◆ EPPO Standards ◆

DIAGNOSTIC PROTOCOLS FOR REGULATED PESTS

TOBACCO RINGSPOT NEPOVIRUS

PM 7/2(1) English

oepp
eppo

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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.

REFERENCES

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

IPPC (1993) *Principles of Plant Quarantine as Related to International Trade.* ISPM no. 1. IPPC Secretariat, FAO, Rome (IT).

IPPC (1999) *Glossary of Phytosanitary Terms.* ISPM no. 5. IPPC Secretariat, FAO, Rome (IT).

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

OEPP/EPPO (1999) EPPO Standards PM 1/2(8) EPPO A1 and A2 lists of quarantine pests. In: *EPPO Standards PM1 General phytosanitary measures*, pp. 5-17. OEPP/EPPO, Paris (FR).

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.

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 a general expert (i.e. an entomologist, mycologist, virologist, bacteriologist etc.) who is not necessarily a specialist on the organism or its taxonomic group. 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

This is a new series.

EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES

PM 7/2(1) English

Diagnostic protocols for regulated pests

TOBACCO RINGSPOT NEPOVIRUS

Specific scope

This standard describes a diagnostic protocol for *Tobacco ringspot nepovirus.*

Specific approval and amendment

First approved in 2000-09.

Introduction

Tobacco ringspot virus (TRSV) is the type member of the genus *Nepovirus*. It has isometric particles of about 28 nm and is readily transmitted by sap inoculation. It is transmitted in nature by the nematode vector *Xiphinema americanum* and closely related *Xiphinema* spp. *Thrips tabaci*, spider mites, grasshoppers and aphids have also been reported as possible vectors (Stace-Smith, 1985) but their significance to the natural spread of TRSV is currently unclear. TRSV is seed-transmitted in several host plants. It naturally infects a wide range of herbaceous and woody hosts causing many serious disease problems in the regions of North America where the nematode vectors occur. The most significant crops affected by TRSV are soybean (*Glycine max),* grapevine (*Vitis vinifera*) and blueberry *(Vaccinium corymbosum)* and, to a lesser extent, tobacco *(Nicotiana tabacum),* a number of ornamentals and *Cucurbitaceae*. Its host range is similar to that of *Tomato ringspot nepovirus* (TomRSV), but it is less important on fruit crops generally; it does, however, pose a significant risk to *Vaccinium* and *Vitis* within the EPPO region. TRSV has been reported from many parts of the world, but most of these reports were associated with material originating from North America. In particular, it has been recorded in several EPPO countries but these reports are either unconfirmed or concern interceptions of imported material, primarily pelargonium. The natural vector of TRSV is confined to the Americas; there has been no evidence of natural spread in any other geographical area.

Identity

Name: *Tobacco ringspot nepovirus*

Synonym: Anemone necrosis virus, blueberry necrotic ringspot virus

Acronym: TRSV

Taxonomic position: Viruses: *Comoviridae: Nepovirus*

Bayer code: TRSV00

Quarantine pest: EPPO A2 list no. 228; EU Annex I/A1

Detection and identification

TRSV generally occurs in all plant parts (including roots and seeds) of its host species. Detection is, however, easiest from soft tissues (e.g. young leaves and shoots). For this reason, it may be important to extract during an appropriate period in the growing season of the host species concerned to ensure an adequate virus concentration. For pelargonium, for example, tests are best done between November and April, or when temperatures are cooler.

For detection and identification of TRSV, the following methods are principally available:

- disease symptoms can be useful in some hosts (e.g. soybean and blueberry) but other hosts (e.g. pelargonium) may show no symptoms;
- inoculation of herbaceous test plants is of limited diagnostic value, even if a reference isolate of TRSV is used. Serological tests are necessary to identify reactions produced on indicator plants as specifically due to TRSV;
- immunoelectron microscopy is very quick and reliable, but only economical when small numbers of samples are to be analysed and a transmission electron microscope is available;
- DAS-ELISA is the preferred method of detection and identification.

Natural hosts and symptomatology

Besides the crops already mentioned, TRSV has been found in *Anemone*, *Malus domestica*, *Solanum melongena*, *Begonia semperflorens, Rubus fruticosus, Capsicum annuum, Prunus avium, Cornus, Vigna unguiculata, Daphne, Fraxinus, Gladiolus, Hydrangea, Iris, Lobelia, Lupinus, Mentha, Narcissus, Carica papaya, Pelargonium, Petunia, Sambucus* and various weeds (EPPO/CABI, 1997). In soybean, terminal buds are curved (bud blight) with other buds progressively becoming brown and brittle. Brown streaks may develop on the stems and petioles of larger leaves and pods are underdeveloped and aborted. Pods that set before infection may develop dark blotches. On tobacco, TRSV causes ring and line patterns on the foliage, and stunting. On blueberry, the symptoms include stunting, twig dieback, necrotic or chlorotic spots, and rings or line patterns on the foliage. In cucurbits, there is stunting with leaf mottle and the fruit is deformed. On grapevine, the new growth is weak and sparse, internodes are shortened, leaves are small and distorted, and plants are stunted with very few deformed berries produced. TRSV has only been seen a few times in cherry: scattered young leaves throughout the crown show irregular blotching over the whole leaf blade and have deformed margins. In wild *R. fruticosus,* there are faint to severe ringspots, mottling and mosaic, yellow line patterns, leaf distortion and stunting of infected foliage (Stace-Smith 1987). TRSV has been found only once in a cultivated *Rubus* sp. No definite symptoms have been associated with TRSV in pelargonium.

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 does not lead to specific TRSV identification when used alone, 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 TRSV concentrations in plant tissue for subsequent identification methods, such as IEM and DAS-ELISA.

Extraction buffers to be used for test-plant inoculations

The following buffers are examples of those which can be used to extract inoculum from various plants prior to mechanical inoculation of test plants:

- For extraction from most plants, the infected material may be ground in 0.02 M Na/K phosphate buffer, pH 7.0, containing 2% (w/v) polyvinylpyrrolidone (PVP) (MW 10 000 to 40 000).
- For extraction from grapevine or blueberry, an aqueous solution of nicotine at 2.5% and 2.0%, respectively, may be used or, alternatively, 0.5 M Tris-HCl buffer, pH 8.2, containing 2% PVP 10 000 to 40 000.
- Inoculum from *Pelargonium* is more infective if leaves are triturated in 0.06 M phosphate buffer, pH 7.6, containing 4% polyethylene glycol (PEG) (M.W. 6000) (OEPP/EPPO, 1990).

Celite is added to the inoculum as an abrasive or carborundum is used for dusting the leaves prior to inoculation. This should be washed off after inoculation to avoid damage to the inoculated leaves which will mask reactions. Inoculated plants should be kept at 18-22°C in a glasshouse or growth chamber.

Recommended test plants and their symptoms

Chenopodium amaranticolor and *C. quinoa*: necrotic local lesions; systemically chlorotic and/or necrotic spots and/or dieback but systemic infections are not always induced.

Nicotiana benthamiana, *N. clevelandii* and *N. tabacum*: necrotic local lesions develop into rings or ringspots; systemically infected leaves usually show ring- or line-patterns.

Phaseolus vulgaris: necrotic spots on inoculated leaves; systemically infected leaves may show spots and rings. The growing tip becomes necrotic.

Cucumis sativus: chlorotic or necrotic local lesions; systemic mottling and dwarfing with apical distortion.

Immunoelectron microscopy (IEM)

In this method (Milne, 1984; Milne & Lesemann, 1984), virions are either first adsorbed to the electron microscopic 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 are then 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 TRSV and normal (pre-immune) serum should be used as positive and negative controls, respectively.

IEM requires considerable expertise and access to a transmission electron microscope. 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 carried out in a very short period of time (less than 1 h). Because not only the virus particles but also the reaction of the antibodies with the virions are visible, it can be an accurate means of diagnosis.

Because 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:

1. Samples are ground in suitable buffers (see mechanical inoculation), using a pestle and mortar.
2. Electron microscope grids are coated with TRSV antibodies by floating them on small drops (10-20 µL) of TRSV antiserum diluted 1:1000 in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS), pH 7.4, at room temperature for 5 min.
3. After washing with 20 drops of TBS or PBS, the grids are drained (but not dried) with blotting paper.
4. For 'trapping' of TRSV virions, grids are floated on 10 to 20-µL drops of plant sap for 15 min or longer (incubation for low-titered viruses: 2 h to overnight).
5. To 'decorate' the virus particles, grids are washed with PBS or TBS as before and, after draining, are floated as previously on TRSV antiserum diluted of 1:50 to 1:100 in phosphate buffer for 15 min.
6. After washing the grids with distilled water, they are stained with 1-2% uranyl acetate and examined under the electron microscope at a magnification of about 40 000×.

DAS-ELISA

Because TRSV is a good immunogen, ELISA can readily be used for detecting and identifying the virus in herbaceous hosts, grapevine and blueberry using appropriate extraction buffers. ELISA kits containing all necessary components needed for the test, are widely available commercially and can be used by those who do not wish, or do not have the facilities, to prepare their own reagents. DAS (double antibody sandwich) ELISA (Clarke & Adams, 1977) is the preferred method for TRSV detection and identification.

A suitable procedure is as follows. ELISA microtitre plates are coated by filling the wells with 100 µL of TRSV-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 PBS plus Tween (PBS-T).

Samples can be leaf, stem, sprout, root or seed material. Each sample is 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 are used, they should be washed thoroughly between samples. Sample buffers can differ with the material being tested and it is usually best to follow the suggestions supplied with the reagent kit.

A general extraction buffer can be prepared by dissolving the following in 1000 mL 1x PBS-T: sodium sulphite (anhydrous) - 1.3 g; polyvinylpyrrolidone (PVP) MW 24-40 000 - 20.0 g; sodium azide (optional) - 0.2 g; powdered egg (chicken) albumin, Grade II - 2.0 g. Adjust to pH 7.4. Store at 4° C. To extract blueberry, the following should be added to the general extraction buffer, to 1000 mL: sodium phosphate, dibasic (anhydrous) - 10.4 g; potassium phosphate, monobasic (anhydrous) - 0.9 g. Store at 4^{o} C. To extract grapevine, a specific buffer should be prepared by dissolving in 900 mL distilled water: Tris - 60.5 g, sodium chloride - 8.0 g, polyvinylpyrrolidone (PVP), MW 24-40 000 - 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^{o} C.

The samples are loaded onto an ELISA plate, 100 µL in each of two wells per sample, and incubated at 4^{o}C overnight.

One positive TRSV control and known healthy controls (preferably 6 different extracts evenly distributed over the plate), 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 may be used. In addition, the plate should include wells containing reagents only.

After washing four times as before, each well is filled with 100 µL of alkaline phosphatase-labeled anti-TRSV IgG diluted (e.g. 1:200) in PBS-Tween-PVP containing 0.2% (w/v) ovalbumin. The ELISA plate is incubated for 3-4 h at 33^{o}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 1-2 h at room temperature, ELISA plates are read at $A_{405\ nm}$. Samples giving a result more than twice the mean of the healthy controls are considered positive.

Comparison with similar species

Because symptoms induced in test plants resemble those caused by other nepoviruses, test-plant reactions are not diagnostic for TRSV. Serological tests are currently the only reliable ones. As TRSV is serologically unrelated to other nepovirus species, 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. In pelargonium, there may be a mixed infection of TRSV and TomRSV; these are usually symptomless and samples should be tested for the two viruses separately.

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

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol should have been followed. TRSV is positively identified by an adequate result in DAS-ELISA or IEM.

In DAS-ELISA, the mean $A_{405\ nm}$ value of the sample should exceed a threshold value that, following a substrate incubation period of 1-2 h at room temperature, is at least 2 times higher than the mean of the healthy controls. Under these conditions, the healthy and positive controls should give $A_{405\ nm}$ values of < 0.07 and > 0.14 respectively.

For IEM, isometric virions, 25-28 nm in diameter and densely decorated with antibodies, should be unequivocally visualized following the use of TRSV antiserum; particle morphology and decoration intensity should be indistinguishable from those obtained with a reference isolate of TRSV; TRSV virions should appear undecorated when normal (pre-immune) serum has been used.

Report on the diagnosis

A report on the execution of the protocol should include:

- information on the origin of the infected material;
- a description of the disease symptoms (including photographs), if any are evident on the sample;
- the number of TRSV-infected plants (samples) out of the total number of plants (samples) tested;
- if IEM is carried out, micrographs showing decorated virions trapped from at least one of the samples shown to be infected with TRSV; for comparison, micrographs of decorated and undecorated particles as visualized in the control experiments;
- records of all $A_{405\ nm}$ values obtained in DAS-ELISA;
- comments on the certainty or uncertainty of the identification

Further information

Further information on this organism can be obtained from:

CSL Diagnostics, Central Science Laboratory, Sand Hutton, York YO41 1LZ (UK) (DAS-ELISA)

Biologische Bundesanstalt für Land- und Forstwirtschaft, Messeweg 11-12, 38104 Braunschweig (Germany) (IEM and DAS-ELISA)

Naktuinbouw Laboratories, Sotaweg 25, P.O.Box 135, 2370 AC Roelofarendsveen (Netherlands) (IEM and DAS-ELISA)

Plantenziektenkundige Dienst, P.O. Box 9102, 6700 HC Wageningen (Netherlands)

Acknowledgements

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