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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 7/33

Organization Européenne et Méditerranéenne pour la Protection des Plantes
1, rue Le Nôtre, 75016 Paris, France

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

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

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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

Potato spindle tuber pospiviroid

Specific scope

This standard describes a diagnostic protocol for *Potato spindle tuber pospiviroid* in microplants and leaves of potato plants for the purpose of post-entry quarantine testing, potato nuclear-stock production testing and field-stock testing. Use for other purposes is not covered (e.g. leaves from field-grown plants, tuber sap, true seed, hosts other than potato).

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 *Pospiviroid Potato tuber spindle tuber viroid* (PSTVd) is an infectious unencapsidated, small, circular, single-stranded RNA, with considerable secondary structure, capable of autonomous replication when inoculated into a host. In potato, it is commonly 359 nucleotides in length (Gross *et al*., 1978) and more rarely 358 or 360 (Herold *et al*., 1992; Lakshman & Tavantzis, 1993). Nucleotide lengths of 356 have been found in a wild *Solanum* spp. (Behjatnia *et al*., 1996) and tomato (Puchta *et al*., 1990). Lengths of 356 and 357 have been reported in pepino (*Solanum muricatum*) (Puchta *et al*., 1990; Shamloul *et al*., 1997). Isolates show few differences in homology but a range of symptoms in potato.

The natural host range of PSTVd is relatively narrow. The primary hosts are potato (and stolon- and tuber-forming *Solanum* spp.) and tomato (Puchta *et al*., 1990; Verhoeven & Roenhorst, 1995). PSTVd has also been found in avocado (*Persea americana*) (Querci *et al*., 1995) and pepino (Puchta *et al*., 1990; Shamloul *et al*., 1997; Verhoeven & Roenhorst, 1995). In 2001, PSTVd was detected in glasshouse tomato crops in New Zealand (Elliot *et al*., 2001). It has a wide experimental host range, infecting 94 species in 31 families (Jeffries, 1998).

PSTVd is the only viroid known to infect cultivated species of potato naturally, but Mexican papita viroid has been found infecting the wild potato species *Solanum cardiophyllum* (papita guera, cimantli) (Martinez-Soriano *et al*., 1996). Experimentally, other viroid species in the Genus *Pospiviroid*, e.g. *Tomato chlorotic dwarf viroid* (Singh *et al*., 1999), *Columnea latent viroid* (Hammond *et al*., 1989) and *Tomato planta macho viroid* (Galindo *et al*., 1982), have been shown to infect cultivated potato. Whether these other viroids will be found infecting potato may depend on the proximity of potato to the natural host, in the field, glasshouse or tissue-culture laboratory, and the measures taken to prevent cross infection.

PSTVd has been found infecting potato in Africa (Nigeria), Asia (Afghanistan, China, India) parts of eastern Europe including the former USSR, North America (EPPO/CABI, 1997) and Central America (Badilla, 1999). Systematic testing over the last 10–20 years has apparently eliminated or significantly reduced PSTVd from commercial seed and ware production schemes in North America and parts of eastern Europe. PSTVd has recently been isolated from a wild *Solanum* spp. growing in the Northern Territory of Australia (Behjatnia *et al*., 1996).

PSTVd is transmitted in true potato seed (0–100% of seed may be infected) (Fernow *et al*., 1970; Singh, 1970) via infected pollen or ovules (Grasmick & Slack, 1986; Singh *et al*., 1992) and by contact, mainly by machinery in the field. Experimental acquisition and transmission of PSTVd by *Myzus persicae* from plants coinfected by *Potato leafroll polerovirus* has been reported (Salazar *et al*., 1995; Querci *et al*., 1996; Syller & Marczewski, 1996; Querci *et al*., 1997), from a small percentage of plants (Singh & Kurz, 1997).

Tomato bunchy top was once thought to be caused by PSTVd. It is now known to be caused by a different viroid, *Tomato bunchy top viroid*.

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

Identity

Name: *Potato spindle tuber pospiviroid*
Synonyms: potato spindle tuber virus, potato gothic virus
Acronym: PSTVd
Taxonomic position: Family Pospiviroidae, Genus *Pospiviroid*
Bayer computer code: PSTVD0
Phytosanitary categorization: EPPO A2 list no. 97; EU Annex 1/A1

Detection

Symptoms

In potato, symptoms of PSTVd depend on strain, cultivar and environment and may vary from severe symptoms (reduction in plant size, uprightness and clockwise phyllotaxy of the foliage when the plants are viewed from above, dark green and rugose leaves; Web Fig. 1) to mild and symptomless infection (Web Fig. 2). Tubers may be reduced in size, misshapen, spindle or dumbbell-shaped, with conspicuous prominent eyes which are evenly distributed (Pfannenstiel & Slack, 1980) (Web Figs 3, 4 and 5).

Identification

Growing conditions for plants and testing programme

Since viroid concentration is affected by temperature and light levels, microplants and glasshouse plants for testing should be grown at temperatures not less than 18 °C (preferably at temperatures higher than 20 °C) and with at least a 16-h photoperiod. Microplants should be tested when at least 4–6 weeks old and with stems of at least 5 cm length. The whole stem should be sampled. Microplants giving a negative test result should be tested after growth in the glasshouse, as soon as the plants are well established but prior to flowering and pollen production. Samples should be taken from a fully expanded leaflet at the top of each stem of the plant.

For post-entry quarantine or nuclear-stock testing systems not based on micropropagation, small glasshouse-grown plants should be tested as soon as they are well established and again when about 25 cm tall, at or near flowering. The test programme as specified in the flow diagram (Figs 6 and 7) should be followed.

Detection methods

The RNA extraction and detection methods described in this protocol were selected after ring-testing the methods currently used within the EU and elsewhere (EU 1997; OEPP/EPPO, 1984, 1998), followed by multilaboratory validation[2]. Other methods may be used provided that they have broad specificity (they should detect all PSTVd isolates and preferably other *Pospiviroid* spp. which may infect potato) and the quality control requirements described in the section quality control are met.

For quarantine testing and nuclear-stock production, the decision scheme (Fig. 6) provides for two methods for initial detection of PSTVd in samples: Return-PAGE (polyacrylamide gel electrophoresis) with silver staining (R-PAGE, Appendix 1) or Digoxigenin-labelled RNA probe (DIG probe, Appendix 2). A second preferably different method should be used to confirm a positive detection result (secondary detection test). R-PAGE, DIG Probe or RT-PCR (Appendix 3) may be used as secondary detection test methods. TaqMan (Appendix 4) may also be used.

For surveys using post-harvest testing of eyeplugs, the decision scheme (Fig. 7) normally requires that R-PAGE or DIG probe should be used. However, RT-PCR or TaqMan may be used if the primers (probes) are known to detect the viroid (i.e. survey as a result of an outbreak). A second, preferably different method should be used to confirm a positive detection result (secondary detection test), chosen from R-PAGE, DIG probe, RT-PCR and TaqMan.

Specificity and sensitivity of detection methods

None of the detection methods described will specifically identify PSTVd. For this the viroid has to be sequenced and analysed. The sensitivity described against each of the methods is based on ring-test results. The first figure is the smallest quantity of infected tissue detected by the best laboratory down to a minimum of 0.0155 mg (this is equivalent to about 17 pg of PSTVd). The second figure is the smallest quantity of leaf tissue detected by most laboratories.

Primary detection methods
The R-Page method will detect most viroids. It is the least sensitive of the methods (1 mg; 5 mg). Dig Probe, which is based on a full-length PSTVd monomer, is specific to pospiviroids. In addition to PSTVd, the probe may also react with *Chrysanthemum stunt viroid* and *Citrus exocortis viroid* (A. Harness, Agdia Inc, US, pers. comm.) and it strongly detects *Tomato chlorotic stunt pospiviroid* (Singh *et al*., 1999). It may hybridize with other *Pospiviroid* spp. The method should detect all PSTVd isolates. It is durable, reliable and sensitive (0.0155 mg; 0.50 mg).

[2]The initial ring test comprised 2 sets of samples, one which required no further extraction and the other which required addition of leaf material and an RNA extraction. Each sample set comprised RNA derived from 8 different concentrations of PSTVd-infected tissue + 2 negative controls. The PSTVd isolate used was of Russian origin (358 nt) with 98% similarity to Accession M25199 (strain *Solanum commersonii*) (van Wezenbeek *et al*., 1982). Although some ^{32}P-labelled RNA probe methods gave excellent results, they were judged unsuitable for multilaboratory validation because of health and safety implications of using ^{32}P. TaqMan was included because of its potential to be used for survey work and direct tuber testing (not in the scope of the current project) despite its not being widely available. Multi-laboratory validation was as described for the initial ring test but with RNA derived from 11 different concentrations of PSTVd-infected tissue concentrations + 4 negative controls.

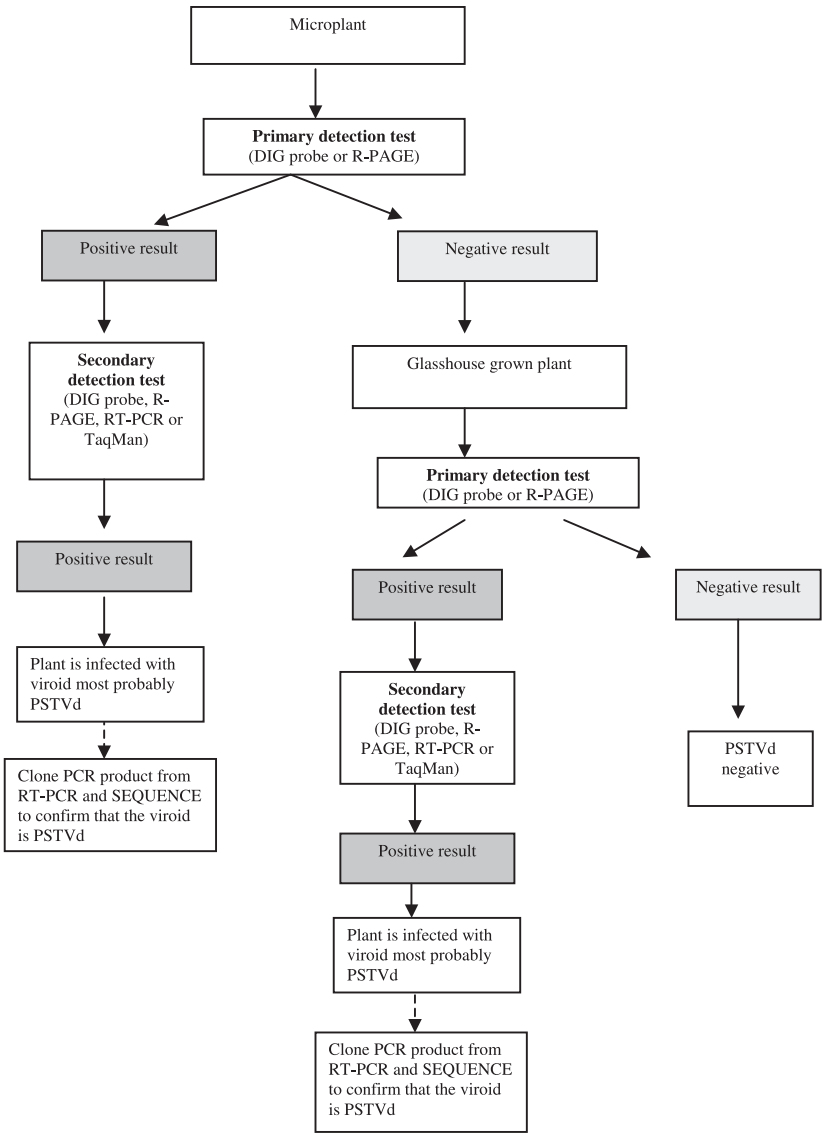

Fig. 6 Decision scheme for testing and confirming presence or absence of PSTVd in post-entry quarantine or potato nuclear-stock production systems based on micropropagation.

Secondary detection methods

In RT-PCR, the primer sequences will probably detect most PSTVd isolates currently listed on sequence databases and some other *Pospiviroid* spp. (Table 1). The method is very sensitive (0.0155 mg; 0.062 mg), but may miss some PSTVd isolates and other closely related viroids. There is a potential risk of false negatives. The PCR product can be sequenced to identify PSTVd. The method is only recommended as a primary detection method when testing is done for isolates that the primers/probe are known to detect (i.e. surveying as a result of an outbreak).

In TaqMan, the primer sequences will probably detect most PSTVd isolates listed on sequence databases and some other *Pospiviroid* spp. (Table 1). It is probably the most sensitive of the methods used in this protocol (0.0155 mg, 0.0155 mg) but, because it is uncertain whether all PSTVd isolates will be detected, TaqMan is not recommended as a primary detection method for post-entry quarantine or nuclear testing in nuclear-stock production. In addition other *Pospiviroid* spp. may not be detected. The method is only recommended as a primary detection method when testing is done for isolates that the primers/probe are known to detect (i.e. surveying as a result of an outbreak).

Sample extraction

Sample extraction is preferably done by grinding (e.g. in a mortar and pestle) rather than by expressing sap (e.g. in a Pollähne press) since lower concentrations of PSTVd may be obtained in expressed sap. However, when testing large numbers of samples, it may be more appropriate to use a Pollähne press. If sap is used, checks are needed to ensure that the viroid is still detected.

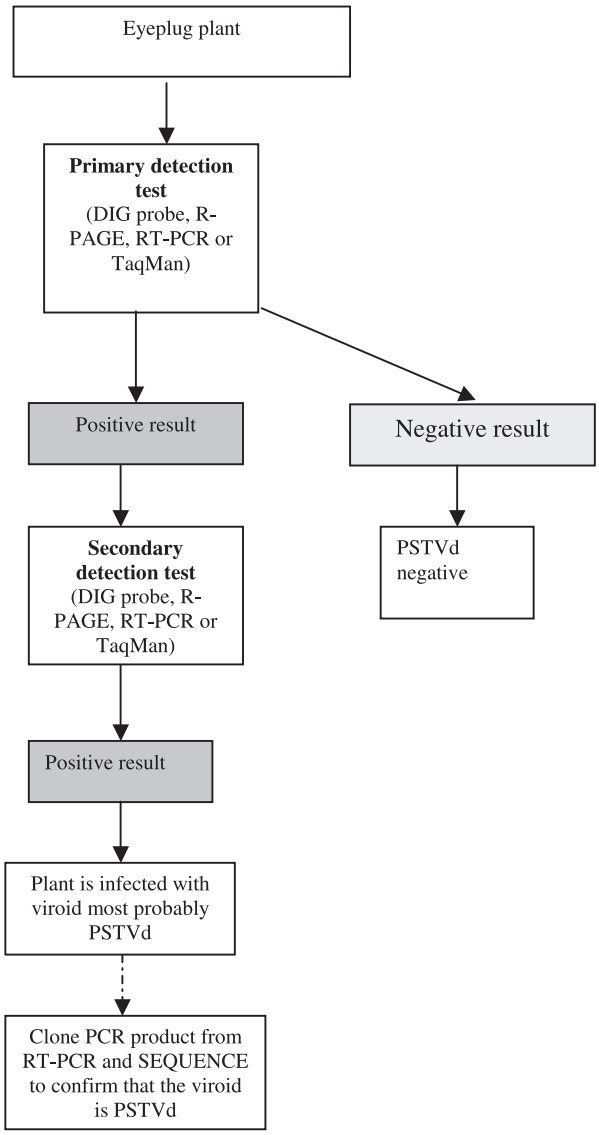

Fig. 7 Decision scheme for testing and confirming presence or absence of PSTVd in eyeplug plants post-harvest conducted as part of a survey where the isolate is known to be detected by the primers/probe used in RT-PCR and TaqMan.

Quality control

Controls

In general, negative potato controls should be used. In order to control the quality of sample preparation and the efficiency of detection, a PSTVd-positive potato control 1 : 10 of the amount of leaf tissue used per plant for the RNA extraction should be used as a standard. For example, where no bulking of samples from different plants is done, for 200 mg of sample used for RNA extraction, 20 mg infected leaf + 180 mg healthy potato tissue should be used for the positive control. If the positive control is not detected, the test should be repeated. The positive potato control should be grown under the same conditions as the plants for test. With all the methods described in this protocol, with the possible exception of R-PAGE, it should be possible to bulk samples 1 : 10. The positive control should be adjusted accordingly, e.g. 10 lots of 20 mg sample bulked for RNA extraction, 2 mg infected leaf + 198 mg healthy potato tissue. If this is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved. For RT-PCR and TaqMan, additional 'internal controls' may be used for primary detection. These are not essential where RT-PCR and TaqMan are used for confirmation since failure to detect should result in further investigation.

Confirmation of positive results

Excess RNA extracts and all plants from which they have been derived (subcultures in the case of microplants) should be kept until the result is confirmed or otherwise. For confirmation, the material should be sampled again. If bulking of samples has been done, each plant should be tested individually to locate the infected plant. A secondary detection method should be used for confirmation that the sample is viroid-positive (most probably PSTVd). For specific identification, the PCR product from the RT-PCR reaction should be sequenced and compared with other sequences on the database. If the sample cannot be confirmed as positive, other plants should be sampled, in case of sampling errors.

Possible confusion with other species

See 'Specificity of detection methods' above.

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 6 or 7, should have been followed.

Report on the diagnosis

The report on the execution of the protocol should include:

- information and documentation on the origin of the infected material
- the type of material in which found, e.g. microplants, glass-house plants grown from tubers, potato seeds
- why the material was being tested, e.g. post-entry quarantine, certification, survey
- the method used for primary detection and confirmation
- results obtained by the recommended procedures, with documentary evidence, e.g. photographs of gels (R-PAGE, RT-PCR), membrane or photograph (DIG Probe) or print-outs (TaqMan), including results for the appropriate negative and positive controls
- comments as appropriate on the certainty or uncertainty of the identification

If sequence information is obtained to prove that the viroid is PSTVd and not another viroid that may be infecting potato, this should also be reported.

Table 1 Results of BLAST search for RT-PCR and Taqman primer/pospiviroid sequence homology: probability of detection on basis of number and position of mismatches

Pospiviroid	No of sequences	RT-PCR	TaqMan
Chrysanthemum stunt viroid	15	None detected	None detected
Citrus exocortis viroid	40	None detected	None detected
Columnea latent viroid (Indian tomato bunchy top viroid)	1	Not detected	Not detected
Iresine viroid	1	Not detected	Not detected
Mexican papita viroid	9	All detected	Possibly detected, depending on isolate (few mismatches, at noncritical positions)
Potato spindle tuber viroid	71	68 detected 1 possibly not detected 1 (infectious viroid RNA replicon evolved from an *in vitro*-generated noninfectious viroid deletion mutant) not detected 1 (strain *S. commersonii*) not detected	59 detected 6 : 1 mismatch 5 : 2 mismatches 1 : 3 mismatches Of the 12 sequences with mismatches, three are from wild *Solanum* spp. Assay should still detect all the variants, as the mismatches are not in critical positions
Tomato apical stunt viroid	4	None detected	None detected
Tomato chlorotic dwarf viroid	1	Detected	Probably detected (3 mismatches at noncritical positions)
Tomato planta macho viroid	1	Not detected	Possibly detected (3 mismatches)

Further information

Further information can be obtained from: C. Jeffries (E-mail: colin.jeffries@sasa.gsi.gov.uk) or T. James (E-mail: tina.james@sasa.gsi.gov.uk), Scottish Agricultural Science Agency, East Craigs, Edinburgh, EH 12 8NJ, United Kingdom.

Acknowledgements

This protocol was originally drafted by: C. Jeffries and T. James, Scottish Agricultural Science Agency, Edinburgh (GB).

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Appendix 1. Digoxigenin-labelled RNA Probe[3]

Introduction

Digoxigenin (DIG) is a system for non-radioactive labelling (Podleckis *et al*., 1993). It offers the advantages of health and safety, that labelled probes can be stored for at least a year, and that hybridization solutions can be reused several times. The probe, not specific for PSTV, is based on a full-length monomer and may hybridize with other Pospiviroids to a greater (*Tomato chlorotic stunt viroid* (Singh *et al*., 1999)) or lesser extent (*Chrysanthemum stunt viroid*, *Citrus exocortis viroid* (A. Harness, Agdia, pers. comm.) depending on sequence similarity. If necessary, consult the Roche DIG online manual at http://biochem.roche.com/prodinfo_fst.htm?/prod_inf/manuals/dig_man/dig_toc.htm

Materials

Ames buffer: distilled water 160.0 mL; NaCl 11.7 g; $MgCl_2$ 0.4 g; sodium acetate 8.21 g; ethanol 40.0 mL; sodium dodecyl sulphate (SDS) 6.0 g. Dissolve NaCl first, then other salts, then ethanol, then SDS. Adjust to pH 6.0 with HCl or NaOH.

20 × SSC: NaCl 173.3 g; sodium citrate 88.2 g. Dissolve in 800 mL distilled water. Adjust to pH 7.0 with 10 M NaOH, make up to 1000 mL with distilled water and autoclave (or use Roche Molecular Biochemicals cat no. 1666 681).

10% SDS: sodium dodecyl sulphate (SDS) 50 g, in 500 mL distilled water.

Wash buffer 1: 20 × SSC 100 mL; 10% SDS 10 mL; make up to 1000 mL with distilled water.

Wash buffer 2: 20 × SSC 5 mL; 10% SDS 10 mL; make up to 1000 mL with distilled water.

RNase A: prepare a 10 mg/mL solution of RNase A in 10 mM tris-HCL pH 7.5, 15 mM NaCl. Store at −20 °C. Before use, heat the solution or an aliquot to 100 °C for 15 min and allow to cool. Store heat-treated RNase at 4 °C.

Materials from Roche Molecular Biochemicals: G Easy Hyb buffer (Catalogue number 1603 558); DIG Luminescent Detection Kit, shipped on dry ice (Catalogue number 1363 514); DIG Wash and Block Buffer Set (Catalogue number 1585 762); Hybridization Bags (Catalogue number 1666 649). Only for use when using a shaking waterbath. If using a hybridization oven, use hybridization tubes. If using a shaking incubator, small dishes can be used instead.

Sample size and positive and negative controls

For testing single plants, use a leaf tissue sample of 200 mg. For the positive control, use no more than 20 mg of PSTVd-infected tissue per 180 mg healthy tissue. The test is sensitive enough always to detect this level. If it is not detected, the test should be repeated. The test is sensitive enough to allow

[3]Based on protocol supplied by Agdia Inc., 30380 County Road 6, Elkhart, Indiana 46514 (US).

bulking of 10 plants (at least 20 mg of tissue per plant). For the positive control, use not more than 2 mg of PSTVd-infected tissue added to 198 mg healthy tissue. One or more positive controls should be used for each batch of extractions and applied to each membrane. If samples are being extracted on separate days and stored, a positive control should be done for each day's extractions. A negative potato control should also be used. Process positive samples last to avoid possible splash contamination of individual tubes.

Sample extraction

Grind the tissue sample in a small amount of Ames Buffer. Add the remaining buffer to a concentration ratio of 1 : 1.5 (sample weight:buffer volume), e.g. 200 mg of tissue per 300 µL of Ames Buffer. Transfer to a 1.5-mL microcentrifuge tube. Cover tubes and incubate at 37 °C for 15 min. Add an equal volume of research-grade chloroform to each tube, close caps tightly, and mix the contents thoroughly by shaking, vortexing, or inverting until an emulsion has formed. Centrifuge the tubes briefly to separate the contents into aqueous (top) and chloroform (bottom) layers, or place the tubes in a refrigerator (4 °C) to separate overnight. The layers should be clearly separated before proceeding since material from the interphase can cause false positives.

Spotting samples

Pipette 3 µL from the aqueous layer onto the membrane (Agdia) and air-dry the membrane at room temperature. Store the sample extracts at 4 °C. If it is necessary to retest any samples, the stored extracts may be used. Chloroformed samples can be retained for several months at least and a spotted, dried membrane may be retained in a dry location at room temperature for several years at least without affecting the results.

Hybridization/Detection

Having established an optimum exposure time for UV exposure, UV-crosslink the air-dry membranes on 20 × SSC wetted filter paper in a UV Crosslinker (or for a transilluminator type light box, place the dry membrane face down and expose to UV light, or bake the membrane at 80 °C in an oven for 2 h). Briefly centrifuge the tube containing the lyophilized DIG-labelled PSTVd probe (Agdia) before opening. Avoid RNase contamination (wear gloves). Resuspend the lyophilized DIG-labelled PSTVd probe in 100 µL DIG Easy-Hyb Buffer (Roche Diagnostics). The ratio of probe to hybridization-buffer volume will be stated on the tubes of probe supplied. Place the membrane in a glass hybridization tube[4]. Add 100 µL of resuspended DIG-labelled probe to 8 mL DIG Easy Hyb Buffer (Roche Diagnostics) and pour sufficient over the membrane to cover it (about 4 mL is needed for 100 cm^2 membrane). The remaining hybridization buffer with added probe can be stored (see 6.7). If the re-suspended probe is added directly to the membrane without dilution in DIG Easy Hyb Buffer, high background will result. Hybridize in a hybridization incubator (or shaking waterbath or shaking incubator) overnight at 55 °C.

For the following procedures, through incubation in Detection Buffer, the membrane should never be allowed to dry. Washes can be done in a heat resistant dish. The next morning carefully draw off the hybridization buffer and store in a sterile tube. The probe in hybridization buffer can be stored at –70 °C and re-used (defrost and denature at 65 °C for 15 min). Wash the membrane for 5 min at room temperature in 200 mL of Wash Buffer 1, then for 15 min at room temperature in 200 mL of Wash Buffer 1 containing 1 µg mL^{-1} RNase A. The addition of RNase A is essential in order to avoid false positives with healthy material. Wash the membrane twice, for 15 min per wash, at 65 °C in 200 mL of preheated Wash Buffer 2. Rinse the membrane in 50–100 mL Maleic Acid Buffer (100 mM maleic acid, pH 7.5; 150 mM NaCl, supplied as 10× Maleic Acid Buffer in the DIG Wash and Block Buffer Set, Roche Diagnostics) for 1 min at room temperature. Pour off the solution. Then block for 1–2 h at room temperature in 25 mL Blocking Solution per membrane (i.e. 2.5 mL of 10 × Blocking Solution + 22.5 mL 10 × Maleic Acid Buffer, supplied in the DIG Wash and Block Buffer Set, Roche Diagnostics) using an orbital shaker (or tilting table) at 100–150 rev min^{-1}. Do not pour off the solution. Centrifuge the anti-DIG-alkaline phosphatase solution (supplied in the DIG Luminescent Detection Kit, Roche Diagnostics) at 10 000–12 000 rev min^{-1} for 5 min, to remove small antibody aggregates that may be present and which can lead to speckling background. Add the anti-DIG-alkaline phosphatase directly to the Blocking Solution used in the previous blocking step at a dilution of 1 : 10 000, taking care not to add the anti-DIG-alkaline phosphatase directly onto the membrane. Use sterile techniques when handling the stock anti-DIG-alkaline phosphatase and CSPD substrate solutions. Incubate the membrane for 30 min (not more) at room temperature on the shaker (or tilting table). Pour off the solution and wash the membrane twice, for 15 min per wash, at room temperature in about 150 mL Maleic Acid Buffer. Use the orbital shaker at 50–80 rev min^{-1} (or tilting table). Dilute CSPD 1 : 100 in 0.5 mL of Detection Buffer (supplied in the DIG Luminescent Detection Kit, Roche Diagnostics. Detection Buffer is 100 mM Tris-HCl, pH 9.5; 100 mM NaCl). Wash the membrane once for 5 min at room temperature in 50–100 mL Detection Buffer. Place the wet membrane, sample side up, on an acetate sheet (or Cling Film) and dribble diluted CSPD all over the membrane using a pipette (about 0.5 mL CSPD per 100 cm^2 membrane). Carefully pick up the sheet and membrane and gently move around to disperse the substrate. Carefully place another acetate sheet (or Cling Film) over the membrane and using the side of fingers, gently remove air bubbles and further disperse the substrate. Place the membrane in an autoradiography cassette. In a dark room, place a film in the cassette. Firmly

[4]Alternatively, use hybridization heat-sealed bags and a shaking waterbath or a dish that can be sealed with 2 layers, important to avoid splitting, of cling film and placed in a shaker incubator. For small filters, it is possible to place a plastic weigh boat inside the dish and add the hybridization buffer to the weigh boat.

close the cassette, put away excess film and turn on the light. Expose membranes to film for 2.5–3.0 h at room temperature or 1.0–1.5 h at 37 °C and then develop the film.

The positive reaction is a very intense to easily visible spot. A faint spot is probably positive. A barely visible spot or spot outline is possibly positive or negative.

Appendix 2. Return Polyacrylamide Gel Electrophoresis

Introduction

Return-polyacrylamide gel electrophoresis (r-PAGE) is based on the difference in conformation of the viroid molecule under native and denaturing conditions (Reisner, 1987). The first run is performed under native conditions (temperature below the main transition temperature), when the electrophoretic mobility of the viroid is similar to that of linear nucleic acid molecules of the plant. However, during the return run under denaturing conditions (temperature above the transition temperature), the viroid assumes its circular form, which results in decreased electrophoretic mobility in comparison with linear molecules. Therefore a clear separation between the viroid band and plant nucleic acids is obtained. The viroid RNA is visualized by staining with silver nitrate. The method is not specific for PSTVd and will probably detect all other viroids.

Materials

Sample extraction

Extraction buffer: LiCl 29.69 g; glycine 4.50 g. Make up to 180 mL with distilled water and, using 10 M or more LiOH, adjust to pH 8.8. Make up to 200 mL with distilled water and if necessary adjust to pH 9.0 using LiOH. The buffer should be clear, with no yellow tinge.

PEG 6000 and PEG Scoop: grind the PEG to a fine powder using a mortar and pestle or a coffee grinder. A 'PEG scoop' can be made to enable rapid dispensing of 0.02 g of powdered PEG into the Eppendorf tubes[5].

Phenol/chloroform mix: phenol crystals 500 g; chloroform 500 mL; octan-1-ol 20 mL; hydroxyquinolene 1.0 g. First mix the phenol and chloroform and leave to dissolve for a few hours before adding other substances. The mix can be stored in a fume cupboard for several months.

10% SDS: sodium dodecyl sulphate (SDS) 40.0 g, made up to 400 mL with distilled water.

Ethanol/ether pellet wash: ethanol 50 mL; ether 50 mL. Store in a screw top bottle in a freezer.

Gel preparation

Acrylamide solution: acrylamide 30.0 g; bis-acrylamide 0.7 g. Make up to 100 mL, dissolve and filter through Whatman filter paper no. 1 paper and use immediately.

Ammonium persulphate: ammonium persulphate 0.1 g; add 900 µL distilled water to a weigh boat and mix gently. Use immediately.

Gel mix: acrylamide stock 83. 5 mL; 5 × TBE 22. 4 mL; temed 3. 4 mL; distilled water 390. 7 mL. Store in the dark at 4 °C.

5 × TBE buffer: bought as a premixed powder (tris-borate-EDTA buffer) (Sigma Cat. No. T 3913). Dissolve contents of pouch in 1 L of distilled water.

Loading and running

Loading buffer: sucrose 12 g; xytene-cyanol-FF 0.125 g; distilled water 30 mL. Autoclave after preparation.

Running buffer: 5X tbe buffer 44 mL; make up to 1 L with distilled water.

Silver staining

Fixing buffer: ethanol 20 mL; acetic acid 1 mL; distilled water 179 mL.

Silver stain: $AgNO_3$ 0.19 g; distilled water 200 mL.

Developer solution: NaOH 1.5 g; formaldehyde 400 µL; sodium borohydride 0.0088 g; make up to 200 mL with distilled water.

Stop solution: Na_2CO_3 1.5 g; distilled water 200 mL.

Sample size and positive and negative controls

As for Appendix I[6]. The bulking rate may have to be reduced until detection is achieved.

Sample extraction

The purpose of the two-step PEG (polyethylene glycol) extraction method described is to extract low-MW RNA from the sample to yield a very clean preparation. This means that very thin gels can be used, which increases the sensitivity of detection.

Grind the tissue samples, e.g. by mortar and pestle with a small pinch of acid-washed sand (white quartz −50 + 70 mesh), in 20 µL of 10% SDS and 180 µL of LiCl Extraction Buffer. Add 400 µL of Phenol/Chloroform Mix and grind further.

[5]Using a felt tip pen, mark the level corresponding to 0.02 g PEG on the outside of the tube. Remove the PEG and using a scalpel cut off the tip of the Eppendorf at the 0.02 g mark. Insert the point of a disposable scalpel into the plastic wall of the tip to act as a handle for the scoop. The operator should practice using the scoop, repetitively weighing out from the scoop until 0.02 g can be measured reliably.

[6]The control is not a control for individual tubes. It acts as a control for factors that could affect the complete batch of tests, such as buffers and final salt concentrations. It is also a visual cross-reference for evaluating results. A vital quality control check for each sample during the extraction procedure is for the operator to be fully aware of the appearance of each step and in particular the final pellet. Although very small, the final pellet should be visible for the test to be valid. Any sample with no pellet should be re-sampled and tested.

Carefully pour the slurry into a 1.5-mL microcentrifuge tube and centrifuge at about 12 000 rev min^{-1} for 20 min. For each sample, set out a further two microcentrifuge tubes each containing 0.02 g of PEG, MW 6000. For speed, add the PEG using a 'PEG scoop' (Table 1). After centrifugation, and using a clean pipette tip for each sample, carefully draw up 180 µL of supernatant and add to one of the two tubes containing PEG (if the sample is less than 180 µL, make up to 180 µL with LiCl buffer). Vortex the tubes until all the crystals have dissolved. It may be helpful to leave the tip in the tube while vortexing to aid mixing. Once mixed, remove the tips and centrifuge at about 12 000 rev min^{-1} for 30 min. Draw up the supernatant, taking care not draw up any of the glycerine-like pellet as this contains the high MW fraction and may cause overloading of the gels. Add the supernatant to the second tube containing PEG. Vortex the tubes until all the crystals have dissolved. It may be helpful to leave the tip in the tube while vortexing to aid mixing. Once mixed, remove the tips and centrifuge at about 12 000 rev min^{-1} for 30 min. A very small pellet will form on one side of the tube. Using a fine-tip plastic pastette, draw off the supernatant and discard. Add about 300 µL of cold Ethanol/Ether Wash to each tube. The remaining PEG will sink to the bottom of the tube. Using another fine-tip pastette, draw up the wash from the bottom of the tube. The pellet may dislodge from the side of the tube, but provided it is not drawn up into the pastette, it will remain intact. The final pellet should be visible. Resample and re-extract any samples with no pellet. Leaving the microcentrifuge tubes open, air-dry the pellets (or dry in a vacuum oven at room temperature for about 5 min, avoiding over-drying).

Gel preparation

The methods described in this protocol are for 1-mm-thick gels 9 × 8 cm used in vertical gel electrophoresis equipment that will operate at 250 V (25 Vcm^{-1}), and will withstand heating to 70 °C. Make up the gels the day before use, according to the instructions supplied with the gel electrophoresis equipment. Clean the glass plates with alcohol before assembly. Insert the comb into the gel cassette. The volumes specified are for use with Atto gel electrophoresis equipment[7] and use of different equipment may require adjustment of the volumes. For different-sized gels, the voltage should be adjusted. The approximate voltage to run at = 25 V × (the length of the gel + the distance between the gel and the negative electrode + the distance between the gel and the positive electrode). Increased voltage may result in localized heating in tanks without integral cooling, causing deformation of the gels. Some experimentation may be required to achieve the optimum voltage.

To prepare two gels, add 134 µL of ammonium persulphate to 20 mL of Gel Mix and stir rapidly. Using a fine-tip plastic pastette, pour the Gel Mix between the vertical plates until the solution is just over the top of the comb (this prevents shrinkage of the wells as the gel sets). Leave the gels to set for 1 h then wrap the cassettes in a good quality cling film (e.g. Saran Wrap) and store at 4 °C. Gels can be stored for 2 days, but storage for longer than this can reduce resolution of the resulting bands and therefore sensitivity at low concentrations of nucleic acid.

Loading and running the gels

The gels are run at a constant 250 V (or other voltage, see above). In return PAGE the first run of the gel is done at room temperature and the second (or reverse) run is done at a high temperature. In order to prepare for the reverse run, heat a benchtop incubator or oven to 70 °C and also heat 500 mL of Running Buffer to 70 °C. Re-suspend each air dried pellet (section 4) in 10 µL of distilled water, add 3 µL of Loading Buffer to each sample and vortex briefly. If only one gel is being run in a two-gel unit, the blank cassette should be used. Remove the clamps, comb and rubber gasket. Check that all the well tops are clear of gel, since this will prevent loading. Fix the cassette into the equipment as described in the instructions supplied with the equipment. For the first run (at room temperature), add sufficient Running Buffer (also at room temperature) to cover the bottom of the gels, clear any air bubbles by tilting the equipment until the bubbles surface, then fill the tank. Fill the top reservoir and gently wash the wells using a fine-tip pastette. Before loading the samples, trail a little Loading Buffer across the wells to make them visible for loading. Using a clean tip for each sample, gently load 13 µL of each sample into each well. Orientate the gel the same way each time, usually by placing the control at one end of the gel.

After loading, connect the electrodes, and run the gel at constant 250 V (or other voltage, see above), until the dye front is 1 cm from the bottom of the gel (about 1 h for 9 × 8 cm gels). Turn off power, pour off the Running Buffer and replace with hot Running Buffer (70 °C). Remove air bubbles as before, reconnect electrodes, reverse the polarity, and place the electrophoresis tank inside an incubator or water bath at 70 °C. Run the gel at constant 250 V (or other voltage, above), until the dye front is at the top of the gel (about 30 min for 9 × 8 cm gels).

Silver staining

Carefully prise the gel plates apart. If the gel has remained on the spacer plate, run a scalpel blade down the side of the spacer to prevent the gel from tearing. Hold the plate gel-side down over a container containing enough Fixing Buffer to cover the gel(s) adequately (about 200 mL), loosen one corner of the gel so that it slowly pulls away into the buffer. Place the container with Fixing Buffer and gel(s) onto an orbital shaker and gently shake for 15 min. Rinse the gel(s) once with distilled water, add sufficient Silver Stain to cover the gel(s) as before and gently shake for 20 min. Thoroughly wash the gel(s) 3 times with distilled water and add sufficient Developer Solution to cover the gel(s) as before. Leave in the developer until the PSTVd

[7]Atto Dual Mini Slab Kit (Catalogue No AE6400); Extra set of plates (*Catalogue No* AE6420); PS304 or PS503 Power Pack (Catalogue no. 160 000 or 172 000). Genetic Research Instrumentation Ltd, Gene House, Queensborough Lane, Rayne, Braintree CM7 8TF (GB).

controls begin to appear, then rinse the gel once with distilled water and add sufficient Stop Solution to cover the gel(s) as before. If the gels are to be stored, remove them from the Stop Solution and carefully wrap in good quality cling film (e.g. Saran Wrap). Store at 4 °C.

Interpretation

In positive samples, the viroid band should appear as a distinct band in the lower two thirds of the gel with no other bands present. The top of the gel should be heavily stained where the denatured nucleic acids have migrated to form dense bands. If bands appear in the lower gel in any of the samples, further investigation should be carried out to confirm whether the samples are positive.

Appendix 3. Two-step reverse transcriptase-polymerase chain reaction (RT-PCR) (incorporating internal controls)

Introduction

The method[8] described is based on that of Seigner (2000). The method is not specific for the detection of PSTVd. The primers will probably allow detection of most PSTVd isolates listed on sequence databases and some other closely related *Pospiviroid* spp. In addition to describing an assay for the specific detection of PSTVd, the protocol also includes a control assay, designed against ribosomal 5S gene sequences. Since these sequences are expressed in all plant tissue types, they can be used to monitor the amount and quality of nucleic acid extracted from each sample, thus helping to avoid the risk of false negatives. However, it should be noted that the assay is not an RNA-specific control and it will also detect DNA that may be copurified through the RNeasy extraction method. Despite this limitation, the use of a control assay significantly enhances the overall reliability of PSTVd detection, as it does indicate that nucleic acid has been extracted and that it is free of inhibitors.

General measures to reduce the risk of contamination

Powder-free gloves should be worn all the time. Latex gloves are sufficient for preventing sample contamination and for protection against most reagents. Nitrile gloves should be worn when using ethidium bromide. Use separate, special labelled pipettes for ethidium bromide. Use sterile, nuclease-free, ultra-pure water if not otherwise mentioned in the text. Use clean, disposable plasticware at all stages. Grinding bags should be clean and 'untouched'. Microcentrifuge tubes should be autoclaved and stored in jars/bags. Plastic ware which is used more than once (plastic pestles, racks) should be decontaminated overnight in a solution containing an oxidizing agent (e.g. HOCl, Cl_2) and rinsed thoroughly with water afterwards. Glassware should be cleaned in hot water with detergent added and afterwards incubated at 180 °C for 2 h or more. Pipette tips should be sterile 'filter tips' and preracked or racked and autoclaved. Pipettes should be regularly decontaminated with 'RNase Away' solution (Fisher Scientific). Use different sets of pipettors for RNA extraction, for pipetting RT- and PCR-components, for pipetting RNA- and cDNA-samples and for loading the electrophoresis gel. Use different centrifuges for RNA extraction and for preparing samples for the RT-reaction and PCR.

Materials

TAE-Electrophoresis buffer 50 ×, for preparing 1 L: 242 g Tris (2 M), 57 L ml glacial acetic acid (1 M), 37.23 g Na-EDTA. $2H_2O$ (0.1 M), pH 8.0; working solution is 1 × concentrated.
Loading buffer 6 ×: 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose in 1 × concentrated TAE buffer.
Primers for PSTVd PCR (Weidemann & Buchta, 1998):
Downstream primer: 5′-CCC TGA AGC GCT CCT CCG AG-3′
Upstream primer: 5′-ATC CCC GGG GAA ACC TGG AGC GAA C-3′
Primers for internal control RT-Reaction (Kolchinsky *et al*., 1991):
Downstream primer: PLANT-UNI 2 5′-TGG GAA GTC CTC GTG TTG CA-3′
Upstream primer PLANT-UNI 1 5′-TTT AGT GCT GGT ATG ATC GC-3′

RNA extraction
For RNA extraction, use the QIAGEN RNeasy plant mini kit. For extraction, use microcentrifuge tubes which are used only once. Before starting the RNA extraction, aliquot sufficient buffer from the bottles supplied by QIAGEN in order to avoid cross-contamination of the original solutions during the extraction procedure. Cross-contamination of samples is a danger when opening the microcentrifuge tubes and spin columns, so a new cotton pad should be used for opening each microcentrifuge tube and spin column. When loading the RNeasy spin columns, do not touch the silica-gel membrane. Use a new collection tube for each step during the RNA extraction. Extraction and RT-PCR should be performed at different places. All centrifugation steps should be done at room temperature, which ensures proper binding of RNA to the silica-gel membrane of the spin columns.

Samples for extraction will include the samples under investigation and negative and positive controls. These may be plant material or, for the positive controls, pre-extracted and stored RNA, which is added to plant sap/leaf tissue and re-extracted. Fresh or frozen material (stored at –20 to –80 °C) can be used. Pre-freezing (at –80 °C or in liquid nitrogen) may help to facilitate grinding of specific tissues but, if using frozen material, it is crucial to avoid any thawing before the addition of Buffer RLC in order to prevent any degradation of RNA by

[8]Supplied by L. Seigner, Bayerische Landesanstalt für Landwirtschaft, Institut für Pflanzenschutz, D-85 354 Freising, Lange Point 10, Germany Buffer RPE is supplied as a concentrate; ensure that 4 vol. of ethanol are added before use.

RNAses. It is advisable to weigh the samples before freezing them.

For small amounts of tissue, place 50 mg plant material (fresh weight) in a microcentrifuge tube, add 450 µL Buffer RLC containing β-mercaptoethanol (β-ME), grind thoroughly with a micro pestle. β-ME is added to a final concentration of 1% (v/v) to an aliquot of Buffer RLC before use. Buffer RLC is stable for one month after addition of β-ME. If larger amounts of plant material are available, use 500 mg to maximize the likelihood of detecting PSTVd. In this case, place 500 mg (fresh weight) in 10 × 10 cm homogenization bags with an integrated plastic inlay, add 4.5 mL buffer RLC containing β-ME (see above) and grind thoroughly using a small hand roller. Transfer 450–700 µL of the extract directly to a QIA shredder spin column supplied with the RNeasy Kit. Do not contaminate the pipette by touching the polyethylene grinding bags. The QIA shredder step ensures a further homogenization of the plant material and removal of cellular debris.

Centrifuge at maximum speed for 2 min. Carefully transfer the supernatant of the flow-through fraction to a new microcentrifuge tube and only use this for subsequent steps. Add 0.5 vol. ethanol (96–100%) and mix immediately by pipetting or vortexing (taking care to avoid contamination). Pipette the sample, including any precipitate which might have formed after the addition of ethanol, to an RNeasy column placed in a 2-mL collection tube and centrifuge for 15–60 s at 8000 *g*. Discard the flow-through and the collection tube. Place the column into a new 2-mL collection tube and open the tube. Add 700 µL buffer RW1 (supplied) to the column. Close the tube gently and centrifuge as above to wash the column. Discard the flow-through and the collection tube. Place the RNeasy column into a new 2-mL collection tube. Add 500 µL Buffer RPE9 to the column and centrifuge as before. Discard the flow-through and the collection tube. Place the column into a new 2-mL collection tube, open the tube and add another 500 µL Buffer RPE to the column and centrifuge for 2 min at 8000 *g* to dry the column. Following the centrifugation, remove the RNeasy spin column from the collection tube carefully so that the column does not touch the flow-through as this will result in a carryover of ethanol. To elute the RNA, transfer the RNeasy column to a new 1.5-mL collection tube (supplied). Pipette 50 µL RNase-free water (supplied) directly onto the RNeasy silica-gel membrane. Do not touch the membrane. Close the tube and centrifuge for 1 min at 8000 *g* to elute. The RNA solution can immediately be used for the RT-reaction or can be kept at –20 °C until used.

Reverse Transcriptase Reaction and Polymerase Chain Reaction (RT-PCR) (Two step protocol)

Test each sample twice. Run 2 separate RT reactions, one for the PSTVd, the other for the internal control.

Measures to reduce the risk of contamination and hints
Master mixes should be set up in a dedicated cabinet or clean bench, which should be cleaned and decontaminated regularly using oxidizing solutions or 'RNase away' solution (Fisher Scientific) and/or UV-C-light. Only use the pipettors dedicated for each process, e.g. master mix set-up or DNA spiking (not the ones used for gel loading or extraction, etc.). Decontaminate pipettes regularly. Only use filtered tips and use a fresh tip for each pipetting step. Close reagent/sample tubes once desired aliquot has been removed. Wear powder-free gloves and change them if they become contaminated. Only use clean sterile plasticware. All new reagents should be aliquoted before regular usage, in a dedicated PCR cabinet, using a clean pipettor set. Aliquoted stocks should be kept in a separate box from all other reagents. All reagents should be kept in a clean drawer in a –20 °C freezer and should be returned to the freezer immediately after use. Plant material as well as cDNA/RNA samples should not be stored in this drawer. Thaw reagents just before use, mix all reagents well (by inverting tubes a number of times or for small volumes by flicking the tube) especially the RNase-inhibitor and the Reverse Transcriptase, because they are delivered in buffer containing glycerol. After mixing, spin briefly (5 s) in a microcentrifuge. dNTPs should not be thawed more than 2–3 times. All reaction tubes should be labelled carefully and/or a carefully designed scheme for the arrangement of the samples in the thermocycler block should be used in order to avoid confusion over sample identity.

Setting up the Reverse Transcriptase Reaction (RT-reaction)
Use a special set of pipettors for RT components and a separate pipette for cDNA samples. Do the calculations (see below) for preparing the RT-master mix before starting. Pre-cool centrifuge and rotor at 4 °C. RNA from known healthy and infected samples are used as controls for RT-reaction. Arrange the correct number of 0.2-mL PCR tubes in a suitable rack. Include enough tubes for each sample and all the controls, in duplicate (each sample is tested twice). Two separate RT reactions are run, one for the PSTVd, the other for the internal control.

Denaturation
Pipette carefully 5 µL of PSTVd or rRNA downstream primers 4 µM (primer for PSTVd RT-reaction: 5′-CCC TGA AGC GCT CCT CCG AG-3′ (Weidemann & Buchta, 1998), primer for Internal control RT-Reaction PLANT-UNI 2: 5′-TGG GAA GTC CTC GTG TTG CA-3′ (Kolchinsky *et al*., 1991)) into the tubes and add 5 µL of each RNA sample (take care to avoid any contamination), vortex and spin briefly (5 s) in a microcentrifuge. Put the tubes in a preheated thermocycler with heated lid and incubate at 90 °C for 5 min to completely denature the RNA and primers. Immediately afterwards, spin briefly (5 s) in a precooled centrifuge (4 °C) and put the tubes on ice or on a precooled rack on a precooled cooling unit (–20 °C). It is important to keep the samples at low temperature to avoid any renaturation (forming new secondary structures) of viroid-RNA and primers.

Preparation of the Reverse Transcriptase (RT)-master mix
During the denaturation step, prepare the RT-master mix (sterile nuclease-free, ultra-pure water 1.9 µL; Superscript II Reverse Trancriptase buffer; supplied with Superscript II RT, Invitrogen) 4 µL; DTT 0,1 M (supplied with Superscript II RT,

Invitrogen) 2 µL; RNAsin (Promega) (40 U µL^{-1}) 0.5 µL; dNTPs (25 mM each) 0.8 µL; Superscript II Reverse Transcriptase (Invitrogen) 200 U µL^{-1} 0.8 µL; volume of RT mix 10 µL; total volume + primer and sample 20 (µL). Remove all reagents from the freezer with exception of the Superscript II-RT (Invitrogen), and allow to thaw. For each sample, 10 µL master mix is required. Make sufficient mix for duplicates of each reaction, i.e. all the samples to be tested, a positive control (at least one), a negative control (at least one). Add one extra volume to allow for pipetting errors. Add the reagents in the order and quantity shown above and keep the tube with the master mix cooled. RNase-inhibitor should be added according to the manufacturer's instructions. Remove the Superscript II-RT from the freezer immediately before pipetting, mix by flicking the tube or pipetting up and down and spin briefly. Dispense the appropriate amount of Superscript II-RT. After completion, close the tube with the RT master mix, mix thoroughly, centrifuge briefly and put on ice. Continue with the cooled samples immediately after the denaturation step. Carefully add 10 µL of RT-master mix to each sample tube. Mix by vortexing and centrifuge briefly in a precooled centrifuge (4 °C). Proceed immediately with the RT-reaction (see below).

RT-reaction

Place the samples into the preheated thermocycler (50 °C) with the heated lid enabled. Incubate for 1 h at 50 °C for cDNA synthesis (50 °C is necessary for proper denaturation of the viroid RNA during the incubation). Then incubate 3 min at 95 °C to destroy the activity of the Superscript II-RT, and centrifuge briefly. Continue with the PCR or keep the cDNA samples at –20 °C until further use.

Polymerase Chain Reaction (PCR)

Setting up the Polymerase chain reaction (PCR)
Use a special set of pipettors for PCR components and a separate pipette for cDNA samples. No precooling of the centrifuge, rotor and Thermocycler to 4 °C is needed, because AmpliTaq Gold DNA Polymerase (Applied Biosystems) is only activated after being heated. Use cDNA from known healthy and infected samples, as well as nuclease-free ultra-pure water, for positive and negative controls, respectively. Perform the calculations (see below) for preparing the PCR master mix before starting (20 µL master mix per sample). Arrange the appropriate number of PCR tubes (duplicates for each cDNA sample and for the controls at least one).

Preparation of the PCR master mix
Remove all reagents from the freezer, with the exception of the AmpliTaq Gold Polymerase (Applied Biosystems), and allow to thaw. Add the reagents in the following order and quantity, keeping the tube with the master mix cooled: sterile nuclease-free, ultra-pure water 9.57 µL; AmpliTaq buffer (without $MgCl_2$) (Applied Biosystems) 2.2 µL; $MgCl_2$ (Applied Biosystems) 25 mM 1.32 µL; DNTPs (1 mM) 4.4 µL; downstream primer (20 µM) 1.1 µL; upstream primer (20 µM) 1.1 µL. Remove the AmpliTaq Gold DNA Polymerase (5 U µL^{-1}) from the freezer. Mix the reagents by flicking the tube, or pipetting up and down, and spin briefly. Pipette the enzyme (0.11 µL) into the master mix. After completion, close tube, mix thoroughly, centrifuge briefly and leave on ice.

Carefully pipette 20 µL PCR master mix into each PCR tube and then add carefully 2.2 µL of cDNA sample (take care to avoid contamination). Close the tubes, mix, spin briefly and put the tubes into the thermocycler with a heated lid. Start the thermocycler programme for PCR as follows: activate AmpliTaq Gold DNA Polymerase + denature cDNA, 94 °C for 9 min 15 s; 40 cycles of 94 °C 45 s, 62 °C 45 s, 72 °C 60 s (for the transition from 62 °C to 72 °C, ramping 1 °C per 4 s); 72 °C 10 min. This thermal cycler programme is mainly according to Weidemann & Buchta (1998) with a single modification (activation of AmpliTaq Gold DNA Polymerase by heating prior to PCR). When the PCR is finished, centrifuge briefly and continue with gel electrophoresis (see below) or keep the samples at –20 °C until further use. The PSTVd PCR product has a length of 359 bp. The rRNA product from potato has a length of about 260 bp.

Agarose gel electrophoresis

Weigh the agarose for preparing a 1.5% (w/v) gel (5 mm thick). Place the agarose into an Erlenmeyer flask and pour in the appropriate volume 1 × concentrated TAE-buffer (diluted from 50 × concentrated TAE stock solution). Dissolve agarose by heating the suspension, cool on a magnetic stirrer to about 55–60 °C and carefully add the correct amount of ethidium bromide stock solution (0.5 µg ethidium bromide per mL gel solution). Pour the gel solution into a prepared, UV-light-transmissible electrophoresis tray, set in the comb to form the sample wells (4 mm deep, 3 mm broad).

Take 10 µL of each PCR sample, add 2 µL of 6 × concentrated loading buffer and mix. Place the gel tray carefully into the electrophoresis chamber, and carefully pipette 9 µL of the samples mixed with loading buffer into the wells of the agarose gel. The first and last lane of each gel at least should be loaded with a DNA standard according to the manufacturer's instructions. Run at 70–120 V until the marker dye has moved about 6 cm. Remove and blot.

A digital camera system is recommended to document the results photographically.

Appendix 4 TAQMAN

Introduction

This method combines RT-PCR with real-time fluorescent detection (Mumford *et al*., 2000). The primers/probe will probably allow detection of most PSTVd isolates listed on sequence databases and some other closely related *Pospiviroids* (e.g. *Tomato chlorotic dwarf viroid*).

In addition to describing an assay for the specific detection of PSTVd, this protocol also includes a control assay, designed against plant cytochrome oxidase (COX) gene sequences. As COX is a universal gene expressed in all plant tissue types, it can be used to monitor the amount and quality of nucleic acid extracted from each sample, thus helping to avoid the risk of false negatives. However, it should be noted that the COX assay is not an RNA-specific control and it will also detect DNA that may be copurified by the CTAB extraction method. Despite this limitation, the use of a control assay significantly enhances the overall reliability of PSTVd detection, as it does indicate that nucleic acid has been extracted and that it is free from inhibitors.

Primers and probe sequences can be obtained from Central Science Laboratory, York (GB).

Nucleic acid extraction

This follows the CTAB method, adapted from Lohdi *et al.* (1994). At all stages clean, disposable plasticware should be used. Grinding bags should be clean and unhandled, microcentrifuge tubes should be autoclaved and stored in jars or bags. Pipette tips should be preracked (e.g. ATLAS tips, Alpha laboratories) or racked and autoclaved (loose non-filter tips). Only a dedicated, extraction pipettor set should be used. Samples for extraction will include the samples under investigation and the negative and positive RNA controls. These may be plant material or for the positive controls pre-extracted and stored RNA which is added to plant sap or leaf tissue and re-extracted. Place tissue, 100–200 mg, in 10 × 15 cm 500 gauge polythene bag and grind using a small hand roller. Pre-freezing (at –80 °C or in liquid nitrogen) may help with the grinding of some tissues. Grind until the tissue forms a smooth paste. Add 1–2 mL (10 vols) of CTAB Extraction Buffer (CTAB 20 g, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, NaCl 81.8 g, distilled water to 1 L, autoclave and store at room temperature; add 1% Na_2SO_3, 2% PVP-40 fresh to aliquot of stock buffer immediately prior to extraction) and mix thoroughly using the roller.

To ensure that RNAses do not degrade the PSTVd RNA, grinding and add buffer within 20–30 s. Decant the ground sap into a 1.5-mL microcentrifuge tube and incubate sap at 65 °C for 30 min. Centrifuge tubes at about 13 000 rev min^{-1} in a microcentrifuge for 5 min (at room temperature). Remove 700 µL of clarified sap, place in a new microcentrifuge tube, add 700 µL of chloroform:isoamyl acetate (IAA) 24 : 1 and mix to an emulsion by inverting the tube. Centrifuge as before. Carefully remove 500 µL of upper (aqueous) layer and transfer to a new tube. Add 500 µL of chloroform:IAA (24 : 1), mix and centrifuge as before. Remove the aqueous layer, taking extra care not to disturb the interphase. Add a 0.5 volume of 5 M NaCl and an equal volume of ice-cold isopropanol (optionally, add 1 µL of dextran blue at 20 mg mL^{-1} in water). Mix well and incubate at –20 °C overnight. Centrifuge for 10 min as before to pellet the nucleic acid. Resuspend the pellet in 200 µL of TE Buffer containing 1% SDS (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% sodium dodecyl sulphate). Add 100 µL of 5 M NaCl and 300 µL of ice-cold isopropanol. Mix well and incubate at –20 °C for 30 min. Centrifuge for 10 min as before to pellet the nucleic acid. Decant off the salt and isopropanol and wash the pellet by adding 400 µL 70% ethanol and centrifuge for 4 min. Decant off the ethanol and dry the pellet to remove residual ethanol. Resuspend the pellet in 100 µL of nuclease-free, ultrapure water.

Setting up TaqMan assays

At all stages while setting up reactions, precautions should be taken to avoid contamination of samples and reagents. All TaqMan reagents (Gold RT-PCR Kit; Applied Biosystems, N8080232) should be stored at –20 °C. Thaw before use, keeping enzymes on ice. Mix all reagents well (by inverting tubes a number of times or for small volumes by flicking the tube) and spin briefly (5 s) in a microcentrifuge.

Take two microcentrifuge tubes (either 0.5- or 1.5-mL as appropriate) and separately make up first-stage (7.5 µm reverse primer 1.0 µL, sterile nuclease-free ultra-pure water 3.0 µL, RNA 1.0 µL) and second-stage (10 × PE *Taq* Gold Buffer A 2.5 µL, 25 mM $MgCl_2$ 5.5 µL, 6.25 mM dNTPs 2.0 µL, 7.5 µM forward primer 1.0 µL, TaqMan probe (FAM labelled) 0.5 µL, MMLV Rtase 0.05 µL, AmpliTaq Gold DNA Polymerase 0.125 µL, sterile nuclease-free ultra-pure water 8.325 (µL) master mixes. Make sufficient mix for duplicates of each reaction, i.e. all the samples to be tested, an RNA positive control (at least one), a negative water control (at least one), a negative control of uninfected RNA (at least one). Add one extra volume to allow for pipetting errors. Add the reagents in the order and quantity shown above. After completion, close tube and leave on ice. Arrange the correct number of 0.2-mL PCR tubes in a suitable rack. Add 1.0 µL of total RNA extract or control material to each tube. Add 4 µL of First-Stage reaction mix to each tube, close the lids and transfer tubes onto the thermal cycler. Run programme 'denature 95' (95 °C, 3 min, 1 cycle). Allow tubes to reach 4 °C and chill for 5–10 min before continuing. Meanwhile, add 20 µL of Second-Stage reaction mix to each well of a 96-well PCR plate. Once denaturation step has finished, pipette the contents of each First-Stage mix tube into the appropriate Second-Stage mix well, ensuring that the mix is dispensed into the bottom of each well. Cap all the filled wells. Transfer the plate to the ABI Prism 7700 machine.

For cytochrome oxidase, only the Single-Master mix is required. The denaturation step is not required. RNA/control is added direct to 24 µL of master mix, containing all the assay reagents (10 × PE *Taq* Gold Buffer A 2.5 µL, 25 mM $MgCl_2$ 5.5 µL, 6.25 mM dNTPs 2.0 µL, 7.5 µM Forward COX primer 1.0 µL, 7.5 µM Reverse COX primer 1.0 µL, COX TaqMan probe (VIC labelled) 0.5 µL, AmpliTaq Gold DNA Polymerase 0.125 µL, MMLV Rtase 0.05 µL, sterile nuclease-free ultrapure water 11.325 µL, RNA 1.0 µL).

Running TaqMan reactions

Follow the manufacturer's instructions.

Fig. 1. Dwarfed plant with upright growth and rugose leaves cv. Norgold Russet. 3rd generation infection. Photo: SA Slack.

Fig. 2. Symptomless infection cv La Chipper. 3rd generation infection. Photo: SA Slack.

Fig. 3. Spindle tubers with deep eyes cv. Russet Burbank (far right healthy). Photo: SA Slack.

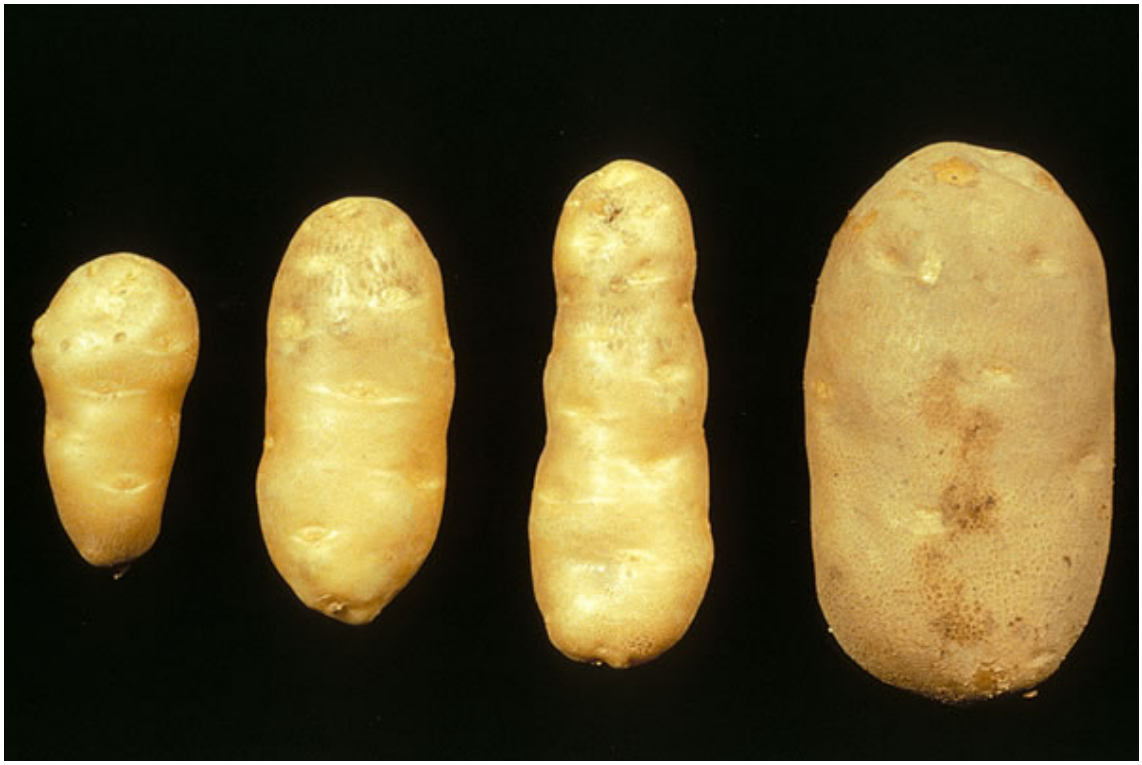

Fig. 4. Spindle tubers top, healthy bottom. Photo: J Bryan.

Fig. 5. Effect of increasing generation of infection on severity of tuber symptoms. Healthy tubers (top row), current season infection (2nd row) tubers 3rd generation infected tubers (3rd row) cvs. Norgold Russet and Russet Burbank. Photos: SA Slack.