100 (ADGEN-1071-03); rabbit antirat AP conjugate (SIGMA) – use at 1 : 5000.

INSV antisera: trapping antiserum (rabbit polyclonal) – use at 1 : 1000 (ADGEN-1028-01); detecting antiserum (INSV monoclonal) – use at 1 : 100 (ADGEN-1028-03); rabbit anti-mouse AP conjugate (SIGMA) – use at 1 : 5000.

WSMoV antisera: trapping antiserum (rabbit polyclonal) – use at 1 : 1000 (DSMZ kit T-0118) or prefix D for DAS-ELISA kit; detecting antiserum (WSMoV monoclonal) – use at 1 : 500 (DSMZ kit T-0118-MAb 1B4); rabbit antimouse AP conjugate (SIGMA) – use at 1 : 5000.

Lateral Flow Devices: INSV 14-768 (10 tests) or 14-769 (100 tests) (ADGEN); TSWV 14-766 (10 tests) or 14-767 (100 tests) (ADGEN).

Indicator plants

TSWV

Selected susceptible host species and symptoms (Plant Viruses Online http://image.fs.uidaho.edu/vide/, van de Wetering, 1999; Gera *et al*., 2000): *Cucumis sativus* – chlorotic spots with necrotic centres in cotyledons, not systemic; *Petunia × hybrida* – necrotic local lesions, not systemic; *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Nicotiana tabacum* (Web Fig. 6), *Nicotiana rustica* – necrotic local lesions, systemic necrotic patterns and leaf deformation; *Impatiens* – chlorotic to necrotic spots or rings on inoculated leaves, systemic chlorotic to necrotic spots; *Datura stramonium* – chlorotic and necrotic spots and rings on inoculated leaves, systemic mosaic and mottle; *Nicotiana benthamiana* – chlorotic to necrotic ring spots on inoculated leaves, systemic chlorosis, stunting; *Lycopersicon esculentum* – chlorotic to necrotic spots and rings on inoculated leaves, systemic mosaic, systemic chlorosis and necrotic spotting.

INSV

Selected susceptible host species and symptoms (Brunt *et al*., 1996): *Impatiens* (as for TSWV); *N. benthamiana* (as for TSWV; Web Fig. 7); *D. stramonium* – local lesions (some isolates); *P. hybrida*: small necrotic spots on inoculated leaves (not systemic); *L. esculentum* – variable between isolates; lesions on inoculated leaves only.

WSMoV

Systemic hosts (Yeh *et al*., 1992): *D. stramonium*, *N. benthamiana* (Web Fig. 8). Local lesion host, not systemic: *P. hybrida*. Other experimentally susceptible hosts (Yeh *et al*., 1992; all systemic except *C. amaranticolor* and *C. quinoa*): *Citrullus lanatus*, *Cucumis metuliferus*, *Cucumis sativus*, *D. stramonium*, *L. esculentum*, *N. glutinosa*, *N. rustica*, *Gomphrena globosa*, *Chenopodium amaranticolor*, *Chenopodium quinoa*.

RT-PCR/MRT-PCR

Oligonucleotide Primer Sequences (Mumford *et al*., 1996b):

Degenerate primers for the detection of TSWV, INSV and WSMoV

S1 UNIVF 5′-TGT A (G/A) TG (T/G)TCCAT(T/A)GCA-3′

S2 UNIVR 5′-AGA GCA AT (T/C) GTG TCA-3′

Specific primers for the detection of TSWV

L1 TSWVR 5′-AAT TGC CTT GCA ACC AAT TC-3′

L2 TSWVF 5′-ATC AGT CGA AAT GGT CGG CA-3′

Specific primers for the detection of INSV

S1 INSVF 5′-AAA TCA ATA GTA GCA TTA-3′

S2 INSVR 5′-CTT CCT CAA GAA TAG GCA-3′

Specific primers for the detection of WSMoV (Chu *et al*., 2001)

H1R 5′-ACA GAA AGG TTA GCA CTG AA-3′

H2F 5′-ACA GAG GAC TCC ACT CCC GG-3′

Internal control primers (Universal plant 5SrRNA primers)

PLANT-UNI F TTT AGT GCT GGT ATG ATC GC

PLANT-UNI R TGG GAA GTC CTC GTG TTG CA

Universal plant 5SrRNA primers, from Kolchinsky *et al*. (1991).

RNA extraction method

This follows Logemann *et al*. (1987). Extraction buffer: 4 M guanidine thiocyanate; 20 mM MES (2(N-morpholino) ethanesulphonic acid) pH 7.0; 20 mM EDTA; 50 mM 2-mercaptoethanol; 1% polyvinylpyrrolidone (w/v). This buffer does not need to be autoclaved. Alternatively the Promega SV RNA kit used as per kit instructions (Promega) is also recommended.

Detection of TSWV from plant samples and thrips (Boonham *et al*., 2001)

TSWV oligonucleotide primers and probe designed within the conserved regions of the TSWV nucleoprotein gene:

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

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

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

Thrips primers and probe (Boonham *et al*., 2001) – designed within the actin gene:

WFT-RNA-25F WFT-actin TCT GGT G-3′	5′-GGT ATC GTC CTG GAC
WFT-RNA-93R WFT-actin-C TTC A-3′	5′-GGG AAG GGC GTA ACC
WFT-RNA-48T WFT-actin TGT CCC CA-3′	5′-CGG TGT CTC CCA CAC

Appendix 2 Detection and identification methods

Triple Antibody Sandwich TAS-ELISA

Extract saps in grinding buffer (PBS-T) 1 : 10 (w/v). Pipette 100 µL of homogeneous sample into a pair of wells on the microtitre plate for testing after step 2.2 of the ELISA test methods below. Conserve the remainder of the extract at 4 °C until testing is completed.

This ELISA, based on the method of Clark & Adams (1977), employs polyclonal antisera to trap the virus and monoclonal antisera to detect the virus (Appendix 3). Microtitre plates (Nunc Maxisorp Immunoplate) are used. Known infected plants are used as positive controls together with healthy plants of the same species as the test plants, where practicable, as negative control.

Coat microtitre plate(s), 100 µL per well, with appropriate polyclonal antisera diluted at a predetermined rate (Appendix 3) in coating buffer. Cover plates and incubate at 33 °C for 3 h. Flick out the contents of the wells. Wash the wells three times with PBS – Tween (Appendix 3) with 3-min soaks between washes. Blot dry on absorbent paper. Add sample homogenate at 100 µL per well, using two wells per test sample. Incubate at 4 °C overnight. Flick out and wash four times as before. Dilute specific monoclonal antibody at a predetermined rate (Appendix 1) in conjugate buffer. Add 100 µL per well. Cover plates and incubate at 33 °C for 2 h. Wash 3 times as before. Add appropriate conjugate, 100 µL per well (Appendix 3). Cover plates and incubate at 33 °C for 2 h. Wash 3 times as before. Add substrate (Appendix 3) at 1 mg mL^{-1} in substrate buffer. Incubate at room temperature for 1 h. Read at 405 nm.

An alternative to the above method for TSWV or INSV is a 'cocktail' ELISA using the antisera and the method as above, but adding monoclonal antisera and conjugate together to conjugate buffer at the predetermined rate (Appendix 3), then adding to plates at 100 µL per well.

Mechanical inoculation of indicator plants

The method follows Mumford (1995). Place selected indicator plants (Appendix 4) in the dark for 24 h prior to inoculation, to enhance susceptibility. Grind infected material with chilled inoculation buffer (0.01 M phosphate buffer, pH 7.0, containing 1% sodium sulphite), using a chilled pestle and mortar. Apply sap extract to the leaves of young plants with a small amount of celite (mixed with sap) or carborundum powder (applied as a light application to leaves). Use a gloved finger, dipped into the sap and gently rubbed down the top surface of the lamina, away from the plant stem. Wash plants carefully to remove any residual abrasive powder.

Lateral flow tests

Remove a leaf or a portion of leaf (about 3×4 cm) with suspicious symptoms. Unscrew lid from extraction bottle and place leaf inside (bottle contains buffer and sodium azide). Replace lid tightly, ensuring dropper cap is on. Shake firmly for about 20 s until a green extract is visible. See also instruction leaflet for lateral flow kit.

Remove a test device from its foil pack, avoiding to touch the viewing window. Remove cap from lid of bottle and discard 2–3 droplets by inverting and gently squeezing bottle. Hold device horizontally and gently squeeze 2 drops onto the sample well of the device. Keep device horizontal until extract is absorbed (about 30 s) and a blue dye appears in the viewing window. Wait until the control line appears (labelled C on the device). The control line should be clearly visible in the viewing window of the device after 3 m, and the test (labelled T on the device) result visible in 1–3 min). Two blue lines (C & T) indicate a positive result, test satisfactory; one blue line (C only) indicates a negative result, test satisfactory; faint blue T line, strong C line indicates a possible positive, test satisfactory. A faint or absent line may indicate a low concentration of the pathogen, uneven distribution within the plant or recent infection. See also instruction leaflet for lateral flow kit and Danks & Barker (2000).

RNA extraction method of Boonham *et al.* (2001)

Macerate 100–200 mg leaf tissue to a fine powder in liquid nitrogen. Mix ground tissue with 1 mL buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% Na_2SO_3, 2% PVP-40). Incubate in microfuge tube at 65 °C for 10–15 min. Extract twice with chloroform: isoamyl alcohol (24 : 1). Precipitate RNA from the aqueous layer overnight with an equal volume of 4 M lithium chloride at 4 °C. Centrifuge for 30 min at 13 000 *g* at 4 °C. Resuspend pellet in 200 µL of TE buffer containing 1% sodium dodecyl sulphate (SDS). Incubate at –20 °C for 30 min with 100 µL of 5 M NaCl and 300 µL of ice-cold isopropanol. Centrifuge for 10 min at 13 000 *g* at 4 °C, wash with 70% ethanol, re-pellet, and dry pellet. Re-suspend pellet in 50 µL DEPC-treated water and store at –20 °C.

RNA extraction method of Logemann *et al.* (1987)

Freeze 200 mg of plant tissue in liquid nitrogen in a polythene bag and homogenize to a fine powder using a hand roller. Add two volumes (400 µL) of Logemann buffer (Appendix 3) and homogenize the tissue further. Decant the homogenate into a 1.5-mL microfuge tube. In a safety cabinet, add an equal volume (400 µL) of phenol:chloroform 5 : 1 (e.g. Amresco), vortex and then centrifuge at 13 000 *g* at 4 °C for 15 min. Without disturbing the interphase, pipette the upper aqueous phase into a new 1.5-mL microfuge tube. To this add 0.1 volumes of 3M

sodium acetate and 2.5 volumes of ice-cold absolute ethanol. Incubate at –20 °C for 1 h. Recover the RNA by centrifugation at 13 000 *g* at 4 °C for 15 min. Discard the ethanol phase. Resuspend the pellet in 200 µL of 3M sodium acetate. Centrifuge for 5 min at 13 000 *g* at room temperature. Discard the supernatant. Wash the RNA by resuspending in 500 µL of ice cold 70% ethanol. Centrifuge at 4 °C for 15 min as previously. Remove the ethanol wash. Dry the pellets in a vacuum desiccator for 10–15 min, or on the bench for 30 min or until pellets are dry. Resuspend in 100 µL of DEPC-treated sterile distilled water and store at –20 °C. Centrifuge for 30 s prior to use. Alternatively, the Promega SV RNA kit used as per kit instructions (Promega – See Appendix 3) is also recommended.

RT-PCR/MRT-PCR Tests

The methods are adapted from Weekes *et al*. (1996) and Mumford *et al*. (1996b). Prepare the RT reaction mix in a microfuge tube: 3.5 mM dNTPs 3 µL (1 mM); Primer 2 µL; 5xM-MLV Buffer 2 µL; distilled H_2O 1.1 µL; 200 U µL^{-1} M-MLV 0.5 µL; 120 U µL^{-1} RNase inhibitor 0.4 µL, for each 10 µL final volume reaction. Add 9 µL of master mix to 1 µL of RNA sample in each microfuge tube. Reaction conditions: 42 °C for 15 min, 99 °C for 5 min, 5 °C for 5 min.

Prepare the PCR reaction mix (kept on ice): Primer 2 µL; 50 mM $MgCl_2$ 1.5 µL; 10× Buffer 5 µL; distilled H_2O 31.25 µL; 5 U µL^{-1} Polymerase 0.25 µL; 10 µL RT prep 10 µL. Add 40 µL of PCR master mix (see Table 2) to each tube, mix thoroughly. Run the following programme on a thermal cycler: 1 cycle (5 min at 94 °C), 29 cycles (1 min at 94 °C, 1 min at 48 °C, 1 min at 72 °C), 1 cycle (1 min at 94 °C, 1 min at 48 °C, 10 min at 72 °C). Use 55 °C anneal for L TSWV and S INSV and control (Plant-uni) reactions, 48 °C anneal for S UNIV reactions, and 50 °C anneal for WSMoV reactions.

For MRT-PCR using the TSWV and INSV specific primers, the reaction is carried out as described above except that 0.5 µL of each RNA sample is used in the RT mix, and PCR is undertaken using the LTSWV primers each at 0.1 µM and SINSV primers each at 0.2 µM (Mumford *et al*., 1996b). It is recommended to include a separate reaction, using the above method and 5SrRNA control primers (designated Plant-uni) (Kolchinsky *et al*., 1991) for use as an RNA extraction quality control (Appendix 3). These primers were designed to ribosomal RNA and produce a product which differs in size according to the host plant. The presence of an amplicon indicates RNA extraction was successful. Run under standard conditions (annealing at 55 °C).

Analysis of PCR product

The PCR fragments are detected by agarose gel electrophoresis and stained with ethidium bromide. Prepare a 1–2% agarose gel by gently bringing to the boil agarose in 1 × BE (Appendix 2). Cool the molten agarose to 50–60 °C, pour into the mould and insert the comb. Allow the gel to set. Remove the comb, submerge the gel in 1 × TBE. Add 10 µL of loading buffer to tubes containing 50 µL of sample, flick to homogenize the solution. Load the wells carefully (12 µL of sample + buffer). Include appropriate markers and positive control amplified DNA. Run gel at 100 V/40mA for 1 h until the gel dye front is within 1 cm of the end of the gel. Remove gel and stain in ethidium bromide solution (0.5 Ug mL^{-1}) for 45 min. Rinse the gel in distilled water. Visualize the amplified DNA fragments by UV *trans*-illumination. Verify results against DNA marker and positive control. Photograph the gel to provide a permanent record.

Interpretation of PCR

For the RNA extraction internal control (Kolchinsky *et al*., 1991), where an amplicon is detected, RNA extraction was successful. Where an amplicon is absent, RNA extraction has failed. For the RT-PCR or MRT-PCR tests using specific primers for TSWV/INSV designated LTSWV or SINSV, respectively (Mumford *et al*., 1996b), the test is negative if the 276 bp fragment (TSWV) or the 602 bp fragment (INSV) is not detected and an amplicon for the positive control is detected. The test is positive if the 276 bp fragment (TSWV) or the 602 bp fragment (INSV) is detected. For the RT-PCR test using specific primers for WSMoV (Chu *et al*., 2001), the WSMoV RT-PCR test is negative if the 700 bp fragment is not detected and an amplicon for the positive control is detected. The WSMoV RT-PCR test is positive if the 700 bp fragment is detected. For the MPCR test for the detection of TSWV, INSV and WSMoV using degenerate primer pair SUNIV (Mumford *et al*., 1996b), the MPCR test is negative if the 871 bp fragment (TSWV, INSV, and other tospoviruses) or the 933 bp fragment (WSMoV) is not detected and an amplicon for the positive control is detected. The MPCR test is positive if the 871 bp fragment (TSWV, INSV, and other tospoviruses) or the 933 bp fragment (WSMoV) is detected.

Detection of TSWV in plants and individual thrips using real time fluorescent RT-PCR (TaqMan)

RNA extraction

To extract from plants (Boonham *et al*., 2001), macerate 100–200 mg leaf tissue to a fine powder in liquid nitrogen. Mix ground tissue with 1 mL buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% Na_2SO_3, 2% PVP-40). Incubate in microfuge tube at 65 °C for 10–15 min. Extract twice with chloroform: isoamyl alcohol (24 : 1). Precipitate RNA from the aqueous layer overnight with an equal volume of 4 M lithium chloride at 4 °C. Centrifuge for 30 min at 13 000 *g* at 4 °C. Resuspend pellet in 200 µL of TE buffer containing 1% sodium dodecyl sulphate (SDS). Incubate at –20 °C for 30 min with 100 µL of 5 M NaCl and 300 µL of ice-cold isopropanol. Centrifuge for 10 min at 13 000 *g* at 4 °C, wash with 70% ethanol, re-pellet, and dry pellet. Re-suspend pellet in 50 µL DEPC-treated water and store at –20 °C.

To extract from thrips (Boonham *et al*., 2001), macerate individual insects in 0.5 mL microfuge tubes, each with 50 µL

DEPC-treated water (on ice). Add chelex resin (Chelex100, Biorad) (50 µL of a 50% w/v slurry) to each sample. Heat at 94 °C for 5 min on a thermocycler. Centrifuge tubes for 5 min at 13 000 *g* at 4 °C. Discard pellet and store supernatant at –20 °C.

Real time fluorescent RT-PCR (TaqMan)

Prepare the TaqMan reaction mix (see Table 3): TSWV-CP-100R 150 nM 0.5 µL; TSWV-CP-17-F 150 nM 0.5 µL; TSWV-CP-73T (FAM label) 150 nM 0.5 µL; WFT-RNA-25F 150 nM 0.5 µL; WFT-RNA-93R-C 150 nM 0.5 µL; WFT-RNA-48T (VIC label) 150 nM 0.5 µL; $MgCl_2$ 25 mM 5.5 µL; 10× Buffer 2.5 µL PCR core reagent kit (PE Biosystems); dNTPs 10 mM 2.0 µL; distilled water 9.875 µL; Ampli Taq Gold Taq Polymerase 0.125 µL; MMLV (1/100 diln) 1.0 µL. Add 24 µL of master mix to 1 µL of RNA sample in each microfuge tube.

Run the following generic programme on, e.g. ABI Prism 7700 Sequence Detection System (PE Biosystems), using real-time data collection (Mumford *et al*., 2000): 1 cycle (30 min at 48 °C), 1 cycle (10 min at 95 °C), 40 cycles (1 min at 60 °C, 15 s at 95 °C). The threshold cycle (CT) is the cycle at which a significant increase in fluorescence occurs, hence a CT value below 40 indicates a positive result. The change in normalized fluorescence (delta Rn) records the amount of product amplified.

Fig 1. Chrysanthemum infected by TSWV. Courtesy CSL, York (GB).

Fig 2. Impatiens infected by INSV. Courtesy CSL, York (GB).

Fig 3. Watermelon infected by WSMoV. Courtesy CSL, York (GB).

Fig 6. *Nicotiana tabacum* infected by TSWV. Courtesy CSL, York (GB).

Fig 7. *Nicotiana benthamiana* infected by INSV. Courtesy CSL, York (GB).

Fig 8. *Nicotiana benthamiana* infected by WSMoV. Courtesy CSL, York (GB).