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

Normes OEPP
EPPO Standards

Diagnostics
Diagnostic

PM 7/49

Organisation 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 Standards on Diagnostics are intended to be used by NPPOs in their capacity as bodies responsible for the application of phytosanitary measures. Standards on diagnostic protocols are concerned with the diagnosis of individual pests and describe different methods which can be used to detect and identify pests of phytosanitary concern for the EPPO region. General Standards on diagnostics are in preparation on: (1) the purpose of diagnostic protocols (which may differ according to the circumstances of their use); and (2) reporting and documentation of diagnoses.

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 EPPO Standards on Diagnostics:

- 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 (FR).

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 Standards on Diagnostics 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

Forty-one 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

PM 7/20 (1) *Erwinia amylovora*. *Bulletin OEPP/EPPO Bulletin* **34**, 159–172

PM 7/21 (1) *Ralstonia solanacearum*. *Bulletin OEPP/EPPO Bulletin* **34**, 173–178

PM 7/22 (1) *Xanthomonas arboricola* pv. *corylina*. *Bulletin OEPP/EPPO Bulletin* **34**, 179–182

PM 7/23 (1) *Xanthomonas axonopodis* pv. *dieffenbachiae*. *Bulletin OEPP/EPPO Bulletin* **34**, 183–186

PM 7/24 (1) *Xylella fastidiosa*. *Bulletin OEPP/EPPO Bulletin* **34**, 187–192

PM 7/25 (1) *Glomerella acutata*. *Bulletin OEPP/EPPO Bulletin* **34**, 193–200

PM 7/26 (1) *Phytophthora cinnamomi*. *Bulletin OEPP/EPPO Bulletin* **34**, 201–208

PM 7/27 (1) *Puccinia horiana*. *Bulletin OEPP/EPPO Bulletin* **34**, 209–212

PM 7/28 (1) *Synchytrium endobioticum*. *Bulletin OEPP/EPPO Bulletin* **34**, 213–218

PM 7/29 (1) *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **34**, 219–228

PM 7/30 (1) *Beet necrotic yellow vein benyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 229–238

PM 7/31 (1) *Citrus tristeza closterovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 239–246

PM 7/32 (1) *Plum pox potyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 247–256

PM 7/33 (1) *Potato spindle tuber pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **34**, 257–270

PM 7/34 (1) *Tomato spotted wilt tospovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 271–280

PM 7/35 (1) *Bemisia tabaci*. *Bulletin OEPP/EPPO Bulletin* **34**, 281–288

PM 7/36 (1) *Diabrotica virgifera*. *Bulletin OEPP/EPPO Bulletin* **34**, 289–294

PM 7/37 (1) *Thaumetopoea pityocampa*. *Bulletin OEPP/EPPO Bulletin* **34**, 295–298

PM 7/38 (1) *Unaspis citri*. *Bulletin OEPP/EPPO Bulletin* **34**, 299–302

PM 7/39 (1) *Aphelenchoides besseyi*. *Bulletin OEPP/EPPO Bulletin* **34**, 303–308

PM 7/40 (1) *Globodera rostochiensis* and *Globodera pallida*. *Bulletin OEPP/EPPO Bulletin* **34**, 309–314

PM 7/41 (1) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *Bulletin OEPP/EPPO Bulletin* **34**, 315–320

Some 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 'inter-comparison' 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 PM 7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostics
Diagnostic

Tomato ringspot nepovirus

Specific scope

This standard describes a diagnostic protocol for *Tomato ringspot nepovirus* (ToRSV).

Specific approval and amendment

Approved in 2004-09.

Introduction

Tomato ringspot virus (ToRSV) is a distinctive member of the genus *Nepovirus* (Stace-Smith, 1996). It has isometric virus particles of about 28 nm and is transmitted by sap inoculation with care. It is transmitted in nature by the nematode vector *Xiphinema americanum sensu lato*. This is a species complex which has not yet been defined and it is not therefore known if all members of the group are vectors. Vectors of ToRSV include *X. americanum*, *X. bricolensis*, *X. californicum*, *X. intermedium*, *X. rivesi* and *X. tarjanensei* (Taylor & Brown, 1997). ToRSV is seed-transmitted in several host plants, can be spread from pollen to seed or by pollen to the pollinated plant. Infected seeds may be important as a continuing source of virus in the soil.

The virus is found in woody and semiwoody hosts, but it can also be found in herbaceous ornamental and weed species. It has a similar host range to *Tobacco ringspot nepovirus* (TRSV) (OEPP/EPPO, 2001) although it is more important on fruit crops. Those most affected include raspberries (*Rubus idaeus*), bramble (*R. laciniatus*); grapevine; peach, cherry and other *Prunus* spp.; blackcurrant, gooseberry, strawberry, blueberry (*Vaccinium corymbosum*), *Pelargonium*, *Hydrangea*, *Gladiolus* and *Fraxinus americana*. Some weeds can constitute reservoirs for the virus, e.g. *Taraxacum officinale*. ToRSV is widespread in the temperate regions of North America where the vector occurs and has been reported from ornamentals and berry crops in other parts of the world.

Identity

Name: *Tomato ringspot nepovirus*
Synonym: Tobacco ringspot No. 2, Nicotiana virus 13, blackberry (Himalaya) mosaic virus, winter peach mosaic virus, prune brown line virus, prunus stem-pitting virus, red currant mosaic virus. Strains: peach yellow bud mosaic virus, grape yellow vein virus, tobacco strain, apple union necrosis nepovirus, euonymus chlorotic ringspot virus (Stace-Smith, 1996)
Acronym: ToRSV
Taxonomic position: Viruses: *Comoviridae*: *Nepovirus*
EPPO computer code: TMRSXX
Phytosanitary categorization: EPPO A2 list No. 102, EU Annex designation I/AI

Detection

For detection of ToRSV, disease symptoms can be useful, but they cannot be taken as proof of the presence of the virus; serological or molecular tests are essential for positive identification. Inoculation of herbaceous test plants has the advantage that it will detect all strains. However, it is essential that at least three plants of an indicator host are used to ensure successful transmission (or two where more than one indicator host is used). Serological or molecular tests are necessary to identify specifically the reactions produced on indicator plants.

Natural hosts and symptomatology

The most serious diseases caused by ToRSV are those on fruit crops, including yellow bud mosaic in peach and almond which causes pale-green to pale-yellow blotches developing along the main vein or large lateral veins of the leaves. Buds can either produce rosettes of small leaves or are pale yellow and die. Fruits may be dwarfed and malformed. Some strains of the virus cause stem pitting and decline in *Prunus* spp. and necrosis of the union in apple (OEPP/EPPO, 1991). Plants infected with ToRSV show distinctive symptoms as a shock reaction while chronically infected plants usually exhibit no obvious symptoms but show a general decline in productivity (Stace-

Smith, 1984). On raspberry, canes are stunted and fruits are crumbly and unmarketable. By the third year of infection, 10–80% of fruiting canes may be killed. On grapevine, severely infected vines have many winter-killed buds and weak, stunted shoot growth. Later shoot and foliage symptoms are very obvious on one or more shoots with leaves developing ringspots and mottle. Fruit clusters are reduced in size with berries aborting. Field-grown tomatoes show conspicuous curling and necrosis of the terminals of actively growing shoots while younger leaves develop brown necrotic rings and sinuous lines. Fruits infected early develop faint to conspicuous grey to brown, corky, superficial concentric rings or parts of rings (EPPO/CABI, 1997). Symptoms on *Pelargonium* have been recorded as ringspots or faint chlorotic flecks which tend to fade. However, these symptoms are rare and more commonly no symptoms are seen at all.

Mechanical inoculation of test plants

Mechanical inoculation to herbaceous test plants is simple, sensitive and reliable. Although it has been a traditional method of virus detection, it cannot specifically identify ToRSV as such as the symptoms produced on test plants are generally the same for all *Nepoviruses*. However, test-plant inoculations can be used for virus detection and isolation as well as for increasing ToRSV concentrations in plant tissue for subsequent identification by other methods, such as IEM, DAS/TAS-ELISA or RT-PCR.

Time of testing can be important and should be considered before sampling is initiated. Experience has shown that, for *Pelargonium*, tests are best done between November and April, or when temperatures are cooler. For woody plants, early spring seems to be best, since flowers or young leaves can be tested. For herbaceous plants, it is also possible to detect ToRSV in root extracts.

Extraction buffers to be used for test-plant inoculations
For inoculum preparation from most plants, infected material should be ground in 0.02 M Na/K phosphate buffer, pH 7.0, containing 2% (w/v) polyvinylpyrrolidone (PVP) (MW 10 000–40 000). Inoculum from *Pelargonium* is more infective when leaves are triturated in 0.06 M phosphate buffer, pH 7.6, containing 4% polyethylene glycol (PEG) (MW 6000) (OEPP/EPPO, 1990). Celite should be added to the inoculum as an abrasive or carborundum used to dust the leaves prior to inoculation. This should be washed off with tap water after inoculation to avoid damage to the inoculated leaves which will mask reactions. Inoculated plants are best kept at a temperature range of 18–22°C in a glasshouse or growth chamber.

Recommended test plants and their symptoms
Chenopodium amaranticolor and *Chenopodium quinoa* – chlorotic or necrotic local lesions; systemic apical necrosis; *Nicotiana clevelandii* and *Nicotiana tabacum* – necrotic local lesions develop into rings or ringspots; systemically infected leaves usually show ring or line patterns; *Phaseolus vulgaris* – chlorotic local lesions; systemic rugosity and necrosis of leaf tips; *Cucumis sativus* – chlorotic local spots; systemic chlorosis and mottle; *Petunia hybrida* – necrotic local lesions; necrotic collapse of young systemically infected leaves.

Identification

Several methods are available for the identification of ToRSV. Immunoelectron microscopy (IEM) is very rapid but only economical when small numbers of samples are to be analysed and a transmission electron microscope (TEM) is available. Low concentrations may be difficult to detect. RT-PCR may suffice as a single method of detection depending on the plant being tested; there may be problems with inhibition, e.g. *Pelargonium*. The method is very sensitive, but expertise is required. ELISA, including DAS-ELISA and TAS/indirect-ELISA, is rapid, easy to learn and use, and minimal equipment is required. Where serologically deviant strains of the virus are likely to occur, an indirect (or TAS) form of ELISA is suggested (OEPP/EPPO, 1991) but some strains may still be missed. These methods can be used to confirm reactions produced on indicator plant hosts as ToRSV. A suggested scheme for use of these methods is given in Fig. 1.

Immunoelectron microscopy

In IEM (Milne, 1984; Milne & Lesemann, 1984), virions are either first adsorbed to the electron microscope grid or specifically trapped from plant extracts onto antiserum-coated grids. If virions homologous to the antiserum used for coating the grid are present in the sample, the latter generally leads to a strikingly higher number of virus particles 'trapped' on the grid. Although this increase in particle numbers can already provide an indication for the presence of a certain virus, the adsorbed or trapped virions can be further incubated with antiserum. If this antiserum is homologous to the adsorbed or trapped virions, they appear to be 'decorated', as they are surrounded by a dense halo of antibodies bound to the virus particles. A reference isolate of ToRSV, and normal (preimmune) serum, should be used as positive and negative controls, respectively.

IEM requires considerable expertise and access to a transmission electron microscope (TEM). When large numbers of samples are to be analysed, it is also labour-intensive and time-consuming. For a small number of samples, however, it does have the advantage that it can be done in a very short period of time (less than 1 h). Since not only the virus particles but also the reaction of the antibodies with the virions are actually visualized, it can be an accurate means of diagnosis. However, low concentrations may be difficult to detect.

Since nepoviruses often occur at low concentrations in field-grown plants and do not readily adsorb to electron microscope grids, the following IEM method is recommended. Samples are ground in suitable buffers (see Mechanical Inoculation), using a pestle and mortar. Electron microscope grids are coated with ToRSV antibodies by floating them on small drops (10–20 µL) of ToRSV antiserum diluted 1 : 500 or 1 : 1000 in phosphate

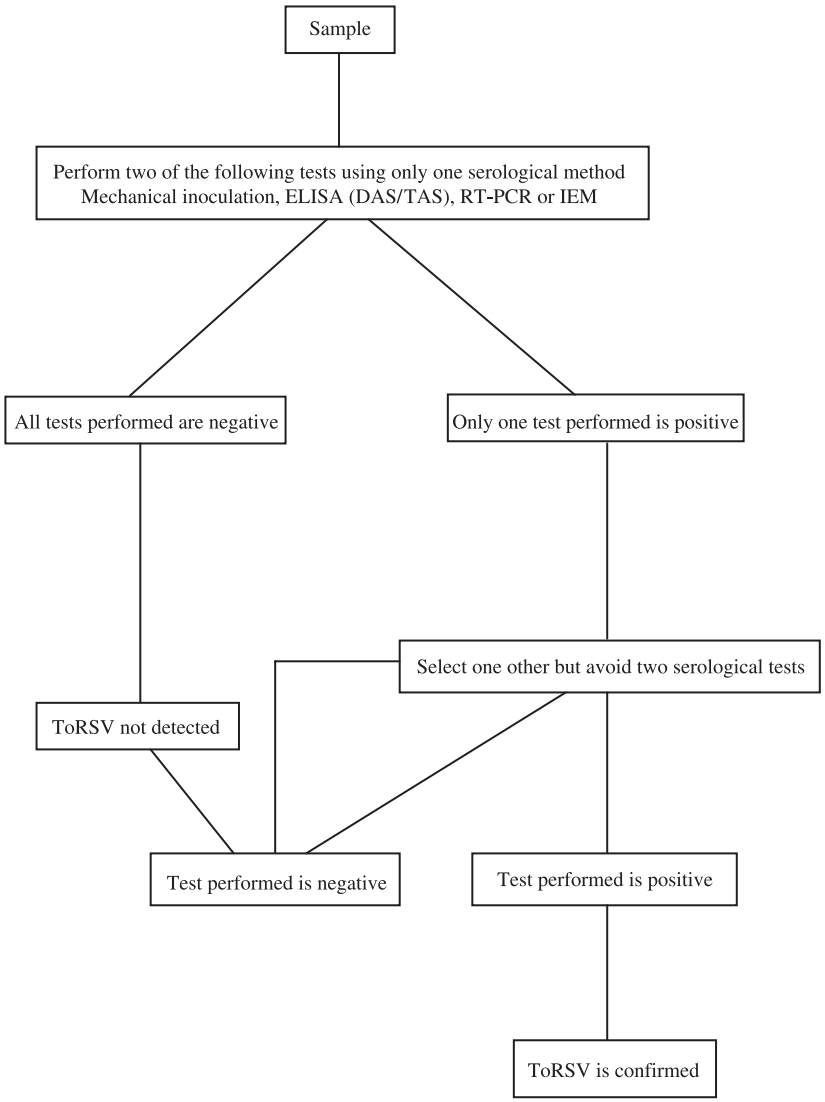

Fig. 1 Decision scheme for the detection and identification of *Tomato ringspot nepovirus* (ToRSV) in samples.

buffered saline (PBS), pH 7.4, at room temperature for 15 min. After washing with 20 drops of PBS, the grids are drained (but not dried) with filter paper and floated on the plant sap. To 'decorate' the virus particles, grids are washed with PBS as before and, after draining, are floated on ToRSV antiserum diluted 1 : 50 to 1 : 100 in phosphate buffer, pH 6.5. After washing the grids with distilled water, they are stained with 3 drops of 1–2% uranyl acetate, using a Pasteur pipette, and examined under the TEM at a magnification of about 40 K.

RT-Polymerase Chain Reaction (PCR)

RNA is extracted from herbaceous and woody plant tissues for testing by RT-PCR. This is a very sensitive molecular method claimed to detect all major serogroups, from A to E (Griesbach, 1995). However, it needs expensive equipment and a great deal of expertise to be able to carry out the various stages to a satisfactory conclusion. Alternatively, there are user-friendly commercial kits for nucleic acid extraction, such as Qiagen and Pure script (Gentra).[1]

Total nucleic acid extraction

Total nucleic acid (TNA) is extracted from samples using the method of Dellaporta as recommended by Griesbach (1995) and Dellaporta *et al*. (1983). Leaf tissue, 0.5–0.75 g, is quickly frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The powder is then transferred with the liquid nitrogen into 30-mL Oakridge tubes. The tissue should not be allowed to thaw out until the buffer is added and the tubes should not be capped until the nitrogen has completely evaporated. 15 mL of extraction buffer (EB) (100 mM

[1]Suggested suppliers for commercial kits for nucleic acid extraction: Qiagen, Qiagen House, Fleming Way, Crawley, West Sussex RH10 9NQ, UK; Pure script (Gentra), Gentra Systems Inc. 13355, 10th Avenue N, Suite 120, Minneapolis, MN 55441, USA.

Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM mercaptoethanol) and 1.0 mL of 20% SDS are added. The tubes are shaken vigorously and incubated at 65°C for 10 min. 5.0 mL of 5 M potassium acetate is added. Tubes are vigorously shaken again, incubated at 0°C for 20 min and centrifuged at 25 000 ***g*** for 20 min. The supernatant is poured through a Miracloth filter into clean 30-mL tubes containing 10 mL isopropanol. The tubes are mixed and incubated at –20°C for 30 min. The TNA is pelleted at 20 000 ***g*** for 15 min. The supernatant is gently poured off and pellets are lightly dried by inverting the tubes on paper towel for 10 min. TNA pellets are re-dissoved with 0.7 mL of 50 mM Tris, 10 mM EDTA pH 8. The solution is transferred to 1.5-mL microfuge tubes, and centrifuged for 10 min to remove insoluble debris. The supernatant is then transferred to a new microfuge tube and 75 µL 3 M sodium acetate and 500 µL isopropanol are added. The TNA is mixed well, then pelleted for 30 s in a microfuge. The pellet is washed by adding 80% ethanol, dried and re-dissolved in 100 µL 10 mM Tris, 1 mM EDTA pH 8.

This method has been validated on certain hosts but, given the range and diversity of hosts infected by ToRSV, there are likely to be species for which the Dellaporta method will not work. A more robust RNA extraction method, known to work on many hosts, including some difficult ones such as strawberry, may be preferable, as follows. Total RNA is extracted from samples using a CTAB-based method, modified from that described by Chang *et al*. (1993). Leaf tissue (100 mg) is ground in 1 mL of grinding buffer (2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M sodium chloride, 1.0% sodium sulphite, 2.0% soluble PVP), transferred into a 1.5-mL microfuge tube and incubated at 65°C for 10–15 min. After incubation, tubes are centrifuged at 12 000 ***g*** for 5 min. The resulting clarified sap is extracted twice with chloroform: isoamyl alcohol (24 : 1 v/v). RNA is then precipitated by adding an equal volume of 4 M LiCl and incubating overnight. The RNA is pelleted by centrifugation for 25 min at 12 000 ***g*** at 4°C. The resulting pellet is then resuspended in 200 µL of TE-SDS buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) SDS). The RNA is then precipitated by adding 100 µL of 5 M NaCl and 300 µL of iso-propanol, before incubating at –20°C for 30–60 min. The sample is then microfuged for 15 min at 12 000 ***g*** at 4°C and the resulting pellet washed (in 70% ethanol), air dried and resuspended in 50 µL of RNase-free water.

For PCR detection of ToRSV in perennials, e.g. grapevine, the method described by Rowhani *et al*. (1993) may be used.

cDNA synthesis

Samples are reverse-transcribed independently of the PCR reaction (Griesbach, 1995) by adding 2 µL of TNA sample to an RT master mix (100 U M-MLV reverse transcriptase per sample, dNTPs, and buffer as indicated in the directions supplied by the manufacturer (Promega Corp., Madison, US) containing 10 µg mL of D1 primer (TCC GTC CAA TCA CGC GAA TA). A 10-µL overlay of mineral oil is added to the total reaction volume of 20 µL and briefly centrifuged at 4000 rev min^{-1}. The preparation is placed in a thermocycler at 37–40°C for 50 min followed by 95°C for 5 min.

PCR amplification

1-2 µL of RT product or control ('infected RNA', 'uninfected RNA' and water) together with 1 µM each of primers U1 (GAC GAA GTT ATC AAT GGC AGC) and D1 (see above) (Rott *et al*., 1991) and 1.5 mM $MgCl_2$ are used for the PCR. The total reaction volume of 75 µL should contain dNTPs, buffer and detergent according to manufacturer's directions (Promega Corp., Madison, US). Samples are preheated to 77–80°C for 3–4 min before 1 U *Taq* polymerase per reaction is added. After the reaction mixture is complete, the temperature is raised to 94°C for 4 min to denature the template. The PCR is then performed using the following programme: 94°C (denature) for 1 min, followed by 55°C (anneal) for 2 min, 72°C (extension) for 2 min, for 35–40 cycles, followed by a 5–10 min extension at 72°C (Griesbach, 1995).

Gel electrophoresis

Following RT-PCR, products are analysed by gel electrophoresis (Griesbach, 1995). 15 µL of each sample is mixed with 10× Ficoll-EDTA-Tris loading buffer and loaded onto a 2.0% Metaphore agarose gel in 0.5× Tris-borate-EDTA buffer. The electrophoresis is run at 40 mA 1–1.75 h in prechilled buffer. After running, the gel is stained in loading buffer or 0.5 µg mL^{-1} ethidium bromide solution for 20–30 min and de-stained twice before visualizing under UV light. Positive samples will give a band of 449 base pairs (bp). An image of the gel (either film or digital) should be captured and included with the final report.

ELISA

ELISA can readily be used for detecting and identifying ToRSV in herbaceous hosts and fruit trees using appropriate extraction buffers. The time of testing may be important and should be considered before sampling is initiated. Experience has shown that, for *Pelargonium*, tests are best done between November and April, or when temperatures are cooler. For woody plants, early spring is the time at which flowers or young leaves can reliably be tested. For herbaceous plants, it is also possible to detect ToRSV in root extracts.

ELISA kits containing all necessary components are widely available commercially.[2] If these are used, the manufacturer's instructions should be carefully followed. Alternatively, the

[2]Some suggested suppliers of antisera and ELISA kits: Advanced Diagnostics International, LLC. 700 Research, Center Blvd, Fayetteville, AR 72701, USA – http://www.adillc.com (ELISA kit); ATCC, Reference Materials, LGC Queens Road, Teddington TW11 0LY, UK – http://www.lgc.co.uk/atcc.asp (antiserum); BIOREBA, Chr. Merian-Ring 7, CH-4153 Reinach BL 1, Switzerland – E-mail: admin@bioreba.ch (ELISA kit); DSMZ, Plant Virus Collection, c/o Biologische Bundesanstalt für Land- und Forstwirtschaft, Messeweg 11/12, D-38104 Braunschweig, Germany – E-mail: S.Winter@bba.de or 100705.337@compuserve.com (antiserum and ELISA kit); Plant Research International PO Box 16, NL-6700 AA Wageningen, the Netherlands – www.plant.wageningen-ur.nl (ELISA kit); Sanofi PASTEUR, Sanofi Pasteur, 3, boulevard Raymond Poincaré – 92430 Marnes-La-Coquette, France – Fax: 01 47 41 91 33 (ELISA kit).

procedures presented here may be followed. DAS-ELISA (Clark & Adams, 1977) is the preferred method for ToRSV detection and identification but, where serologically deviant strains of the virus are likely to occur, an indirect (or TAS) form of ELISA is suggested (OEPP/EPPO, 1991).

DAS-ELISA

ELISA microtitre plates are coated by filling the wells with 100 µL of ToRSV-specific gamma globulin (IgG) used at 1–2 µg mL^{-1} in a 0.05 M sodium carbonate buffer, pH 9.6. The plates are incubated for 2–4 h at 33°C, then washed three times with phosphate-buffered saline plus Tween (PBS-T). Samples can be leaf or cambial and inner bark and/or bud tissues of scions and rootstocks of all hosts. In the latter case, knives or cork borers are used to remove pieces of bark from scion and rootstock portions, and phloem and cambial tissue is scraped from the exposed wood. These tissue samples or excised buds are homogenized in sample buffer (at a ratio of, e.g. 1 : 10), either in a plastic bag using a roller or with a pestle and mortar. If the latter is used, pestle and mortar should be washed thoroughly between samples. Sample buffers may differ with the material being tested and it is usually best to follow the suggestions supplied with the reagent kit. Recommended sample buffers are indicated in Appendix I. The samples are loaded onto an ELISA plate, 100 µL in each of two wells per sample, and incubated at 4°C overnight. Positive ToRSV and known negative controls, ideally in material that is similar to that being tested, should be included in the ELISA plate. If such controls are not available, herbaceous test plants infected with ToRSV can be used. After washing four times as before, each well is filled with 100 µL of alkaline phosphatase-labelled anti-ToRSV IgG diluted (e.g. 1 : 200) in PBS-Tween-PVP to which may be added 0.2% (w/v) ovalbumin or 0.5% semiskimmed milk powder if required. The ELISA plate is incubated for 3–4 h at 33°C. After washing three times, the ELISA plate wells are filled with 100 µL of freshly prepared substrate solution containing 1 mg mL^{-1} p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8. Following a substrate incubation period of about 1 h at room temperature, ELISA plates are read at 405 nm.

TAS- (indirect) ELISA

ELISA microtitre plates are coated by filling the wells with 100 µL of ToRSV-specific gamma globulin (IgG) used at 1–2 µg mL^{-1} in a 0.05 M sodium carbonate buffer, pH 9.6. The plates are incubated for 2–4 h at 33°C. They are then washed three times with phosphate-buffered saline plus Tween (PBS-T). The samples are loaded onto an ELISA plate, 100 µL in each of two wells per sample, and incubated at 4°C overnight. Wells are filled with mouse anti-ToRSV IgG (or monoclonal antibody (Mab)) appropriately diluted in PBS and plates incubated for 2–4 h at 33°C. Wells are filled with commercially rabbit antimouse IgG conjugated to alkaline phosphatase and diluted as before. Plates are incubated at 33°C for 2–4 h. Positive ToRSV and known negative controls, ideally in material that is similar to that being tested, should be included in the ELISA plate. If such controls are not available, herbaceous test plants which have been infected with ToRSV should be used.

Ideally ToRSV is positively identified when two of the following results have been obtained (of which preferably only one should be serological)[3]:

- reactions on test plants indicating that a *Nepovirus* is present
- DAS/TAS-ELISA (A405 nm) value of a sample exceeding a threshold value that, following a substrate incubation period of 1–2 h at room temperature, is at least twice the mean of the healthy control(s) or the mean of the negative control values plus 2 or 3 standard deviations. Under these conditions, the negative and positive controls should give A405 nm values of < 0.07 and > 0.3, respectively, blanked against buffer. Questionable and very weak reactions should be verified in a separate test (e.g. IEM or mechanical inoculation) or re-examined by DAS/TAS-ELISA or by DAS/TAS-ELISA using extracts from symptomatic leaves of herbaceous test plants inoculated with the questionable samples
- in IEM, isometric virions measuring 25–28 nm in diameter, densely decorated with antibodies, are unequivocally visualized. Particle morphology and decoration intensity should be indistinguishable from those obtained with a reference isolate of ToRSV. ToRSV virions should appear undecorated when normal (preimmune) serum has been used
- in RT-PCR, a product of 449 base pairs (bp) is obtained. Positive control reactions should give a product of the same size, while negative controls should yield no amplification products of this size.

Since ToRSV is serologically unrelated to other *Nepovirus* spp. (Stace-Smith, 1996), ELISA and IEM reactions usually provide a clear indication for the presence or absence of the virus, particularly in combination with inoculations to test plants used for increasing virus titres. However, some strains may be missed by using serological techniques alone and it may be preferable to use molecular tests to avoid this.

Reference material

Reference isolates (and antiserum) of ToRSV can be obtained from: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Messeweg 11–12, 38104 Braunschweig, Germany.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information on this organism can be obtained from:
CSL Diagnostics, Central Science Laboratory, Sand Hutton,

[3]In survey situations, where many samples may be tested, it may not be practical to do this, and the most reliable test, according to the experience of the testing laboratory, should then be used.

York YO41 1LZ (UK) (IEM, DAS/TAS-ELISA and RT-PCR)

Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11-12, 38104 Braunschweig (Germany) (IEM and DAS/TAS-ELISA)

Naktuinbouw, PO Box 135, 2370 AC Roelofarendsveen, Netherlands; Plant Protection Service, PO Box 9102, 6700 HC Wageningen (Netherlands) (DAS-ELISA, indicator plants).

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Appendix I

Recommended sample buffers for ELISA

General: dissolve, in 1000 mL 1× PBS-T, sodium sulfite (anhydrous) 1.3 g, polyvinylpyrrolidone (PVP) MW 24 000–40 000 20.0 g, sodium azide (optional*) 0.2 g, powdered egg (chicken) albumin, Grade II 2.0 g. Adjust to 7.4 and store at 4°C.

Blueberry: dissolve, in general extraction buffer to 1000 mL, Na_2HPO_4 (anhydrous) 10.4 g, KH_2PO_4 (anhydrous) 0.9 g. Store at 4°C.

Grapevine: dissolve, in 900 mL distilled water, Tris (hydroxymethyl) aminomethane (Tris) 60.5 g, sodium chloride 8.0 g, polyvinylpyrrolidone(PVP), MW 24 000–40 000 20.0 g, polyethylene glycol 10.0 g, sodium azide (optional*) 0.2 g, Tween-20 0.5 g. Adjust to pH 8.2 with hydrochloric acid. Adjust volume to 1000 mL with distilled water. Store at 4°C.

Pelargonium: dissolve, in distilled water to 1000 mL, Tris (hydroxymethyl) aminomethane (Tris) 12.1 g, polyvinylpyrrolidone (PVP), MW 24 000–40 000 20 g, Tween 20 5 mL, Gelatine 1 g. Adjust pH to 8.6.