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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 7/32

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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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

Plum pox potyvirus

Specific scope
This standard describes a diagnostic protocol for *Plum pox potyvirus*.

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

Sharka (plum pox) is considered one of the most devastating diseases of stone fruits in terms of agronomic impact and economic importance (Dunez & Sutic, 1988; Németh, 1994). The disease is very detrimental in apricot, peach and plum trees because it produces reduced quality and premature dropping of fruits. It is caused by *Plum pox virus* (PPV), a member of the genus *Potyvirus* in family *Potyviridae* (López-Moya & García, 1999).

The PPV epidemic originated in eastern Europe. The disease was described for the first time around 1917 on plums and in 1933 on apricots in Bulgaria (Atanasoff, 1932, 1935). Since then, the virus has progressively spread to a large part of the European continent, around the Mediterranean basin and Near and Middle East. It has been found also in India and in America (Chile, USA and Canada).

The introduction of infected plant propagation material is considered the most important means of long-distance spread of PPV. In addition, the virus is non-persistently transmitted by a number of aphid species existing in each region (Kunze & Krczal, 1971; Labonne *et al*., 1995). Non-aphid transmissible isolates have been described (Maiss *et al*., 1989; López-Moya *et al*., 1995).

PPV particles are flexuous rods about 700 × 11 nm composed of a single mono-stranded RNA molecule close to 10 000 nucleotides coated by up to 2000 subunits of a single coat protein (CP). The expression strategy of PPV, as for other potyviruses, includes translation of a unique long open-reading frame (ORF) further processed to yield viral products. In recent years, knowledge of the molecular biology of PPV in particular, and of potyviruses in general (Riechmann *et al*., 1992; Shukla *et al*., 1994; Revers *et al*., 1999), has experienced a huge increase. Different biotechnological aspects related to PPV, including novel diagnostic techniques that facilitate detection and typing of virus isolates, strategies to implement pathogen-derived resistance through plant transformation, potential use of genetic elements derived from the virus, and PPV-based expression vectors (López-Moya *et al*., 2000).

The numerous PPV isolates differ in biological and epidemiological properties such as aggressiveness, aphid transmissibility and symptomatology. Two main groups Dideron (D) and Marcus (M) have been established serologically (Kerlan & Dunez, 1976). PPV isolates belonging to D or M groups show different epidemiological behaviour. The M isolates are spread more readily by aphids than D isolates and cause more severe symptoms in peach. The D isolates are able naturally to infect apricot and plum, and rarely spread from these hosts to peach. The existence of these two groups is also based on: (1) different serological pattern or reaction with D- or M-specific monoclonal antibodies (Cambra *et al*., 1994; Boscia *et al*., 1997); (2) electrophoretic mobility of the viral CP (Ravelonandro *et al*., 1988; Bousalem *et al*., 1994; Pasquini & Barba, 1994); (3) nucleotide sequence information, either complete or partial including the 3′ terminal region of the genome of several PPV isolates (Ravelonandro *et al*., 1988; Laín *et al*., 1989; Maiss *et al*., 1989; Teycheney *et al*., 1989; Wetzel *et al*., 1991a; Cervera *et al*., 1993; Palkovics *et al*., 1993); (4) sequence analysis of polymerase chain reaction (PCR) fragments corresponding to the C-terminal region of the PPV CP gene and RsaI restriction fragment length polymorphism (RFLP) (Wetzel *et al*., 1991b; Bousalem *et al*., 1994; Candresse *et al*., 1994); (5) different variants of PCR, hemi-nested PCR, nested PCR and Co-operational PCR (Co-PCR) using specific primers (Candresse *et al*., 1994; Olmos *et al*., 1997, 1999, 2002) including colorimetric detection of the

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

amplicons with D- or M-specific probes. Two additional groups of PPV isolates are: El Amar (EA) and Cherry (C). PPV-EA isolates are different in nucleotide sequence (Wetzel *et al*., 1991a) and contain specific epitopes (Myrta *et al*., 1998). Group C was described more recently, after the discovery that some PPV isolates, able to infect cherry, were molecularly and serologically different from the other groups (Nemchinov & Hadidi, 1996; Nemchinov *et al*., 1996) and gave specific reactions with monoclonal antibodies (Myrta *et al*., 2000).

Detection of any PPV isolate (universal) can be achieved by using monoclonal antibody 5B-IVIA (Cambra *et al*., 1994) or polyclonal antibodies. Selective and specific detection of PPV-D isolates (Cambra *et al*., 1994), PPV-M (Boscia *et al*., 1997), PPV-C (Myrta *et al*., 2000) and PPV-EA (Myrta *et al*., 1998) is possible using available ELISA kits. Molecular hybridization techniques (Varveri *et al*., 1988) and different PCR-based assays have been developed for the detection (Korschineck *et al*., 1991; Wetzel *et al*., 1991b, 1992; Candresse *et al*., 1994, 1995; Levy *et al*., 1994; Olmos *et al*., 1996) and for the simultaneous detection and typing of PPV isolates (Olmos *et al*., 1997). Different systems of viral target preparation prior to PCR have been developed based on immunocapture (Wetzel *et al*., 1992) or on print and squash capture (Olmos *et al*., 1996), which removes the need for extract preparation. Use of immobilized targets on paper (Cambra *et al*., 1997) has allowed detection of PPV in single aphids (Olmos *et al*., 1997) by squash-capture PCR. Nested PCR in a single closed tube (Olmos *et al*., 1999) has been applied for sensitive detection of PPV targets in plant material and in single aphids. A Co-PCR system using a universal probe for hybridization (Olmos *et al*., 2002) has been described, giving a sensitivity similar to that of nested PCR. Serological and molecular characterization of PPV isolates correlates very well (Candresse *et al*., 1998).

Identity

Name: *Plum pox potyvirus*
Synonyms: Sharka virus
Acronym: PPV
Taxonomic position: Potyviridae, *Potyvirus*
Bayer computer code: PPV000
Phytosanitary categorization: EPPO A2 list no. 96, EU Annex designation II/A2.

Detection

Under natural conditions, PPV readily infects fruit trees of the genus *Prunus* used as commercial varieties or rootstocks: apricot (*Prunus armeniaca*), European plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), peach (*Prunus persica*), *Prunus cerasifera* and *Prunus cerasifera x munsoniana* cv. Marianna. Sour (*Prunus cerasus*) and sweet (*Prunus avium*) cherries and almond (*Prunus dulcis*) may be infected occasionally. The virus also infects many wild or ornamental *Prunus* species such as *Prunus besseyi*, *Prunus insititia*, *Prunus tomentosa*, *Prunus triloba* and *Prunus spinosa*.

PPV can be artificially transmitted to numerous *Prunus* spp., *Sorbus domestica* and several herbaceous plants. *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Pisum sativum* and *Chenopodium foetidum* are frequently used as experimental host plants for different purposes.

Symptoms

Symptoms may appear on leaves, petals, fruits and stones (Web Figs 1, 2). They are particularly clear on leaves in spring: mild light green discoloration, chlorotic spots, bands or rings, vein clearing or yellowing, or even leaf deformation. Flower symptoms can occur on petals (discoloration) of some peach varieties. Infected fruits show chlorotic spots or lightly pigmented yellow rings or line patterns. Fruits may become deformed or irregular in shape and develop brown or necrotic small areas. Diseased fruits may show internal browning of the flesh and reduced quality. In some cases the diseased fruits drops prematurely from the tree. In general early varieties are much more sensitive for symptom expression on fruits than late varieties. Stones from diseased apricot fruits show pale rings or spots.

Identification

Sampling

Appropriate sample selection is critical for serological or molecular detection. If typical symptoms are present, symptomatic flowers, leaves or fruits should be collected. In symptomless plants, a standard sample should be taken of 5 shoots or 10 fully expanded leaves collected around the canopy of each individual tree from the middle of each scaffold branch, until the appearance of high temperatures at the beginning of summer. Sampling from July to the beginning of September should be avoided in Mediterranean climates. Plant material should preferably be selected from the internal structure of the tree. Samples in spring can be flowers, young shoots or small fruits. Mature leaves can be collected for analysis in autumn. Samples can be stored at 4 °C for not more than 7 days before processing. Fruits can be stored for 1 month at 4 °C. Dormant buds or bark from shoots or branches can be selected in winter.

Preparation of the sample for testing
Approximately 1 g of plant material is weighed, cut into small pieces and placed in a suitable tube or plastic bag for processing. Approximately 20 volumes of extraction buffer are added (Cambra *et al*., 1994) and the sample is homogenized in tubes using a Polytron (Kinematica) or similar apparatus. Alternatively, the sample can be homogenized in plastic bags using the Homex 6 machine (Bioreba) or a manual roller, hammer, or similar implement. The composition of the extraction buffer is: phosphate-buffered saline (PBS) pH 7.2–7.4 (Appendix 1), supplemented with 2% Polyvinylpyrrolidone (PVP-10) and 0.2% sodium diethyl dithiocarbamate (DIECA). Samples for serological testing can be prepared in tubes or in

plastic bags. Samples for molecular testing should be prepared in individual plastic bags.

Biological testing

The object of a biological test is to detect the presence of PPV in plant accessions or selections, or in samples whose sanitary status is to be assessed. The main indicator plants used for PPV testing are: seedlings of *Prunus persica* GF 305 or Nemaguard, or *Prunus tomentosa*. The indicators should be graft-inoculated according conventional methods (Desvignes, 1999), with 4–6 replicates, and held under standard conditions. Symptom onset should be compared with positive and negative control plants.

Serological tests

DASI-ELISA (Double Antibody Sandwich Indirect ELISA), or Triple Antibody Sandwich (TAS), is performed according to Cambra *et al*. (1994) using the detailed protocol described in Appendix 3 and materials (specific monoclonal antibody 5B-IVIA) described in Appendix 1, for universal detection of any PPV isolate. Characterization or typing of PPV-D or PPV-M can be performed following the protocol described in Appendix 3 and materials (specific D or M monoclonal antibodies) described in Appendix 1.

Conventional (biotin/streptavidin system) DAS-ELISA is performed according to Clark & Adams (1977) using the detailed protocol described in Appendix 3 and materials (specific monoclonal antibody 5B-IVIA or polyclonal antibodies) described in Appendix 1, for universal detection of any PPV isolate.

Molecular tests

Immunocapture RT-PCR (IC-RT-PCR)

The immunocapture phase and the RT-PCR are performed according to Wetzel *et al*. (1991b, 1992), Olmos *et al*. (1997) and Rosner *et al*. (1998), using the detailed protocol described in Appendix 4 and materials described in Appendix 2 (oligonucleotide primer sequences and buffers), for universal detection of any PPV isolate.

Characterization or typing of PPV-D or PPV-M can be performed following the protocol described in Appendix 4 and materials (PD and PM specific primers) described in Appendix 2. Characterization can also be done by molecular hybridization of IC-RT-PCR amplification products (P1/P2 primers) using specific D and M probes as described in Appendix 4 and Appendix 2.

Co-operational amplification (Co-PCR)

Co-PCR using purified RNA is performed according to Olmos *et al*. (2002) using the detailed protocol described in Appendix 4 and materials described in Appendix 2 (oligonucleotide primer sequences, buffers and probe), for universal detection of any PPV isolate.

Characterization or typing of PPV-D or PPV-M can be performed following the protocol described in Appendix 4 and materials (specific D and M probes) described in Appendix 2.

Possible confusion with similar species

None.

Requirements for a positive diagnosis

If PPV is diagnosed for the first time, or in critical cases (import/export), the following should be performed and provided (see also Fig. 3):

- the original sample (with labels, if applicable) should be kept under proper conditions as long as possible. Sample extract and PCR amplification product should be kept at –80 °C for 3 months (or longer for legal purposes)
- the combination of two different screening methods, based on biological tests (inoculation of woody indicator plants), on serological tests or on molecular detection (with the validated protocols and reagents) is required to support a positive PPV detection
- to type a PPV isolate from a PPV-infected host, a characterization method based on DASI-ELISA, IC RT-PCR or molecular hybridization, using specific reagents, is required. For first findings of PPV, at least PPV coat protein (CP) should be sequenced for comparison with previously described D, M, EA and C PPV types.

Report on the diagnosis

The report on the execution of the protocol should include:

- results obtained by the recommended procedures
- information and documentation on the origin of the infected plant material
- a description of the disease symptoms
- an indication of the magnitude of the infection
- comments as appropriate on the certainty or uncertainty of the identification.

Further information

Further information on this organism can be obtained from: Instituto Valenciano de Investigaciones Agrarias (IVIA), Department Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia) Spain. E-mail: *mcambra@ivia.es*

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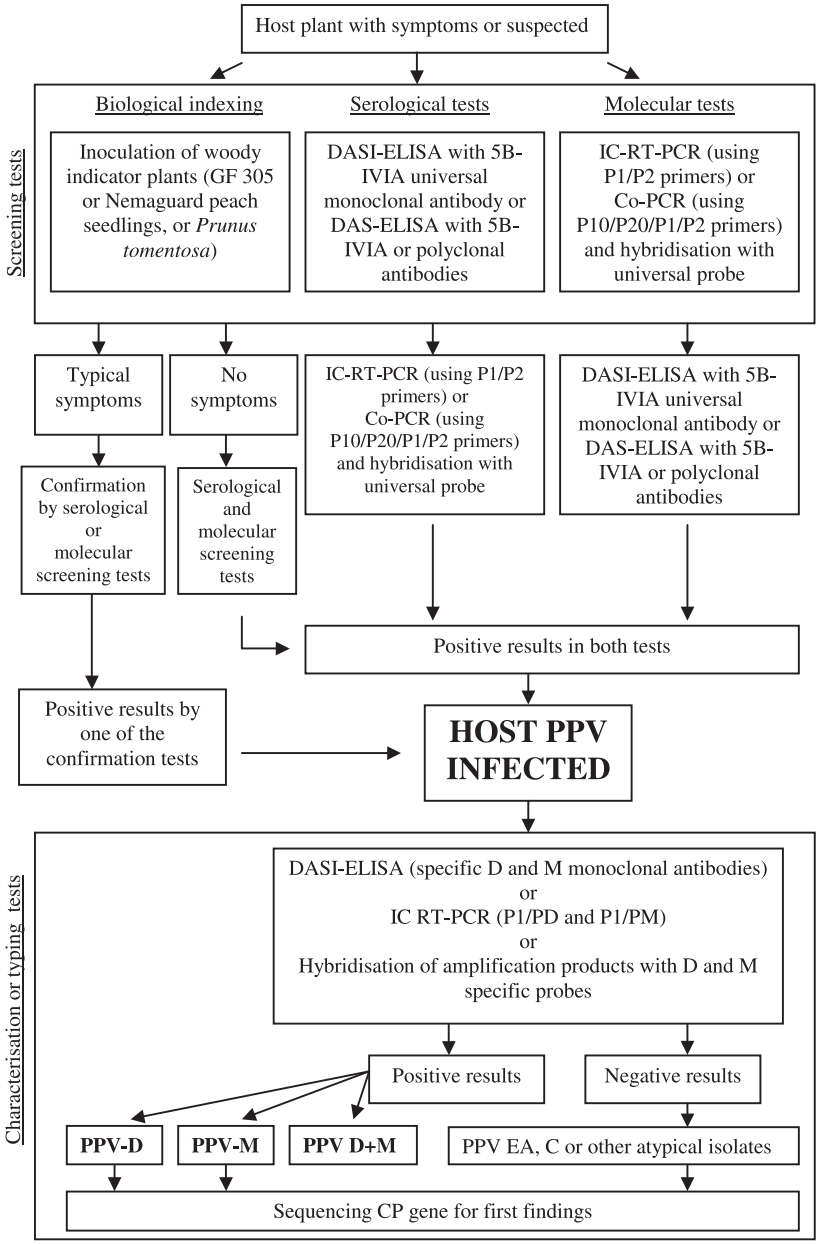

Fig. 3 Decision scheme for detection and identification of *Plum pox potyvirus*.

This protocol was ring-tested in different European laboratories[2].

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Appendix 1. Materials

Materials for detection and characterization of PPV in plant tissues by serological tests

Standard PPV-infected and healthy controls and PPV-specific monoclonal antibodies are commercially available, or can alternatively be obtained by non-profit institutions at Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 5. 46113 Moncada (Valencia), Spain and at Instituto di Virologia Vegetale del CNR, Sezione di Bari, via Amendola 165/A, I-70126 Bari, Italy. (Dr D. Boscia, E-mail: *csvvdb08@area.ba.cnr.it*)

Complete DAS-ELISA or DASI-ELISA kits based on specific monoclonal antibodies 5B-IVIA (PPV universal), 4D (PPV-D specific) and AL (PPV-M specific), for PPV detection and characterization, are commercially available from REAL (validated in ring tests), CE Durviz S.L., Parque Tecnológico de Valencia, Leonardo Da Vinci 10, 46980 Paterna (Valencia), Spain *http://www.durviz.co*; Grittiest S.R. (validated in ring tests), Str. Prove. Per Casamassima Km. 3, I-70010 Valenzano, Italy *http://www.agritest.it*; Agdia Incorporated, 30380 County Road 6, 46514 Elkart, USA *http://www.agdia.com*.

Complete DAS-ELISA kits (conventional) for universal PPV detection, based on polyclonal or monoclonal antibodies (different from 5B-IVIA) are commercially available from: Adgen Limited, Nellies Gate.Anchincruive, Ayr KA6 5HW (GB) *http://www.adgen.co.uk*; BIORAD Laboratories-SANOFI, Rue Raimond Poincaré 3-BD, 92430 Marnes La Coquette (FR) *http://www.bio-rad.com*; Bioreba, Chr. Merian-Ring 7, 4153 Reinach BL1 (CH) *http://www.bioreba.ch*; DSMZ, Deustche Sammlung von Mikroorganismen und Zellkulturen GmbH, Messenweg 11/12, 38104 Braunschweig (DE) *http://www.dsmz.de/nf-plvirus*; Hortitech, Hortitech Diagnostic & Crop Protection Services, Horticulture Research International, Stockbridge House, Cawood, YO8 3TZ Selby (GB) E-mail: *plantclinic.sh@hri.ac.uk*; LOEWE Biochemica GmbH, Mühiweg 2a, D-82054 Sauerlach (DE) *http://www.loewe-info.com*; Plant Research International B.V., PO Box 16, 6700 AA Wageningen (NL) *http://www.plant.wageningen-ur.nl*.

For the biotin/streptavidin system, they are available from: INGENASA, Hermanos García Noblejas 41, 2ª planta, 28037 Madrid (ES) *http://www.ingenasa.es*.

Alkaline-phosphatase linked goat antimouse immunoglobulins Cat No A-3562 are available from Sigma (Steinhein), Germany, and streptavidin alkaline phosphatase linked. goat antimouse immunoglobulins Cat no. 1089 161 from Roche Diagnostics GmbH, Mannheim, Germany.

Buffers

Phosphate-Buffered Saline (PBS) pH 7.2–7.4: NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \times 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, distilled water 1 L.
Carbonate buffer pH 9.6: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, distilled water 1 L.
Washing buffer (PBS, pH 7.2–7.4 with 0.05% Tween 20): NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \times 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, Tween 20 500 µL, distilled water 1 L.
Substrate buffer for alkaline phosphatase: diethanolamine 97 mL; dilute in 800 mL of distilled water; adjust pH 9.8 with concentrated HCl; adjust to 1000 mL with distilled water.

Materials for detection and characterization of PPV in plant tissues by molecular tests

Standard PPV-infected and healthy controls and PPV-specific oligonucleotide primer and probe sequences are available for non-profit institutions at Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 5. 46113 Moncada (Valencia), Spain and at Institute National de Recherche Agronomique (INRA), Centre de Bordeaux, Equipe de Virologie UMR GD2P, IBVM., BP 81, 33883 Villenave d'Ornon Cedex, France. (Dr Thierry Candresse, E-mail: *tc@bordeaux.inra.fr*).

RNA purification kit (validated in ring tests) is available from Rneasy Plant Mini Kit – Cat no. 74904 – Qiagen GmbH (Hilden), Germany.

The oligonucleotide primer sequences (validated in ring tests) are:

- P10: 5′–3′ GAG AAA AGG ATG CTA ACA GGA
- P20: 5′–3′ AAA GCA TAC ATG CCA AGG TA
- P1: 5′–3′ ACC GAG ACC ACT ACA CTC CC
- P2: 5′–3′ CAG ACT ACA GCC TCG CCA GA
- PD: 5′–3′ CTT CAA CGA CAC CCG TAC GG
- PM: 5′–3′ CTT CAA CAA CGC CTG TGC GT

The oligonucleotide 3′DIG labelled probe sequences (validated in ring tests) are:

- PPV Universal Probe: TCG TTT ATT TGG CTT GGA TGG AA-Digoxigenin
- PPV-D Specific Probe: CTT CAA CGA CAC CCG TAC GGG CA-Digoxigenin
- PPV-M Specific Probe: AAC GCC TGT GCG TGC ACG T-Digoxigenin

Colorimetric detection of amplicons (validated in ring tests) is available from Roche Diagnostics GmbH, Cat no. 1585 762 (DIG Wash and Block Buffer Set), Cat no. 1093 274 (Anti-Digoxigenin-AP Fab fragments), Cat no. 1465 341 (Multicolor Detection Set) – Mannheim, Germany.

Buffers and substrate solutions

Carbonate buffer pH 9.6: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, distilled water 1 L
Washing buffer (PBS, pH 7.2–7.4 with 0.05% Tween 20): NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \times 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, Tween 20 500 µL, distilled water 1 L.
50X TAE buffer: Tris 242 g, 0.5 M Na_2EDTA pH 8.0100 mL, glacial acetic acid 57.1 mL, distilled water to 1 L.
Loading buffer: 0.25% bromophenol blue, 30% glycerol in H_2O

Buffer 20X SSC: 3M NaCl, 300 mM sodium citrate, pH 7.0; dissolve 175.3 g NaCl and 88.2 g sodium citrate-$2H_2O$ in 800 mL H_2O, 2 g KCl, Na_2HPO_4-$7H_2O$ and 2.4 g KH_2PO_4 in 800 mL H_2O; adjust pH 7.4 with HCl; adjust volume to 1 L; sterilize by autoclaving.

SDS 10% (w/v) in sterile water, filtered through a 0.2–0.45 µm membrane; dissolve 100 g sodium dodecyl sulphate crystals (SDS) in 900 mL H_2O; heat to 68 °C to dissolve crystals; adjust pH to 7.2 with HCl (about 50 µL): adjust volume to 1 L with H_2O.

Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5; this buffer 10X is available in a ready-to-use form in the Roche DIG Wash and Block Buffer Set (Cat. no. 1585762); bottle (2).

Washing buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% (v/v) Tween 20; this buffer 10X is available in a ready-to-use form in the Roche DIG Wash and Block Buffer Set (Cat. no. 1585762); bottle (1).

Blocking reagent solutions: for stock solution 10% (w/v), dissolve 10 g blocking reagent (Roche, Cat. no. 1096 176) in 100 mL maleic acid buffer with several 30-s heat pulses in the microwave (3–4 min total); avoid boiling; alternatively dissolve 10 g blocking reagent in 100 mL maleic acid buffer; heat at 60 °C for approximately 1 h until completely dissolved; sterilize by autoclaving; dilute as appropriate with maleic acid buffer; the stock solution 10X (10% w/v) is available in a ready-to-use form in the Roche DIG Wash and Block Buffer Set (Cat. no. 1585762); bottle (3)

TE buffer pH 8.0: 10 mM Tris-HCl, 1 mM EDTA.

Washing solution 2X: 2X SSC with 0.1% SDS.

Washing solution 0.5X: 0.5X SSC with 0.1% SDS.

N-lauroylsarcosine: 10% (w/v) in sterile water filtered through 0.2–0.45 µM membrane

Formamide: 500 mL formamide, 50 g ion exchange: AG 501-X8 Resin (Bio-Rad); stir 30 min slowly on a stirrer, then remove resin by filtration and store the deionized formamide at 20 °C.

Standard hybridization buffer: 5X SSC, 0.1% lauroylsarcosine, 0.02% SDS, 1% w/v blocking solution.

Standard hybridization buffer + 30% formamide: 5X SSC, 30% deionized formamide, 0.1% sodium lauroylsarcosine, 0.02% SDS, 2% w/v blocking solution.

Standard hybridisation buffer + 50% formamide: 5X SSC, 50% deionized formamide, 0.1% sodium lauroylsarcosine, 0.02% SDS, 2% w/v blocking solution.

Anti-digoxigenin-AP stock solution: 750 units/mL antidigoxigenin, Fab fragments (available in Roche Cat. no. 1093274) conjugated to alkaline phosphatase; working concentration 150 mU/mL; dilute antidigoxigenin-AP stock solution 1 : 5000.

Detection buffer pH 9.5: 100 mM Tris-HCl, 100 mM NaCl. this buffer 10X is available in a ready-to-use form in the Roche DIG Wash and Block Buffer Set (Cat. no. 1585762; bottle 4).

NBT solution: 75 mg/mL nitro blue tetrazolium salt in 70% (v/v) dimethylformamide.

BCIP solution: 50 mg/mL 5-bromo-4-chloro-3indolyl phosphate (BCIP) toluidinium salt in 100% dimethylformamide; mix 45 µL NBT solution and 35 µL BCIP solution in 10 mL of detection buffer.

Appendix 2. Detailed protocols for serological tests

DASI-ELISA (Cambra *et al.*, 1994)

1. Prepare an appropriate dilution of rabbit-PPV polyclonal immunoglobulins (usually 1–2 µg mL^{-1}) in carbonate buffer pH 9.6 (Appendix 1). Add 200 µL to each well. Incubate at 37 °C for 4 h or at 4 °C for 16 h. Wash the wells three times with PBS-Tween (washing buffer) (Appendix 1).
2. Add 200 µL per well of the plant extract (see sample preparation). Use two wells of the plate for each sample or positive controls and at least two wells for negative controls. Incubate at 4 °C for 16 h. Wash as before.
3. Add specific monoclonal antibodies for universal detection of any PPV isolate or for selective detection of PPV-D or PPV-M (Appendix 1: prepare an appropriate dilution of the monoclonal antibodies 5B-IVIA (0.1 µg mL^{-1} in PBS with 0.5% bovine serum albumin-BSA) for universal PPV detection (Appendix 1. For specific PPV-D detection with 4D monoclonal antibody or PPV-M detection with AL monoclonal antibody (Appendix 1), proceed as above. Add 200 µL to each well. Incubate at 37 °C for 2 h. Wash as before.
4. Add antimouse immunoglobulins conjugated with alkaline phosphatase: prepare an appropriate dilution of antimouse immunoglobulins conjugated with alkaline phosphatase (Appendix 1) in PBS plus 0.5% BSA. Add 200 µL to each well. Incubate at 37 °C for 2 h. Wash as before.
5. Prepare 1 mg mL^{-1} alkaline phosphatase solution (p-nitrophenylphosphate) in substrate buffer (Appendix 1). Add 200 µL to each well. Incubate at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA test is negative if the absorbance of the sample is less than two times the absorbance of the healthy control. The ELISA test is positive if the absorbance of the sample is equal or greater than two times the absorbance of the healthy control.

DAS-ELISA (Clark & Adams, 1977) conventional or biotin/streptavidin system

1. Prepare an appropriate dilution of polyclonal antibodies or monoclonal antibody 5B-IVIA (Appendix 1) (usually 1–2 µg mL^{-1}) in carbonate buffer pH 9.6 (Appendix 1). Add 200 µL to each well. Incubate at 37 °C for 4 h or at 4 °C for 16 h. Wash the wells three times with PBS-Tween (washing buffer) (Appendix 1).
2. Add 200 µL per well of the plant extract (see sample preparation). Use two wells of the plate for each sample or positive controls and at least two wells for negative controls. Incubate at 4 °C for 16 h. Wash as before.
3. Add specific monoclonal antibodies 5B-IVIA or polyclonal antibodies linked with alkaline phosphatase or biotin (Appendix 1) for universal detection of any PPV isolate:

prepare an appropriate dilution of the conjugated antibodies (about 0.1 µg mL^{-1} in PBS with 0.5% bovine serum albumin-BSA). Add 200 µL to each well. Incubate at 37 °C for 3 h. Wash as before.

4. When antibodies are linked with biotin, use an appropriate dilution of streptavidin-alkaline phosphatase conjugated (Appendix 1). Add 200 µL to each well. Incubate at 37 °C for 30 min and wash as before. For both methods (conventional or biotin/streptavidin), prepare 1 mg mL^{-1} alkaline phosphatase solution (p-nitrophenylphosphate) in substrate buffer. Add 200 µL to each well. Incubate at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA test is negative if the absorbance of the sample is less than two times the absorbance of the healthy control. The ELISA test is positive if the absorbance of the sample is equal or greater than two times the absorbance of the healthy control.

Appendix 3. Detailed protocols for molecular tests

IC-RT-PCR (Wetzel *et al*., 1991b, 1992; Olmos *et al*., 1997)

Immunocapture phase (IC) according to Wetzel *et al.* (1992)

Prepare coated Eppendorf tubes: prepare a dilution (1 µg/mL) of polyclonal antibodies or monoclonal antibody (5B-IVIA) PPV specific in carbonate buffer pH 9.6 (Appendix 2). Dispense 100 µL of the diluted antibodies into the Eppendorf tubes. Incubate at 37 °C for 3 h. Wash the tubes twice with 150 µL of sterile washing buffer (Appendix 2).

Clarify 100 µL plant extract previously obtained (see sample preparation) by centrifugation (5 min at 13 000 rev min^{-1}), and submit to an immunocapture phase for 2 h on ice (Rosner *et al*., 1998) or alternatively at 37 °C (Wetzel *et al*., 1992), in coated Eppendorf tubes. Wash Eppendorf tubes three times as before.

Amplification by RT-PCR

General PPV detection (P1–P2 primers) according to Wetzel *et al*. (1991b):

- P1: 5′–3′ ACC GAG ACC ACT ACA CTC CC
- P2: 5′–3′ CAG ACT ACA GCC TCG CCA GA

Ingredients for the cocktail reaction (µL) are: H_2O 15.55; 10X-Taq Polymerase Buffer 2.5; 25 mM $MgCl_2$ 1.5 (1.5 mM); 5 mM dNTPs 1.25 (250 µM); 4% Triton X-100 2 (0.3%); 25 µM primer P1(1 µM); 25 µM primer P2 1 (1 µM); 10 U µL^{-1} AMV 0.1; 5 U µL Taq Polymerase 0.1, to total volume of 25 µL. Add 25 µL of the cocktail reaction mixture directly to the washed tubes.

Conditions for RT-PCR: 42 °C for 45 min; 92 °C for 2 min; 40 cycles (at 92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min); 72 °C for 10 min; 4 °C hold.

Specific PPV-D detection (P1-PD primers) (Olmos *et al*., 1997):

- P1: 5′–3′ ACC GAG ACC ACT ACA CTC CC
- PD: 5′–3′ CTT CAA CGA CAC CCG TAC GG

Ingredients (µL): H_2O 15.05; 10X-Taq Polymerase Buffer 2.5; 25 mM $MgCl_2$ 1.5 (1.5 mM); 5 mM dNTPs 1.25 (250 µM); 4% Triton X-100 2 (0.3%); 25 µM primer P1 1 (1 µM); 25 µM primer PD 1 (1 µM); formamide 0.5 (2%); 10 U µL^{-1} AMV 0.1; 5 U µL Taq Polymerase 0.1; total volume 25 µL. Add 25 µL of the cocktail reaction mixture directly to the washed tubes.

Conditions for RT-PCR: 42 °C for 45 min; 92 °C for 2 min; 40 cycles (92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min); 72 °C for 10 min; 4 °C hold.

Specific PPV-M detection (P1-PM primers) (Olmos *et al*., 1997):

- P1: 5′–3′ ACC GAG ACC ACT ACA CTC CC
- PM: 5′–3′ CTT CAA CAA CGC CTG TGC GT

Ingredients (µL): H_2O 15.05; 10X-Taq Polymerase Buffer 2.5; 25 mM $MgCl_2$ 1.5 (1.5 µM); 5 mM dNTPs 1.25 (250 µM); 4% Triton X-100 2 (0.3%); 25 µM primer P1 1 (1 µM); 25 µM primer PD 1 (1 µM); formamide 0.5 (2%); 10 U µL^{-1} AMV 0.1; 5 U µL^{-1} Taq Polymerase 0.1. Total volume 25 µL. Add 25 µL of the cocktail reaction mixture directly to the washed tubes.

Conditions for RT-PCR: 42 °C for 45 min; 92 °C for 2 min; 40 cycles (92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min); 72 °C for 10 min; 4 °C hold.

Electrophoresis of PCR products

Prepare 2% agarose gel in 0.5X TAE buffer (Appendix 2). Place droplets of about 3 µL of loading buffer (Appendix 2) on parafilm, add 20 µL of PCR product and mix by gently aspirating with the pipette before loading. Load the wells of the gel and include positive and negative controls. Include DNA marker 100 bp in the first well of the gel. Run the gel for 20 min at 120 V (medium gel tray: 15 × 10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15 × 25 cm) in 0.5X TAE buffer. Soak the gel in ethidium bromide solution (0.5 µg mL^{-1}) for 20 min. Visualize the amplified DNA fragments by UV transillumination. Observe specific amplicons of 243 bp (general detection) or 198 bp (specific detection).

PPV detection by Co-operational amplification (Co-PCR) following Olmos *et al*. (2002).

Viral RNA purification with RNeasy Plant Mini Kit (Qiagen) (Appendix 2)

Use 200 µL plant extract (see sample preparation) as sample and mix it with RLT buffer (350 µL). Vortex vigorously. Pipette the lysate directly onto a QIA shredder spin column (lilac) placed in 2-mL collection tube, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant of the flow-through fraction to a new microcentrifuge tube without disturbing the cells-debris pellet in the collection tube. Only use this supernatant in subsequent steps. Add 0.5 volume ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay with following step. Apply sample including any precipitate that may have formed to an RNeasy mini column (pink) placed in a

2-mL collection tube. Close the tube gently and centrifuge for 15 s at ≥ 8000 *g*. Discard the flow-through. Add 700 µL buffer RW1 to the RNeasy column. Close the tube gently and centrifuge for 15 s at ≥ 8000 *g* to wash the column. Discard the flow-through and collection tube. Transfer the RNeasy column into a new 2 mL-collection tube. Pipette 500 µL of RPE buffer onto the RNeasy column. Close the tube gently and centrifuge for 15 s at ≥ 8000 *g* to wash the column. Discard the flow-through. Add another 500 µL buffer RPE to the RNeasy column. Close the tube gently and centrifuge for 2 min at ≥ 8000 *g* to dry the RNeasy silica gel membrane. Continue directly with the following step to eliminate any chance of possible buffer RPE carryover. Place the RNeasy column in a new 2 mL-collection tube and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min. To elute transfer the RNeasy column to a new 1.5 mL collection tube. Pipette 50 µL RNeasy-free water directly onto the RNeasy silica gel membrane. Close the tube gently, and centrifuge for 1 min at ≥ 8000 *g* to elute.

Amplification by Co-PCR (Olmos *et al.*, 2002):

- P10: 5′–3′ GAG AAA AGG ATG CTA ACA GGA
- P20: 5′–3′ AAA GCA TAC ATG CCA AGG TA
- P1: 5′–3′ ACC GAG ACC ACT ACA CTC CC
- P2: 5′–3′ CAG ACT ACA GCC TCG CCA GA

Ingredients (µL): H_2O 8.33; 10X-Taq Polymerase Buffer 2.5 (1X Buffer); 25 mM $MgCl_2$ 3 (3 mM); 5 mM dNTPs 2 (400 µM); 4% Triton X-10 1.87 (0.3%); 10 µM primer P10 0.25 (0.1 µM); 10 µM primer P20 0.25 (0.1 µM); 10 µM primer P1 0.125 (0.05 µM); 10 µM primer P2, 0.125 (0.05 µM); DMSO 1.25 (5%); 10 U µL^{-1} AMV 0.2; 5 U µL^{-1} Taq Polymerase 0.2. Total volume 20 µL. Add 20 µL of the cocktail reaction mixture and 5 µL of RNA sample directly to new sterile tubes.

Conditions for Co-PCR: 42 °C for 45 min; 94 °C for 2 min; 60 cycles (94 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s), 72 °C for 10 min; 4 °C hold

Colorimetric detection of amplicons (Olmos *et al.*, 2002; according to Roche Molecular Biochemicals):

PPV Universal Probe: TCG TTT ATT TGG CTT GGA TGG AA-Digoxigenin

PPV-D Specific Probe: CTT CAA CGA CAC CCG TAC GGG CA-Digoxigenin

PPV-M Specific Probe: AAC GCC TGT GCG TGC ACG T-Digoxigenin

Prepare the following required solutions and buffers (Appendix 2): buffer 20X SSC, SDS 10% (w/v) in sterile water, maleic acid buffer, washing buffer, blocking reagent solutions, TE buffer pH 8.0, washing solution 2X, washing solution 0.5X, N-lauroylsarcosine, formamide, standard hybridization buffer, standard hybridization buffer + 30% formamide, standard hybridization buffer + 50% formamide, antidigoxigenin-AP stock solution, detection buffer pH 9.5, substrate solutions.

Amplified cDNA is denatured at 95 °C for 5 min and placed on ice immediately. Three different positively charged nylon membranes (Roche) are used, one for the universal PPV detection, one for the specific PPV-D detection and one for the specific PPV-M detection. 1 µL of sample is added to each nylon membrane. After spotting on the samples, membranes are dried at room temperature and UV cross-linked in a transilluminator for 4 min at 254 nm.

For general PPV detection (universal probe), the membrane is placed into a hybridization tube and submitted to a prehybridization phase at 60 °C during 1 h using the standard hybridization buffer. After prehybridization, the solution is discarded and the hybridization is performed mixing-3′DIG labelled probe (universal PPV probe) with the standard hybridization buffer at a final concentration of 10 pmol mL^{-1}. Hybridize during 2 h at 60 °C. The membrane is washed at room temperature twice for 15 min with washing solution 2X and twice for 15 min with washing solution 0.5X, and then equilibrated for 2 min in washing buffer. Block for 30 min with blocking solution 1% (w/v). Incubate membrane at room temperature with antidigoxigenin-alkaline phosphatase conjugate antibodies (Appendix 2) for 30 min at a working solution of 1 : 5000 (150 mU mL^{-1}) in blocking solution 1% (w/v) for 30 min. Wash twice for 15 min with washing buffer. Equilibrate for 2 min with detection buffer. Prepare substrate: mix 45 µL NBT solution and 35 µL BCIP solution in 10 mL of detection buffer (alternatively dilute a substrate tablet, included in the Roche Multicolor Detection Kit, in 10 mL detection buffer. After 1 h substrate incubation, stop reaction by washing with tap water.

For specific PPV-D detection (*PPV-D specific probe*): the membrane is placed into a hybridization tube and submitted to a prehybridization phase at 50 °C during 1 h using the standard hybridization buffer + 30% formamide. After prehybridization, the solution is discarded and the hybridization is performed mixing-3′DIG labelled PPV-D specific probe with the standard hybridization buffer + 30% at a final concentration of 10 pmol mL^{-1}. Hybridize during 2 h at 50 °C. Wash membrane at room temperature twice for 15 min with washing solution 2X and twice for 15 min with washing solution 0.5X, and equilibrate for 2 min in washing buffer. Block for 30 min with blocking solution 2% (w/v). Incubate at room temperature with antidigoxigenin-alkaline phosphatase conjugate antibodies (Appendix 2) for 30 min at a working solution of 1 : 5000 (150 mU mL^{-1}) in blocking solution 2% (w/v) for 30 min. Wash twice for 15 min with washing buffer, and equilibrate for 2 min with detection buffer. Prepare substrate: mix 45 µL NBT solution and 35 µL BCIP solution in 10 mL of detection buffer (alternatively dilute a substrate tablet, included in the Roche Multicolor Detection, in 10 mL detection buffer). After 1 h substrate incubation, stop reaction by washing with tap water.

For specific PPV-M detection (PPV-M specific probe): the membrane is placed into a hybridization tube and submitted to a prehybridization phase at 50 °C during 1 h using the standard hybridization buffer + 50% formamide. After prehybridization, the solution is discarded and the hybridization is performed mixing-3′DIG labelled PPV-M specific probe with the standard

hybridization buffer + 50% at a final concentration of 10 pmol mL^{-1}. Hybridize during 2 h at 50 °C. Wash membrane at room temperature twice for 15 min with washing solution 2X and twice for 15 min with washing solution 0.5X, and equilibrate for 2 min in washing buffer. Block for 30 min with blocking solution 2% (w/v). Incubate at room temperature with antidigoxigenin-alkaline phosphatase conjugate antibodies (Roche; Ref: 1093 274) for 30 min at a working solution of 1 : 5000 (150 mU mL^{-1}) in blocking solution 2% (w/v) for 30 min. Wash twice for 15 min with washing buffer, and equilibrate for 2 min with detection buffer. Prepare substrate: mix 45 µL NBT solution and 35 µL BCIP solution in 10 mL of detection buffer (alternatively dilute a substrate tablet, included in the Roche Multicolor Detection, Cat. no. 1465 341, in 10 mL detection buffer). After 1 h substrate incubation, stop reaction by washing with tap water.

Figure 1. A: Symptoms of vein clearing on peach leaves cv. Royal Gem caused by PPV-M, **B:** Typical symptoms of PPV on apricot leaves, and **C:** Symptoms of PPV on Japanese plum leaves. Courtesy M.Cambra, IVIA, Moncada (Valencia), ES.

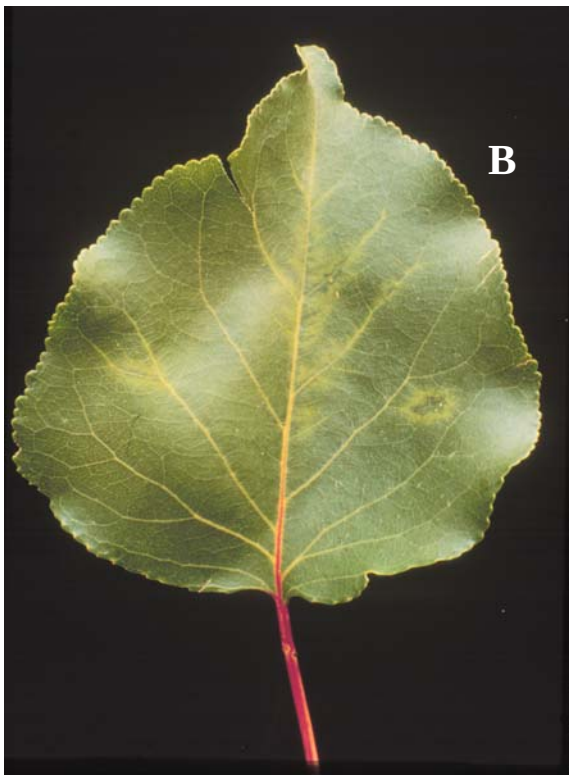

Figure 2. A: Petal discoloration caused by PPV-M on flowers of peach cv. Baby Gold (Courtesy J.C. Desvignes, CTIFL, FR), **B** and **C**: Symptoms of PPV-D and PPV-M on Spring Crest and Arm King peach varieties, respectively (Courtesy M. Cambra, IVIA, Moncada (Valencia) ES). **D** and **E**: Symptoms of PPV on apricot fruits and pale rings in the stones of the same fruits (Courtesy. M.A. Cambra, DG Aragón, Zaragoza, ES). **F** and **G**: Symptoms of PPV-D on fruits of Red Beaut Japanese plum, note that in G the diseased fruits are compared with two healthy fruits on the left (Courtesy M.Cambra, IVIA, Moncada (Valencia) ES).