INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

DRAFT ANNEX to ISPM 27:2010

Plum pox virus

(201-)

Comment [AMJ1]: Laurene Levy—redrafting parts of this document and submitting to Julie A. by September 13

DRAFT DOCUMENT

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Main discussion points during development of the diagnostic protocol	• The minimum requirements for the identification of PPV and in particular strains of PPV • Inclusion of requirements for identification of PPV in different circumstances, e.g. “routine diagnosis of a pest widely established in a country” as opposed to “detection of a pest in a consignment originating in a country where the pest is declared to be absent”.
Notes	File template: IPPCStyles, April 2010. Auto marginal numbering.

Adoption

This diagnostic protocol was adopted by the Commission on Phytosanitary Measures in 20--.

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[1] **1. Pest Information**

[2] Sharka (plum pox) is one of the most serious diseases of stone fruit in terms of agronomic impact and economic importance. The disease, caused by *Plum pox virus* (PPV), affects the genus *Prunus*~~; it is particularly detrimental in apricot (*P. armeniaca*), European plum (*P. domestica*), Japanese plum (*P. salicina*) and peach (*P. persicae*) because it reduces quality and causes premature fruit drop. Estimated costs associated with sharka management worldwide since the 1970s exceed 10 000 million euros.~~.

[3] Sharka was first reported in eastern Europe, in Bulgaria in 1917–1918, and was described as a viral disease in 1932. Since then, the virus has spread progressively to a large part of Europe, around the Mediterranean basin and the Near and Middle East. It has been found with a restricted distribution in South and North America and Asia (EPPO, 2006; CABI, 2008).

[4] PPV is a member of the genus *Potyvirus* in the family *Potyviridae*. The virus particles are flexuous rods about 700 nm × 11 nm composed of a single-stranded RNA molecule consisting of almost 10 000 nucleotides coated by up to 2 000 subunits of a single coat protein (García and Cambra, 2007). ~~PPV is transmitted in the field by aphids in a non-persistent manner, but movement of infected propagative plant material has been and is the main way in which sharka is spread over long distances.~~

Comment [AMJ2]: These three paragraphs should be simplified; the background information on PPV is not needed.

[5] PPV isolates can be classified into seven types or strains: D (Dideron), M (Marcus), C (Cherry), EA (El Amar), W (Winona) and Rec (Recombinant) and T (Turkish) (Candresse and Cambra, 2006; James and Glasa, 2006; Ulubaş Serçe *et al.*, 2009). Most PPV isolates belong to the D and M types. PPV D and M strains can easily infect apricots and plums but differ in their ability to infect peach cultivars. M isolates cause, in general, faster epidemics and more severe symptoms in peach than D isolates. El Amar isolates (PPV-EA) are geographically restricted to Egypt and little information is available about their epidemiology and biological properties. Cherries were not considered a host of PPV for a long time. However, a number of PPV isolates infecting sour cherry (*P. cerasus*) and sweet cherry (*P. avium*) have been identified in several European countries and Turkey. These isolates form a distinct type that has been defined as PPV-C. An atypical PPV was isolated from European plum in Canada (PPV-W) representing a distinct PPV type. In addition, natural recombinants between the D and M types of PPV have been described as PPV-Rec showing an epidemiological behaviour similar to the D type. Recently a second type of recombinant isolate has been reported in Turkey (T type).

Comment [AMJ3]: Laurene is working on this paragraph.

[6] Further information about PPV, including illustrations of disease symptoms, can be found in CABI (2008), EPPO (2004), EPPO (2006) and García and Cambra (2007).

Comment [AMJ4]: Add PPQ photo gallery website to paragraph 6 (and/or 10).

[7] **2. Taxonomic Information**

Name:	*Plum pox virus* (acronym PPV)
Synonyms:	Sharka virus
Taxonomic position:	*Potyviridae*, *Potyvirus*
Common names:	Sharka, plum pox.

[8] **3. Detection and Identification**

[9] Under natural conditions, PPV readily infects fruit trees of the genus *Prunus* used as commercial varieties or rootstocks: apricot, European and Japanese plums, peach, Chinese wild peach (*P. davidiana*), Mahaleb cherry (*P. mahaleb*), Marianna plum (*P. marianna*) and Myrobalan flowering plum (*P. cerasifera*). Sour and sweet cherries, and almond (*P. dulcis*) may be infected occasionally. The virus also infects many wild or ornamental *Prunus* species such as western sand cherry (*P. besseyi*), purple-leaved sand cherry (*P. cistena*), dwarf flowering almond (*P. glandulosa*), Damson plum (*P. insititia*), cherry laurel (*P. laurocerasus*), blackthorn (*P. spinosa*), nanking cherry (*P. tomentosa*) and flowering almond (*P. triloba*). Under experimental conditions, PPV can be transmitted

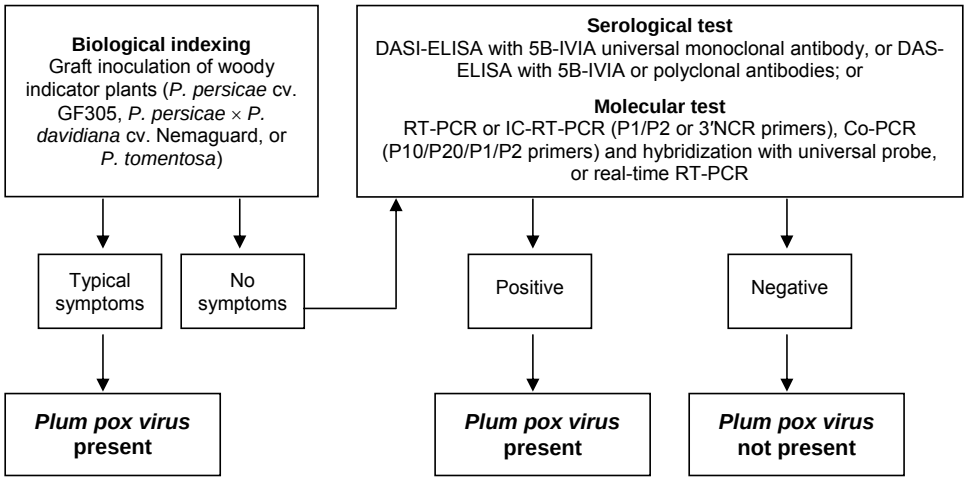
mechanically to numerous *Prunus* spp. and several herbaceous plants (*Chenopodium foetidum*, *Nicotiana benthamiana*, *N. glutinosa*, *N. clevelandii* and *Pisum sativum*).

[10] PPV can be visually diagnosed in the field using symptoms that may~~PPV symptoms may~~ appear on leaves, petals, fruits and stones. They are particularly clear on leaves in springtime and include mild light-green discoloration; chlorotic spots, bands or rings; vein clearing or yellowing; or even leaf deformation. Some of these leaf symptoms are similar to those caused by other viruses, such as *American plum line pattern virus*. Flower symptoms can occur on petals (discoloration) of some peach cultivars when infected with PPV-M or in *P. glandulosa* infected with PPV-D. 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 areas under the discoloured rings. Stones from diseased apricot fruits show typical pale rings or spots. Diseased fruits may show internal browning and gummosis of the flesh and reduced quality. The alcohol or spirits produced from this fruits are unmarketable owing to an undesirable flavour. In severe cases the diseased fruits drop prematurely from the tree. In general the fruit of early cultivars show more marked symptoms than those of late cultivars.

Comment [AMJ5]: Add PPQ symptom photo gallery website to this paragraph.

[11] Appropriate sample selection is critical for PPV detection. If typical symptoms are present, collect flowers, leaves or fruits showing symptoms. In symptomless plants samples should be taken from at least one-year-old shoots with mature leaves or fully expanded leaves collected from the middle of each of the main branches. Sampling must be done avoiding months with the highest temperatures. Plant material should preferably be collected from the internal parts of the canopy of the tree. In springtime, samples can be flowers, young shoots with fully expanded leaves or fruits. In summer ~~and autumn~~ mature leaves and the skin of mature fruits collected from the field or packinghouses can be used for analysis. Testing of leaves should be avoided in autumn unless absolutely necessary because of the chance for false positives in ELISA. Flowers, leaves, shoots and fruit skin can be stored at 4 °C for not more than 7 days before processing. Fruits can be stored for one month at 4 °C before processing. In winter dormant buds or bark tissues from the basal part of twigs, shoots or branches, or complete spurs or dards can be selected.

[12] Detection and identification of all PPV isolates can be achieved using biological, serological or molecular tests following the flow diagram shown in Figure 1. These tests are the minimum requirements to detect and identify PPV (e.g. during routine diagnosis of a pest widely established in a country), but further tests may be required where the national plant protection organization (NPPO) requires additional confidence or to identify the strain of PPV. In all cases, positive and negative controls must be included in the tests. The recommended techniques are described in the following sections.



[13] **Figure 1:** Minimum requirements for the detection and identification of *Plum pox virus* (e.g. during routine diagnosis of a pest widely established in a country)

Comment [AMJ6]: Add: Optional to run test if typical symptoms present. If no symptoms, clarify serological OR molecular. Plum box present box—dashed line up to serological/molecular testing to validate---don't base presence just on symptoms. If visual on biological indicator, strongly suggested to confirm it using serological/molecular test.

[14] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. Use of names of chemicals or equipment in these diagnostic protocols 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.

[15] In instances where the NPPO requires additional confidence in the identification of PPV (e.g. detection in an area where the virus is not known to occur), further tests may be done. Where the initial identification was done using a molecular method, subsequent tests should use serological techniques and vice versa. Further tests may also be done to identify the strain of PPV present (Figure 2).

[16] ## 3.1 Biological detection and identification

[17] Budwood to be grafted must be collected from at least three different branches (this is critical because of the uneven distribution of PPV). The main indicator plants used for PPV indexing are seedlings of *P. persicae* cv. GF305, *P. persicae* × *P. davidiana* cv. Nemaguard, or *P. tomentosa*. Indicator plants are raised from seed, planted in a well-drained soil mixture and maintained in an insect-proof greenhouse between 18 °C and 25 °C until they are large enough to graft (usually 25–30 cm high with a diameter of 3–4 mm). The indicators must be graft-inoculated according to conventional methods such as bud grafting (Desvignes, 1999), using at least four replicates per indicator plant. Following grafting the indicator plants are maintained in the same conditions and after 3 weeks are pruned to a few centimetres above the top graft (Gentit, 2006). Symptoms, in particular chlorotic banding and patterns, are observed on the new growth after 3–4 weeks and must be compared with positive and healthy controls.

[18] There is no quantitative data published on the specificity, sensitivity or reliability of grafting. The method is used widely in certification schemes and is considered to be a reliable and sensitive method of detection. However, it is not a rapid test (symptom development requires several weeks post-inoculation), it requires dedicated facilities such as temperature-controlled greenhouse space, and the symptoms may be confused with those of other graft-transmissible agents.

[19] ## 3.2 Serological detection and identification

Comment [AMJ7]: Add sentence about antibody we're currently using (CFIA-developed antibody).

[20] Enzyme-linked immunosorbent assays are highly recommended for screening large numbers of samples.

[21] For sample processing, approximately 1 g of fresh plant material is weighed, cut into small pieces and placed in a suitable tube or plastic bag. About 20 volumes of extraction buffer are added and the sample is homogenized in tubes using a Polytron (Kinematica) or similar apparatus. Alternatively, the sample can be homogenized in plastic bags using a tissue homogenizer such as 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, containing 2% polyvinylpyrrolidone and 0.2% sodium diethyl dithiocarbamate (Cambra *et al.*, 1994). Plant material should be homogenized thoroughly and used fresh.

[22] **Double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA)**

[23] DASI-ELISA, also called triple-antibody sandwich (TAS)-ELISA, should be performed according to Cambra *et al.* (1994) using the specific monoclonal antibody 5B-IVIA following the manufacturer's instructions.

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[24] 5B-IVIA is the only monoclonal antibody that has been demonstrated to detect all strains of PPV, and does so with high reliability, specificity and sensitivity (Cambra *et al.*, 2006a). In a DIAGPRO ring-

test done by 17 laboratories using a panel of 10 samples (PPV-infected (PPV-D, PPV-M and PPV-D+M) and healthy) from France and Spain, DASI-ELISA using the 5B-IVIA monoclonal antibody was 95% accurate (number of true negatives and true positives diagnosed by the technique/number of samples tested). This accuracy was greater than that achieved with either immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) which was 82% accurate, or co-operational RT-PCR (Co-RT-PCR) which was 94% accurate (Cambra *et al*., 2006b; Olmos *et al*., 2007). The proportion of true negatives (number of true negatives diagnosed by the technique/number of healthy plants) identified by DASI-ELISA using the 5B-IVIA monoclonal antibody was 99.0%, compared with real-time RT-PCR using purified nucleic acid (89.2%) or spotted samples (98.0%), or immunocapture RT-PCR (96.1%). Capote *et al.* (2009) also reported that there is a 98.8% probability that a positive result obtained in winter with DASI-ELISA using the 5B-IVIA monoclonal antibody was a true positive.

[25] DASI-ELISA kits based on the 5B-IVIA monoclonal antibody are available from AC Diagnostics, Inc. (Fayetteville, USA), Agritest (Valenzano, Italy), AMR Lab (Barcelona, Spain) and Real/Durviz (Valencia, Spain).

[26] **Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)**

[27] Conventional or biotin/streptavidin system of DAS-ELISA should be performed using kits based on the specific monoclonal antibody 5B-IVIA or on polyclonal antibodies that have been demonstrated to detect all strains of PPV without cross-reacting with other viruses or healthy plant material. The test should be done according to the manufacturer's instructions.

[28] Whereas the 5B-IVIA monoclonal antibody detects specifically, sensitively and reliably all PPV strains, some polyclonal antibodies are not specific and have limited sensitivity (Cambra *et al.*, 1994; Cambra *et al.*, 2006a). Therefore the use of additional methods is recommended in instances where polyclonal antibodies have been used and the NPPO requires additional confidence in the identification of PPV.

[29] DAS-ELISA kits (conventional or biotin/streptavidin system) for PPV detection are available from AC Diagnostics, Inc. (Fayetteville, USA), Adgen (Auchincruive, United Kingdom), Agdia Incorporated (Elkart, USA), Agritest (Valenzano, Italy), AMR Lab (Barcelona, Spain), Bio-Rad (Marnes La Coquette, France), Bioreba (Reinach, Switzerland), DSMZ (Braunschweig, Germany), Hortitech (Selby, United Kingdom), Ingenasa (Madrid, Spain), Loewe Biochemica (Sauerlach, Germany), Plant Research International (Wageningen, the Netherlands), and Real/Durviz (Valencia, Spain).

[30] ## 3.3 Molecular detection and identification

Comment [AMJ8]: Add text to the effect of "At time of writing, these are the protocols….literature should always be consulted for additional protocols."

[31] Molecular methods may be more expensive and/or time consuming than serological techniques, especially for large-scale testing. However, molecular methods, especially real-time RT-PCR, are generally more sensitive than serological techniques. The use of real-time RT-PCR also avoids the need for any post-amplification processing (e.g. gel electrophoresis) and is therefore quicker with less opportunity for contamination than conventional PCR.

[32] ~~Fresh or frozen (stored between −20 °C and −80 °C for periods of at least one year) plant extracts can be used for molecular tests.~~ With the exception of IC-RT-PCR (for which RNA isolation is not required), RNA extraction should be done using the RNeasy Plant Minikit (Qiagen), the UltraClean™ Plant RNA Isolation Kit (Mo Bio) or other appropriately validated protocols, according to the manufacturer's instructions. Alternatively for real-time RT-PCR, spotted plant extracts (5 µl) or printed tissue sections can be immobilized on Whatman 3MM paper or nylon membranes and analysed by real-time RT-PCR (Olmos *et al.*, 2005; Osman and Rowhani, 2006; Capote *et al.*, 2009). It is not recommended to use spotted or tissue-printed samples in conventional PCR because of the lesser sensitivity compared with real-time RT-PCR.

[33] ### 3.3.1 Reverse transcription-polymerase chain reaction (RT-PCR)

Comment [AMJ9]: Laurene will draft chapeau paragraph for each section under 3.3.

[34] The RT-PCR primers used in this assay are either the primers of Wetzel *et al.* (1991):

P1 (5'-ACC GAG ACC ACT ACA CTC CC-3′)

P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

or the primers of Levy and Hadidi (1994):

3′NCR sense (5′-GTA GTG GTC TCG GTA TCT ATC ATA-3′)

3′NCR antisense (5′-GTC TCT TGC ACA AGA ACT ATA ACC-3′).

[35] The 25 µl reaction mixture is composed as follows: 1 µM of each primer (P1/P2 or the 3′NCR primer pair), 250 µM dNTPs, 1 unit AMV reverse transcriptase, 0.5 units Taq DNA polymerase, 2.5 µl 10 × Taq polymerase buffer, 1.5 mM MgCl2, and 0.3% Triton X-100. The reaction is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 ºC, 40 cycles of 30 s at 94 °C, 30 s at either 60 °C (P1/P2 primers) or 62 °C (3′NCR primers), 1 min at 72 °C, followed by a final extension for 10 min at 72 °C and quickly cooled to room temperature. The PCR products are analysed by gel electrophoresis. The P1/P2 and 3′NCR primers produce a 243 base pair (bp) and 220 bp amplicon, respectively.

[36] The method of Wetzel *et al.* (1991) was evaluated by testing PPV isolates from Mediterranean areas (Cyprus, Egypt, France, Greece, Spain and Turkey). The assay was able to detect 20 fg of viral RNA, corresponding to 2000 viral particles (Wetzel *et al.*, 1991). The method, designed by Levy and Hadidi (1994), was evaluated using PPV isolates from Egypt, France, Germany, Greece, Hungary, Italy, Spain and Romania.

[37] ### 3.3.2 Immunocapture RT-PCR

[38] The immunocapture phase should be performed according to Wetzel *et al.* (1992), using plant sap extracted as in section 3.2.

[39] Prepare a dilution (1 µg ml^{-1}) of polyclonal antibodies or PPV-specific monoclonal antibody (5B-IVIA) in carbonate buffer pH 9.6. Add 100 µl of the diluted antibodies into microfuge tubes and incubate at 37 °C for 3 h. Wash the tubes twice with 150 µl of sterile PBS-Tween (washing buffer). Clarify 100 µl of plant extract (see section 3.2) by centrifugation (5 min at 15 500 × g), and add the supernatant to the coated microfuge tubes. Incubate for 2 h on ice or at 37 °C. Wash the tubes three times with 150 µl of sterile PBS-Tween (washing buffer). Prepare the RT-PCR reaction mixture as described in section 3.3.1 using the primers of Wetzel *et al.* (1992), and add directly to the coated microfuge tubes. Perform the amplification as described in section 3.3.1.

[40] Inherent problems in IC-RT-PCR (inhibition and false positives due to contamination) make this method less reliable than other recommended serological and molecular tests. IC-RT-PCR using the 5B-IVIA monoclonal antibody has been validated in a DIAGPRO ring-test showing an accuracy of 82% for PPV detection (Cambra *et al.*, 2006b; Olmos *et al.*, 2007). The method also requires the use of specific antibodies and can be time consuming. Capote *et al.* (2009) reported that there is a 95.8% probability that a positive result obtained in winter with IC-RT-PCR using the 5B-IVIA monoclonal antibody was a true positive.

[41] ### 3.3.3 Co-operational RT-PCR

Comment [AMJ10]: Might not be necessary?

[42] The RT-PCR primers used in this assay (Co-RT-PCR) are the primers of Olmos *et al.* (2002):

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

P10 (5′-GAG AAA AGG ATG CTA ACA GGA-3′)

P20 (5′-AAA GCA TAC ATG CCA AGG TA-3′).

[43] The 20 µl reaction mixture is composed as follows: 0.1 µM of P1 and P2 primers, 0.05 µM of P10 and P20 primers, 400 µM dNTPs, 2 units AMV reverse transcriptase, 1 unit Taq DNA polymerase, 2 µl 10 × reaction buffer, 3 mM MgCl2, 5% DMSO, and 0.3% Triton X-100. The reaction mixture and 5 µl of RNA sample are added to a sterile microfuge tube. The RT-PCR is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 °C, 60 cycles of 15 s at 94 °C, 15 s at 50 °C, 30 s at 72 °C, followed by a final extension for 10 min at 72 °C and quickly cooled to room temperature.

[44] The RT-PCR reaction is coupled to a colorimetric detection of amplicons using a 3´digoxigenin-labelled PPV universal probe (5′-TCG TTT ATT TGG CTT GGA TGG AA-Digoxigenin-3′; Roche Molecular Biochemicals) as follows. Denature the amplified cDNA at 95 °C for 5 min and immediately place on ice. Place 1 µl of sample on a nylon membrane. Dry the membrane at room temperature and UV cross-link in a transilluminator for 4 min at 254 nm. For pre-hybridization, place the membrane in a hybridization tube at 60 °C for 1 h using a standard hybridization buffer. Discard the solution and perform the hybridization by mixing the 3′DIG-labelled probe with standard hybridization buffer at a final concentration of 10 pmol ml^{-1}, before incubating for 2 h at 60 °C. Wash the membrane twice for 15 min at room temperature with 2 × washing solution, and twice for 15 min at room temperature with 0.5 × washing solution. Equilibrate the membrane for 2 min in washing buffer before soaking for 30 min in blocking solution 1% (w/v). Incubate the membrane at room temperature with anti-digoxigenin-alkaline phosphatase conjugate antibodies at a working concentration of 1:5 000 (150 units $litre^{-1}$) in blocking solution 1% (w/v) for 30 min. Wash the membrane twice for 15 min with washing buffer, and equilibrate for 2 min with detection buffer. The substrate solution is prepared by mixing 45 µl NBT solution (75 mg ml^{-1} nitro blue tetrazolium salt in 70% (v/v) dimethylformamide) and 35 µl BCIP solution (50 mg ml^{-1} 5-bromo-4chloro-3indolyl phosphate toluidinium salt in 100% dimethylformamide) 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 incubation with the substrate stop the reaction by washing with water.

[45] This method was 100 times more sensitive than RT-PCR using the assay of Wetzel *et al.* (1991) (Olmos *et al.*, 2002). The method was validated in the DIAGPRO ring-test and had an accuracy of 94% (Cambra *et al.*, 2006b; Olmos *et al.*, 2007).

[46] 3.3.4 Real-time RT-PCR

[47] Real-time RT-PCR can be performed using either TaqMan or SYBR Green I. Two TaqMan methods have been described for universal detection of PPV (Schneider *et al.*, 2004; Olmos *et al.*, 2005). The primers and TaqMan probe used in the first assay are those reported by Schneider *et al.* (2004):

Forward primer (5′-CCA ATA AAG CCA TTG TTG GAT C-3′)

Reverse primer (5′-TGA ATT CCA TAC CTT GGC ATG T-3′)

TaqMan probe (5′-FAM-CTT CAG CCA CGT TAC TGA AAT GTG CCA-TAMRA-3′).

[48] The 25 µl reaction mixture is composed as follows: 1 × reaction mix (0.2mM of each dNTP and 1.2 mM MgSO4), 200 nM of forward and reverse primers, 100 nM TAMRA probe, 4.8 mM MgSO4 and 0.5 µl RT/Platinum® Taq mix (Superscript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen)). The reaction mixture and 300 pg of RNA template are added to a sterile microfuge tube or equivalent. The RT-PCR is performed under the following thermocycling conditions: 15 min at 52 °C, 5 min at 95 °C, 60 cycles of 15 s at 95 °C, 30 s at 60 °C, and quickly cooled to room temperature. The PCR products are analysed in real-time according to the manufacturer's instructions.

[49] The method of Schneider *et al.* (2004) was evaluated by testing PPV isolates from the United States, strains PPV-C, PPV-D, PPV-EA and PPV-M, and eight other viral species. The method was specific and able to detect consistently 10–20 fg of viral RNA (Schneider *et al.*, 2004). The method could also detect PPV in a number of hosts and in the leaves, stems, buds and roots of *P. persica*.

[50] The primers and TaqMan probe used in the second assay are those reported by Olmos *et al.* (2005):

P241 primer (5′-CGT TTA TTT GGC TTG GAT GGA A-3′)

P316D primer (5′-GAT TAA CAT CAC CAG CGG TGT G-3′)

P316M primer (5′-GAT TCA CGT CAC CAG CGG TGT G-3′)

PPV-DM probe (5′-FAM-CGT CGG AAC ACA AGA AGA GGA CAC AGA-TAMRA-3′).

[51] The 25 µl reaction mixture is composed as follows: 1 µM of P241 primer, 0.5 µM each of P316D and P316M primers, 200 nM TAMRA probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems). The reaction mixture and 5 µl of RNA template are added to a sterile microfuge tube or equivalent. The RT-PCR is performed under the following thermocycling conditions: 30 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and quickly cooled to room temperature. The PCR products are analysed in real-time according to the manufacturer's instructions.

[52] The method of Olmos *et al.* (2005) was evaluated using three isolates each of PPV-D and PPV-M, and was 1 000 times more sensitive than DASI-ELISA using the 5B-IVIA monoclonal antibody. The proportion of true positives (number of true positives diagnosed by the technique/number of healthy plants) identified correctly by real-time RT-PCR using TaqMan (Olmos *et al.*, 2005) and purified nucleic acid was 97.5%, compared with real-time RT-PCR using spotted samples (93.6%), immunocapture RT-PCR (91.5%) or DASI-ELISA using the 5B-IVIA monoclonal antibody (86.6%) (Capote *et al.*, 2009).

[53] Varga and James (2005) described a SYBR Green I method for the simultaneous detection of PPV and identification of D and M strains:

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PPV-U (5′-TGA AGG CAG CAG CAT TGA GA-3′)

PPV-FD (5′-TCA ACG ACA CCC GTA CGG GC-3′)

PPV-FM (5′-GGT GCA TCG AAA ACG GAA CG-3′)

PPV-RR (5′-CTC TTC TTG TGT TCC GAC GTT TC-3′).

[54] The following internal control primers may be included to ensure the correct performance of the assay:

Nad5-F (5′-GAT GCT TCT TGG GGC TTC TTG TT-3′)

Nad5-R (5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′).

[55] A two-step RT-PCR protocol is used. The RT reaction is composed as follows: 2 µl of 10 µM P1 primer, 2 µl of 10 µM Nad5-R primer, 4 µg total RNA and 5 µl water. Incubate 72 °C for 5 min, place on ice. Add 4 µl 5 × first strand buffer (Invitrogen), 2 µl 0.1 M DTT, 1 µl 10 mM dNTPs, 0.5 µl RNaseOUTTM (40 units μl^{-1}) (Invitrogen), 1 µl Superscript™ II (Invitrogen) and 2.5 µl water. Incubate 42 °C for 60 min followed by 99 °C for 5 min. The 25 µl PCR reaction mixture is composed as follows: 400 nM PPV-U primer, 350 nM PPV-FM primer, 150 nM PPV-FD primer, 200 nM PPV-RR primer, 100 nM Nad5-F primer, 100 nM Nad5-R primer, 200 µM dNTPs, 2mM MgCl2, 1 × Karsai buffer (Karsai *et al.*, 2002), 1:42 000 SYBR Green I (Sigma) and 0.1 µl Platinum® Taq DNA high fidelity polymerase (Invitrogen). The reaction mixture and 1 µl of diluted cDNA (1:4) are added to a sterile microfuge tube or equivalent. The PCR is performed under the following thermocycling conditions: 2 min at 95 °C, 39 cycles of 15 s at 95 °C, 60 s at 60 °C, and cooled quickly to room temperature. Melting curve analysis is done by incubation at 60 °C to 95 °C at 0.1 °C s^{-1} with a smooth curve setting averaging 1 point. The melting temperatures for each product are:

Universal PPV detection (74 bp fragment): 80.08–81.52 °C

D strains (114 bp fragment): 84.3–84.43 °C

M strains (380 bp fragment): 85.34–86.11 °C

Internal control (181 bp fragment): 82.45–82.63 °C.

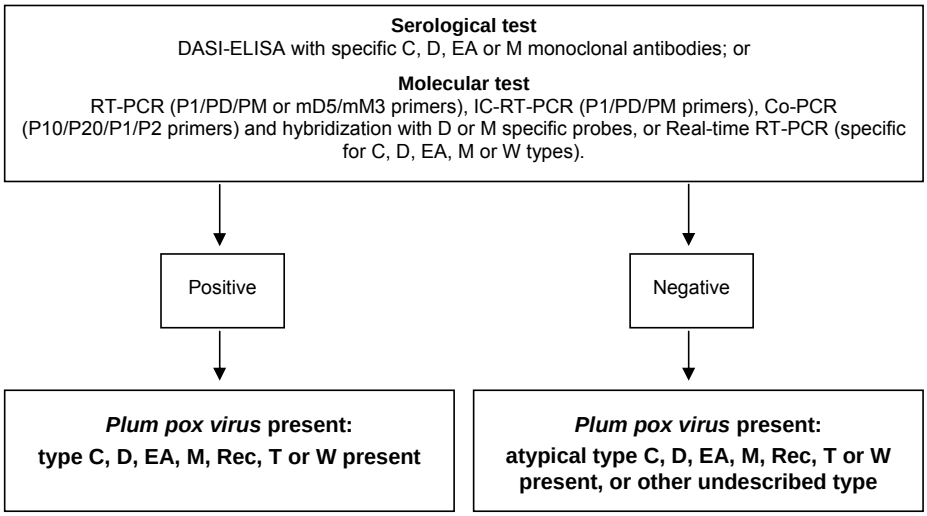
[56] The method of Varga and James (2005) was evaluated using isolates of PPV-C, PPV-D, PPV-EA and PPV-M, and an uncharacterized strain, in Nicotiana and Prunus species.

[57] 4. Identification of Strains

Comment [AMJ11]: Explain why you would want to strain-type, not just how (epidemiology, eradication). Important for managing/eradicating the disease. So other plant protection agencies know what's in your country. Location of nurseries if a certain strain; other internal concerns. Add sentence above each explaining (see prev.)

[58] The methods described in sections 3.2 and 3.3 for serological and molecular detection can also be used for identification of the virus. This section describes additional methods (DASI-ELISA, RT-PCR, Co-RT-PCR and real-time RT-PCR) for identification of PPV strains (see Figure 2). It is not necessary to determine which strain is present in order to identify PPV, but an NPPO may wish to determine the identity of the strain, for example, to assist in predicting its epidemiological behaviour.

[59] Given the variability of PPV, techniques other than sequencing or some PCR-based assays (see below) may provide erroneous results with a small percentage of isolates. However, it is generally possible to discriminate the D and M types of PPV using the serological or molecular techniques described below (Candresse and Cambra, 2006; Cambra *et al.*, 2006a; Capote *et al.*, 2006).



[60] **Figure 2:** Methods for the identification of strains of *Plum pox virus*

[61] Further tests may be done in instances where the NPPO requires additional confidence in the identification of PPV type. Sequencing of the complete or partial CP gene may also be done, especially where atypical or undescribed types are present.

[62] 4.1 Serological identification of strains

[63] DASI-ELISA for differentiation between the two main PPV types (D and M) should be performed according to Cambra *et al.* (1994), using D- and M-specific monoclonal antibodies (Cambra *et al.*, 1994; Boscia *et al.*, 1997), according to the manufacturer's instructions.

[64] This method has been validated in the DIAGPRO ring-test showing an accuracy of 84% for PPV-D detection and 89% for PPV-M detection (Cambra *et al.*, 2006b; Olmos *et al.*, 2007). The 4D monoclonal antibody is PPV-D specific but does not react against all PPV-D isolates. In addition, the AL monoclonal antibody reacts with isolates belonging to strains M, Rec and T since these groups share the same coat protein sequence. Therefore a molecular test is required to discriminate M, Rec and T types detected using an M-specific monoclonal antibody.

[65] DASI-ELISA kits based on the 4D (PPV-D specific) and AL (PPV-M, Rec and T specific) monoclonal antibodies are available from Agritest (Valenzano, Italy), AMR Lab (Barcelona, Spain) and Real/Durviz (Valencia, Spain).

[66] Serological identification of PPV isolates from EA and C groups is done by DASI-ELISA using the EA- and/or the C-specific monoclonal antibodies described by Myrta *et al.* (1998, 2000). These tests have not been validated and the antibodies are not commercially available.

[67] 4.2 Molecular identification of strains

[68] 4.2.1 RT-PCR

[69] PPV-D and PPV-M are identified using the primers described by Olmos *et al.* (1997):

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PD (5′-CTT CAA CGA CAC CCG TAC GG-3′) or PM (5′-CTT CAA CAA CGC CTG TGC GT -3′).

[70] The 25 µl reaction mixture is composed as follows: 1 µM of P1 primer, 1 µM of either PD or PM primer, 250 µM dNTPs, 1 unit AMV reverse transcriptase (10 units μl^{-1}), 0.5 units Taq DNA polymerase (5 units μl^{-1}), 2.5 µl 10 × Taq polymerase buffer, 1.5 mM MgCl2, 0.3% Triton X-100 and 2% formamide. The RT-PCR is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, followed by a final extension for 10 min at 72 °C and quickly cooled to room temperature. The PCR products are analysed by gel electrophoresis. The P1/PD and P1/PM primers produce a 198 bp amplicon. The method was evaluated using six isolates of PPV-D and four PPV-M isolates.

[71] PPV-Rec is identified using the mD5/mM3 Rec-specific primers described by Šubr et al. (2004):

mD5 (5′-TAT GTC ACA TAA AGG CGT TCT C-3′)

mM3 (5′-CAT TTC CAT AAA CTC CAA AAG AC-3′).

[72] The 25 µl reaction mixture is composed as follows (modified from Šubr *et al.*, 2004): 1 µM of each primer, 250 µM dNTPs, 1 unit AMV reverse transcriptase (10 units μl^{-1}), 0.5 units Taq DNA polymerase (5 units μl^{-1}), 2.5 µl 10 × Taq polymerase buffer, 2.5 mM MgCl2, 0.3% Triton X-100 and 5 µl of extracted RNA (see section 3.3). The PCR product of 605 bp is analysed by gel electrophoresis.

[73] 4.2.2 Immunocapture RT-PCR

[74] The immunocapture phase should be performed as described in section 3.2. The PCR reaction mixture is added directly to the coated microfuge tubes. Identification of PPV-D and PPV-M detection is done as described in section 4.2.1.

[75] 4.2.3 Co-operational RT-PCR

[76] Identification of PPV-D or PPV-M should be done as described in section 3.3.3 using 3′DIG-labelled probes specific for D and M strains (Olmos *et al.*, 2002):

PPV-D Specific Probe: 5′-CTT CAA CGA CAC CCG TAC GGG CA-Digoxigenin-3′

PPV-M Specific Probe: 5′-AAC GCC TGT GCG TGC ACG T-Digoxigenin-3′.

[77] The prehybridization and hybridization steps are performed at 50 °C with standard prehybridization and hybridization buffers + 30% formamide (for PPV-D identification) and + 50% formamide (for PPV-M identification). The blocking solution is used at 2% (w/v).

[78] **4.2.4 Real-time RT-PCR**

[79] PPV-D and PPV-M are specifically identified using either SYBR Green I chemistry according to the method of Varga and James (2005) (see section 3.3.4) or the TaqMan method described by Capote *et al.* (2006).

[80] The primers and TaqMan probes used in the method of Capote et al. (2006) are

PPV-MGB-F primer (5′-CAG ACT ACA GCC TCG CCA GA-3′)

PPV-MGB-R primer (5′-CTC AAT GCT GCT GCC TTC AT-3′)

MGB-D probe (5′- FAM-TTC AAC GAC ACC CGT A-MGB-3′)

MGB-M probe (5′-FAM-TTC AAC AAC GCC TGT G-MGB-3′).

[81] The 25 µl reaction mixture is composed as follows: 1 µM of each primer, 150 nM MGB-D or MGB-M FAM probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems). The reaction mixture and 5 µl of RNA template (see section 3.3), or spotted plant extracts (5 µl) or printed tissue sections (see section 3.3), are added to a sterile microfuge tube or equivalent. The RT-PCR is performed under the following thermocycling conditions: 30 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and quickly cooled to room temperature. The PCR products are analysed in real time according to the manufacturer's instructions. The method has been evaluated using 12 isolates each of PPV-D and PPV-M, and 14 samples co-infected with both types.

[82] PPV-C, PPV-EA and PPV-W are specifically identified using SYBR Green I chemistry according to the method of Varga and James (2006). The primers used in this method are

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PPV-U (5′-TGA AGG CAG CAG CAT TGA GA-3′)

PPV-RR (5′-CTC TTC TTG TGT TCC GAC GTT TC-3′).

[83] The following internal control primers may be included to ensure the correct performance of the assay:

Nad5-F (5′-GAT GCT TCT TGG GGC TTC TTG TT-3′)

Nad5-R (5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′).

[84] The 25 µl RT-PCR reaction is composed as follows: 2.5 µl of a 1/10 water dilution of extracted RNA (see section 3.3) and 22.5 µl of master mix. The master mix has the following composition: 2.5 µl of Karsai Buffer (Karsai *et al.*, 2002); 0.5 µl each of 5 µM primers PPV-U, PPV-RR or P1, Nad5R and Nad5F; 0.5 µl of 10 mM dNTPs; 1 µl of 50 mM MgCl2; 0.2 µl of RNaseOUT™ (40 units μl^{-1}; Invitrogen); 0.1 µl of Superscript™ III (200 units μl^{-1}; Invitrogen); 0.1 µl of Platinum® Taq DNA high fidelity polymerase (5 units μl^{-1}, Invitrogen); and 1 µl of 1:5 000 (in TE, pH 7.5) SYBR Green I (Sigma) in 16.1 µl water. The reaction is performed under the following thermocycling conditions: 10 min at 50 °C, 2 min at 95 °C, 29 cycles of 15 s at 95 °C, and 60 s at 60 °C. Melting curve analysis is performed by incubation at 60 °C to 95 °C at 0.1 °C s^{-1} melt rates with a smooth curve setting averaging 1 point. The melting temperatures for each product are

C strain (74 bp fragment): 79.84 °C

EA strain (74 bp fragment: 81.27 °C

W strain (74 bp fragment): 80.68 °C.

[85] This method was evaluated using an isolate each of PPV-C, PPV-D, PPV-EA and PPV-W.

[86] **5. Records**

[87] The records required to be kept are listed in section 2.5 of ISPM 27.

[88] In instances where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the virus is found in an area for the first time, the following additional material should be kept:

- The original sample (labelled appropriately for traceability) should be kept frozen at −80 °C or freeze-dried and kept at room temperature.
- If relevant, RNA extractions should be kept at −80 °C and/or spotted plant extracts or printed tissue sections paper on membranes should be kept at room temperature.
- If relevant, RT-PCR amplification products should be kept at −80 °C.

[89] 6. Contact Points for Further Information

Comment [AMJ12]: Add USDA-PPQ as a point of contact.

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~~[97]~~[98] 7. Acknowledgements

~~[98]~~[99] This diagnostic protocol was drafted by Drs. M. Cambra, A. Olmos and N. Capote, IVIA (see preceding section), Dr. N.L. Africander, National Department of Agriculture, 63 Kipling Street, Kraaifontein 7570, South Africa; Dr. L. Levy, United States Department of Agriculture, Beltsville, Maryland, United States of America; Dr. S.L. Lenardon, IFFIVE-INTA, Cno. 60 Cuadras Km 51/2, Córdoba X5020ICA, Argentina; Dr. G. Clover, Plant Health & Environment Laboratory, Ministry of Agriculture and Forestry, PO Box 2095, Auckland 1140, New Zealand; and Ms. D. Wright, Plant Health Group, Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom.

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