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[25]**Discipline leads history**	[26]2016-11 Mr Delano JAMES (CA, discipline lead) [27]2016-11 Mr Brendan RODONI (AU, referee)
[28]**Consultation on technical level**	[29]First revision of the draft written by: - [30]Mr Delano JAMES (Canadian Food Inspection Agency, TPDP member, Canada) - [31]Mr Mariano CAMBRA (Consellería de Agricultura y Pesca, Instituto Valenciano de Investigaciones Agrarias (IVIA), Spain). - [32]Mr Antonio OLMOS (Consellería de Agricultura y Pesca, Instituto Valenciano de Investigaciones Agrarias (IVIA), Spain).
[33]**Main discussion points during development of the diagnostic protocol** [34]	[35]The TPDP proposed to the SC November 2016 the following revision: [36]The DP should be updated to indicate the new strains of PPV described recently (PPV CR and PPV An) and to include the RT-PCR for specific identification of PPV CR. [37]Sections of DP 2: *Plum pox virus* that may need to be updated include: - [38]Section 1. Pest information to include information on recently described strains. - [39]Section 3.2.1 to indicate the conditions required for the detection of PPV strain CR by ELISA. - [40]Section 4. Identification of Strains. Strain CR should be added to Figure 1, since there is a RT-PCR developed for the identification of CR isolates. - [41]Section 4.2.1. The specific RT-PCR for identification of CR should be added to this section [42]Other notes: - [43]Footnotes and brand names (based on SC decision and according to TPDP instruction to authors): If in the DP there is more than one mention to a brand name, the second mention (and the subsequent mentions) to a brand name shall be associated with the footnote number with the full text (e.g. If the first mention to a brand name is “footnote 1”, the subsequent mentions to brand names should be accompanied by the same footnote number, i.e., “footnote number 1”).
[44]**Notes**	[45]This is a draft document. The final formatting will be adjusted at later stage. [46]2017-03 Edited [47]**Please note that some paragraph numbers may be missing from the document or not be in a chronological order. This is due to technical problems in the OCS but it does not affect the integrity of the content of the document.**

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[49]**CONTENTS**

[50][To be added]

[51]1. Pest Information

[52]Sharka (plum pox) is one of the most serious diseases of stone fruit. The disease, caused by *Plum pox virus* (PPV), affects plants of the genus *Prunus*. It is particularly detrimental in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina* because it reduces quality and causes premature fruit drop. It is estimated that the costs of managing sharka worldwide since the 1970s exceed 10 000 million euros (Cambra *et al*., 2006b).

[53]Sharka was first reported in *P. domestica* 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 East. It has been found with a restricted distribution in South and North America and Asia (EPPO, 2006; CABI, 2016).

[54]*Plum pox virus* is a member of the genus *Potyvirus* in the family *Potyviridae*. The virus particles are flexuous rods of approximately 700 nm × 11 nm, and are 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 is the main way in which PPV is spread over long distances.

[55]*Plum pox virus* isolates can be classified currently into nine strains: D (Dideron), M (Marcus), C (Cherry), EA (El Amar), W (Winona), Rec (Recombinant), T (Turkish), CR (Cherry Russian) and An (Ancestor Marcus) (James *et al*., 2013;). Most PPV isolates belong to the D and M strains. PPV D and M strains can easily infect *P. armeniaca* and *P. domestica* but differ in their ability to infect *P. persica* cultivars. The strains vary in their pathogenicity; for example M isolates generally cause faster epidemics and more severe symptoms than D isolates in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina*. EA isolates are geographically restricted to Egypt and little information is available about their epidemiology and biological properties. PPV isolates infecting *P. avium* and *P. cerasus* have been identified in several European countries. These isolates form two distinct strains that have been defined as PPV-C and PPV-CR. An atypical PPV was detected in *P. domestica* in Canada (PPV-W) representing a distinct PPV strain. PPV W has since been detected in several countries in Europe (James *et al.*, 2013). In addition, natural recombinants between the D and M strains of PPV have been described as PPV-Rec, these showing an epidemiological behaviour similar to the D strain. A second type of recombinant strain has been reported in Turkey (T strain, Ulubaş Serçe *et al*., 2009). A single isolate of PPV An has been described and it has been proposed as a potential ancestor of PPV M (Palmisano *et al*., 2012). A novel sour cherry-adapted Tat strain, neither C nor CR, has also been proposed (Chirkov *et al*., 2016).

[56]Further information about PPV, including illustrations of disease symptoms, can be found in Barba *et al*. (2011), CABI (2016), EPPO (2004, 2006), García and Cambra (2007), and PaDIL (2017).

[57]2. Taxonomic Information

[58]**Name:** *Plum pox virus* (acronym PPV)

[59]**Synonym:** Sharka virus

[60]**Taxonomic position:** *Potyviridae*, *Potyvirus*

[61]**Common names:** Sharka, plum pox.

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[62]3. Detection and Identification

[63]Under natural conditions, PPV readily infects fruit trees of the genus *Prunus* used as commercial varieties or rootstocks: *P. armeniaca*, *P. cerasifera*, *P. davidiana*, *P. domestica*, *P. mahaleb*, *P. marianna*, *P. mume*, *P. persica*, *P. salicina*, and interspecific hybrids between these species. *Prunus avium*, *P. cerasus* and *P. dulcis* may be infected occasionally. The virus also infects many wild and ornamental *Prunus* species such as *P. besseyi*, *P. cistena*, *P. glandulosa*, *P. insititia*, *P. laurocerasus*, *P. spinosa*, *P. tomentosa* and *P. triloba*. Under experimental conditions, PPV can be transmitted mechanically to numerous *Prunus* spp. and several herbaceous plants (*Arabidopsis thaliana*, *Chenopodium foetidum*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa* and *Pisum sativum*).

[64]Sharka symptoms may appear on leaves, shoots, bark, petals, fruits and stones in the field. They are usually distinct on leaves early in the growing season and include mild light-green discoloration; chlorotic spots, bands or rings; vein clearing or yellowing; or leaf deformation. Some of these leaf symptoms are similar to those caused by other viruses, such as *American plum line pattern virus*. *Prunus cerasifera* cv. GF 31 shows rusty-brown corking and cracking of the bark. Flower symptoms can occur on petals (discoloration) of some *P. persica* 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. Some fruit deformations, especially in *P. armeniaca* and *P. domestica*, are similar to those caused by *Apple chlorotic leaf spot virus*. Diseased fruits may show internal browning and gummosis of the flesh and reduced quality. In severe cases the diseased fruits drop prematurely from the tree. In general, the fruits of early maturing cultivars show more marked symptoms than those of late maturing cultivars. Stones from diseased fruits of *P. armeniaca* show typical pale rings or spots. The alcohol or spirits produced from diseased fruits are unmarketable owing to an undesirable flavour. Symptom development and intensity depend strongly on the host plant and climatic conditions; for example the virus may be latent for several years in cold climates.

[65]General guidance on sampling methodologies is provided in ISPM 31 (*Methodologies for sampling of consignments*). Appropriate sample selection is critical for PPV detection. Sampling should take into account virus biology and local climatic conditions, in particular the weather conditions during the growing season. If typical symptoms are present, samples should be collected of flowers, leaves or fruits showing symptoms. In symptomless plants, samples should be taken from shoots that are at least one year old and have mature or fully expanded leaves, collected from the middle of each of the main branches (detection is not reliable in shoots less than one year old). Samples should be collected from at least four different sites (e.g. four branches or four leaves) in each plant; this is critical because of the uneven distribution of PPV. Sampling should not be done during months with the highest temperatures. Tests on samples collected in the autumn are less reliable than tests done on samples collected earlier in the spring. Plant material should preferably be collected from the internal parts of the tree canopy. In springtime, samples can be flowers, shoots with fully expanded leaves, or fruits. In summer and autumn, mature leaves and the skin of mature fruits collected from the field or packing houses can be used for analysis. Flowers, leaves, shoots and fruit skin can be stored at 4 °C for not more than 10 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 can be selected.

[66]Detection of PPV can be achieved using a biological, serological or molecular test; identification requires either a serological or molecular test. A serological or molecular test is the minimum requirement to detect and identify PPV (e.g. during routine diagnosis of a pest widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of PPV (e.g. detection in an area where the virus is not known to be present or detection in a consignment originating in a country where the pest is declared to be absent), further tests may be done. Where the initial identification was done using a molecular method, subsequent tests should use serological methods and vice versa. Further tests may also be done to identify the strain of PPV present. In all cases, positive and negative controls must be included in the tests. The recommended techniques are described in the following sections.

[67]In some circumstances (e.g. during the routine diagnosis of a pest widely established in a country) multiple plants may be tested simultaneously using a bulked sample derived from a number of plants. The decision to test individual or multiple plants depends on the virus concentration in the plants and the level of confidence required by the NPPO.

[68]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. The use of names of reagents, 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.

[69]3.1Biological detection

[70]The main indicator plants used for PPV indexing are seedlings of *P. cerasifera* cv. GF31, *P. persica* cv. GF305, *P. persica* × *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). Alternatively, seedlings of other *Prunus* species may be grafted with indicator plant scions. The indicators must be graft-inoculated according to conventional methods such as bud grafting (Desvignes, 1999), using at least four replicates per indicator plant. The grafted indicator plants are maintained in the same conditions and, after three weeks, are pruned to a few centimetres above the top graft (Gentit, 2006). The grafted plants should be inspected for symptoms for at least six weeks. 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. Illustrations of symptoms caused by PPV on indicator plants can be found in Damsteegt *et al*. (1997, 2007) and Gentit (2006).

[71]There are no quantitative data published on the specificity, sensitivity or reliability of grafting. The method is used widely in certification schemes and is considered a sensitive method of detection. However, it is not a rapid test (symptom development requires several weeks post-inoculation), it can only be used to test budwood, it requires dedicated facilities such as temperature-controlled greenhouse space, and the symptoms observed may be confused with those of other graft-transmissible agents. Moreover, there are asymptomatic strains that do not induce symptoms and thus are not detectable on indicator plants.

[72]3.2Serological detection and identification

[73]Enzyme-linked immunosorbent assays (ELISA) are highly recommended for screening large numbers of samples.

[74]For sample processing, approximately 0.2–0.5 g of fresh plant material is cut into small pieces and placed in a suitable tube or plastic bag. The sample is homogenized in approximately 4–10 ml (1:20 w/v) of extraction buffer using an electrical tissue homogenizer, or a manual roller, hammer or similar tool. 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), or an alternative suitably validated buffer. Plant material should be homogenized thoroughly and used fresh.

[75]*3.2.1Double-antibody sandwich indirect enzyme-linked immunosorbent assay*

[76]Double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA), also called triple-antibody sandwich (TAS)-ELISA, should be performed according to Cambra *et al*. (1994) using a specific monoclonal antibody such as 5B-IVIA, following the manufacturer's instructions.

[77]The only monoclonal antibody currently demonstrated to detect all strains of PPV with high reliability, specificity and sensitivity is 5B-IVIA (Cambra *et al.*, 2006a). Optimal detection of isolates of strain CR requires adjustment of the extraction buffer to pH 6.0 (Chirkov *et al.*, 2013; Glasa *et al.*,

2013). In a DIAGPRO[1] ring-test conducted by 17 laboratories using a panel of 10 samples, including both PPV-infected (PPV-D, PPV-M and PPV-D+M) and healthy samples 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, divided by the 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 (Olmos *et al*., 2007; Cambra *et al*., 2008). The proportion of true negatives (number of true negatives diagnosed by the technique, divided by the 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 IC-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.

[79]*3.2.2Double-antibody sandwich enzyme-linked immunosorbent assay*

[80]The conventional or biotin–streptavidin system of double-antibody sandwich (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 (Cambra *et al*., 2006a; Capote *et al*., 2009). The test should be done according to the manufacturer's instructions.

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

[82]3.3Molecular detection and identification

[83]Molecular methods using reverse transcription-polymerase chain reaction (RT-PCR) may be more expensive or time consuming than serological methods, especially for large-scale testing. However, molecular methods, especially real-time RT-PCR, are generally more sensitive than serological methods. 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 (with the target DNA) than conventional PCR.

[84]With the exception of IC-RT-PCR (for which RNA isolation is not required), RNA extraction should be conducted using appropriately validated protocols. The samples should be placed in individual plastic bags to avoid cross-contamination during extraction. Alternatively, for real-time RT-PCR, spotted plant extracts, printed tissue sections or squashes of plant material can be immobilized on blotting 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 that spotted or tissue-printed samples be used in conventional PCR because of the lower sensitivity compared with real-time RT-PCR.

[85]Each of the following methods describes the volume of extracted sample that should be used as a template. Depending on the sensitivity of the method, the minimum concentration of template required to detect PPV varies as follows: RT-PCR, 100 fg RNA template/ml; Co-RT-PCR, 1 fg RNA template/ml; and real-time RT-PCR, 2 fg RNA template/ml.

[86]*3.3.1Reverse transcription-polymerase chain reaction*

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

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[78][1] DIAGPRO was a project, funded by the European Union, to develop diagnostic protocols for quarantine pests.

[88]P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

[89]P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

[90]or the primers of Levy and Hadidi (1994):

[91]3′NCR sense (5′-GTA GTG GTC TCG GTA TCT ATC ATA-3′)

[92]3′NCR antisense (5′-GTC TCT TGC ACA AGA ACT ATA ACC-3′).

[93]The 25 µl reaction mixture is composed as follows: 1 µM of each primer (P1 and P2, or the 3′NCR primer pair), 250 µM dNTPs, 1 unit *Avian myeloblastosis virus* (AMV) reverse transcriptase, 0.5 units Taq DNA polymerase, 2.5 µl 10× Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.3% Triton X-100 and 5 µl RNA template. The reaction 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 either 60 °C (P1 and P2 primers) or 62 °C (3′NCR primers), and 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products are analysed by gel electrophoresis. The P1/P2 pair of primers produces a 243 base pair (bp) amplicon and the 3′NCR primers produce a 220 bp amplicon.

[94]The method of Wetzel *et al.* (1991) was evaluated by testing PPV isolates from Mediterranean areas (Cyprus, Egypt, France, Greece, Spain and Turkey). It was able to detect 10 fg of viral RNA, corresponding to 2 000 viral particles (Wetzel *et al.*, 1991). Levy and Hadidi (1994) evaluated their method using PPV isolates from Egypt, France, Germany, Greece, Hungary, Italy, Spain and Romania.

[95]***3.3.2 Immunocapture reverse transcription-polymerase chain reaction***

[96]The immunocapture phase should be performed according to Wetzel *et al.* (1992), using plant sap extracted as in section 3.2 using individual tubes or plastic bags to avoid contamination.

[97]A dilution (1 µg/ml) is prepared of polyclonal antibodies or PPV-specific monoclonal antibody (5B-IVIA) in carbonate buffer pH 9.6. Aliquots of 100 µl diluted antibody are dispensed into PCR tubes and incubated at 37 °C for 3 h. The tubes are then washed twice with 150 µl sterile PBS-Tween (washing buffer), and rinsed twice with RNase-free water. Plant extract (100 µl; see section 3.2) is clarified by centrifugation (5 min at 15 500 *g*), and the supernatant added to the coated PCR tubes. The tubes are incubated for 2 h on ice or at 37 °C, and then washed three times with 150 µl sterile PBS-Tween. The RT-PCR reaction mixture is prepared as described in section 3.3.1 using the primers of Wetzel *et al.* (1992), and added directly to the coated PCR tubes. The amplification is performed as described in section 3.3.1.

[98]In general, IC-RT-PCR requires the use of specific antibodies, although direct-binding methods may eliminate this requirement. 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 (Olmos *et al.*, 2007; Cambra *et al.*, 2008). 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.

[99]***3.3.3 Co-operational reverse transcription-polymerase chain reaction***

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

[101]Internal primer P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

[102]Internal primer P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

[103]External primer P10 (5′-GAG AAA AGG ATG CTA ACA GGA-3′)

[104]External primer P20 (5′-AAA GCA TAC ATG CCA AGG TA-3′).

[105]The 25 µl reaction mixture is composed as follows: 0.1 µM each of P1 and P2 primers, 0.05 µM each 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 $MgCl_2$, 5% dimethyl sulphoxide, 0.3% Triton X-100 and

5 µl RNA template. 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, and 30 s at 72 °C, followed by a final extension for 10 min at 72 °C.

[106]The RT-PCR reaction is coupled to a colorimetric detection of amplicons using a 3′digoxigenin (DIG)-labelled PPV universal probe (5′-TCG TTT ATT TGG CTT GGA TGG AA-DIG-3′) as follows. The amplified complementary (c)DNA is denatured at 95 °C for 5 min and immediately place on ice. A 1 µl aliquot of sample is placed on a nylon membrane. The membrane is then dried at room temperature and UV cross-linked in a transilluminator for 4 min at 254 nm. For pre-hybridization, the membrane is placed in a hybridization tube at 60 °C for 1 h using a standard hybridization buffer. The solution is discarded and the hybridization performed by mixing the 3′DIG-labelled probe with standard hybridization buffer at a final concentration of 10 pmol/ml, before incubating for 2 h at 60 °C. The membrane is washed twice for 15 min at room temperature with 2× washing solution, and twice for 15 min at room temperature with 0.5× washing solution. The membrane is then equilibrated for 2 min in washing buffer before soaking for 30 min in sterilized 1% blocking solution (1 g blocking reagent dissolved in 100 ml maleic acid buffer). The membrane is incubated at room temperature with anti-DIG-alkaline phosphatase conjugate antibodies at a working concentration of 1:5 000 (150 units/litre) in 1% blocking solution (w/v) for 30 min. The membrane is then washed twice for 15 min with washing buffer, and equilibrated for 2 min with detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The substrate solution is prepared by mixing 45 µl NBT solution (75 mg/ml nitro blue tetrazolium salt in 70% (v/v) dimethylformamide) and 35 µl BCIP solution (50 mg/ml 5-bromo-4chloro-3indolyl phosphate toluidinium salt in 100% dimethylformamide) in 10 ml detection buffer. After incubation with the substrate, the reaction is stopped by washing with water.

[107]This method has been found to be 100 times more sensitive than the RT-PCR method of Wetzel *et al.* (1991) (Olmos *et al.*, 2002). The method was validated in the DIAGPRO ring-test, where it had an accuracy of 94% (Olmos *et al.*, 2007; Cambra *et al.*, 2008).

[108]*3.3.4 Real-time reverse transcription-polymerase chain reaction*

[109]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 method are those reported by Schneider *et al.* (2004):

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

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

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

[113]The 25 µl reaction mixture is composed as follows: 1× reaction mix (0.2 mM of each dNTP and 1.2 mM $MgSO_4$), 200 nM each of forward and reverse primers, 100 nM TaqMan probe, 4.8 mM $MgSO_4$, 0.5 µl RT/Platinum® Taq mix (Superscript® One-Step RT-PCR with Platinum® Taq DNA polymerase; Invitrogen)[2] and 5 µl RNA template. 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, and 30 s at 60 °C. The PCR products are analysed in real-time according to the equipment manufacturer's instructions.

[115]The method of Schneider *et al.* (2004) was evaluated by testing PPV isolates from the United States of America, 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*.

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[114][2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. The use of names of reagents, 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 these are adequately validated.

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

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

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

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

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

[121]The 25 µl reaction mixture is composed as follows: 1 µM P241 primer, 0.5 µM each of P316D and P316M primers, 200 nM TaqMan probe, 1× TaqMan Universal PCR Master Mix (Applied Biosystems)[2], 1× MultiScribe™ and RNase Inhibitor Mix (Applied Biosystems)[2] and 5 µl RNA template. 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, and 60 s at 60 °C. The PCR products are analysed in real-time according to the equipment manufacturer's instructions.

[122]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, divided by the number of PPV-infected 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).

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

[124]P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

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

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

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

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

[129]The following internal control primers may be included to ensure the validity of the test results:

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

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

[132]A two-step RT-PCR protocol is used. The RT reaction mixture is composed as follows: 2 µl 10 µM P1 primer, 2 µl 10 µM Nad5-R primer, 4 µg total RNA and 5 µl water. The mixture is incubated at 72 °C for 5 min, then placed on ice. The following are then added: 4 µl 5× first strand buffer (Invitrogen)[2], 2 µl 0.1 M dithiothreitol (DTT), 1 µl 10 mM dNTPs, 0.5 µl RNaseOUT™ (40 units/µl) (Invitrogen)[2], 1 µl Superscript™ II reverse transcriptase (Invitrogen)[2] and 2.5 µl water. The mixture is incubated at 42 °C for 60 min followed by 99 °C for 5 min. The 24 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, 2 mM $MgCl_2$, 1× Karsai buffer (Karsai *et al.*, 2002), 1:42 000 SYBR® Green I (Sigma)[2] and 0.1 µl Platinum® Taq DNA high fidelity polymerase (Invitrogen)[2]. The PCR reaction mixture and 1 µl diluted cDNA (1:4) are added to a sterile PCR tube. The PCR is performed under the following thermocycling conditions: 2 min at 95 °C, 39 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 melt rates, with a smooth curve setting averaging 1 point. Following the conditions of Varga and James (2005), the melting temperatures for each product are:

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

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

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

[257]

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

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

[138]4. Identification of Strains

[139]This section describes additional methods (using DASI-ELISA, RT-PCR, Co-RT-PCR and real-time RT-PCR) for identification of PPV strains (see Figure 1). Strain identification is not an essential component of PPV identification but an NPPO may wish to determine the identity of the strain to assist in predicting its epidemiological behaviour.

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

[141]

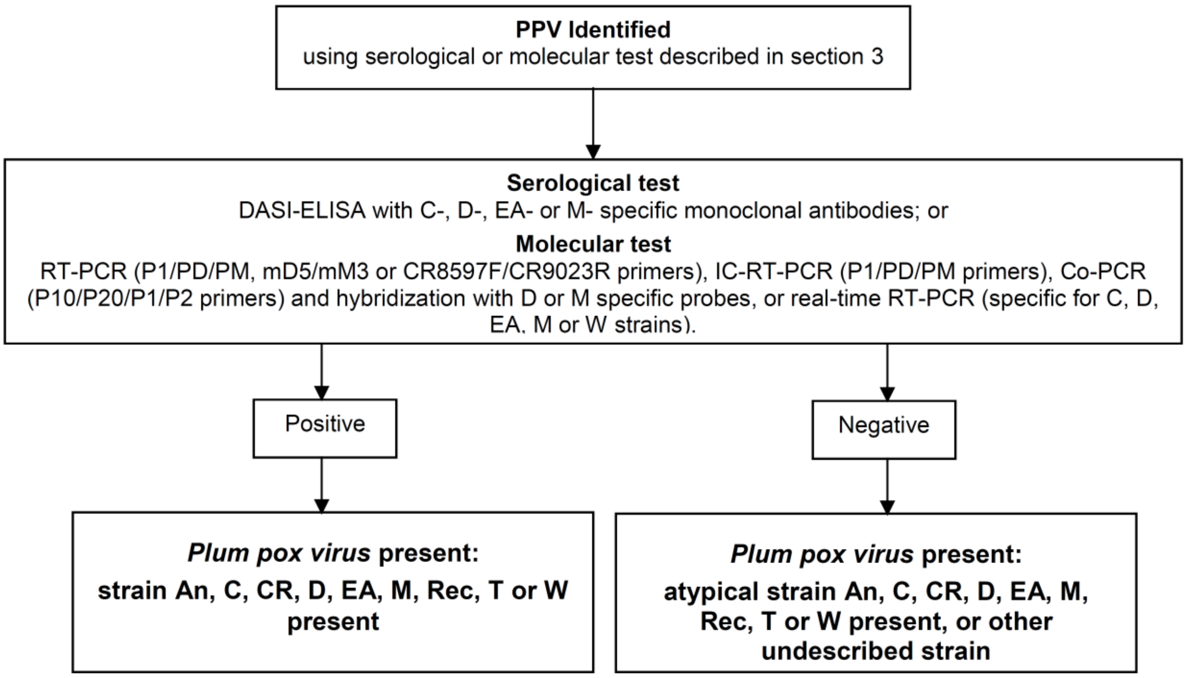

Figure 1. Methods for the identification of strains of *Plum pox virus*.

[142]

[143]Further tests may be done in instances where the NPPO requires additional confidence in the identification of the PPV strain. Sequencing of the complete PPV genome, or complete or partial coat

protein, P3-6K1 and cytoplasmic inclusion protein genes should also be done where atypical or undescribed strains are present.

[144]4.1 Serological identification of strains

[145]DASI-ELISA for differentiation between the two main PPV strains (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.

[146]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 (Olmos *et al.*, 2007; Cambra *et al.*, 2008). The 4D monoclonal antibody is PPV-D specific but does not react with all PPV-D isolates. Furthermore, the AL monoclonal antibody used for PPV-M detection reacts with isolates belonging to strains M, Rec and T because these groups share the same coat protein sequence. A molecular test is therefore required to differentiate between M, Rec and T strains detected using an M-specific monoclonal antibody.

[147]Serological identification of PPV isolates from EA and C groups may be done by DASI-ELISA using the EA- or the C-specific monoclonal antibodies described by Myrta *et al.* (1998, 2000). However, these tests need to be validated.

[148]4.2 Molecular identification of strains

[149]*4.2.1 Reverse transcription-polymerase chain reaction*

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

[151]P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

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

[153]The 25 µl reaction mixture is composed as follows: 1 µM P1 primer, 1 µM of either PD or PM primer, 250 µM dNTPs, 1 unit AMV reverse transcriptase (10 units/µl), 0.5 units Taq DNA polymerase (5 units/µl), 2.5 µl 10× Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.3% Triton X-100, 2% formamide and 5 µl RNA template. 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, and 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products are analysed by gel electrophoresis. The P1/PD pair of primers, and the P1/PM pair of primers, both produce a 198 bp amplicon. Olmos *et al.* (1997) evaluated their method using six isolates of PPV-D and four PPV-M isolates.

[154]The real-time reverse transcription-polymerase chain reaction with SYBR® Green I by Varga and James (2005) described in detail above in section 3.3.4 is also suitable for the identification of D and M strains of PPV.

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

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

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

[158]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), 0.5 units Taq DNA polymerase (5 units/µl), 2.5 µl 10× Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.3% Triton X-100 and 5 µl extracted RNA (see section 3.3). The PCR product of 605 bp is analysed by gel electrophoresis.

[159]PPV-CR is identified using the CR8597F and CR9023R primers described by Glasa *et al.* (2013):

[160]CR8597F (5′-ATG ATG TGA CGT TAG TGG AC-3′)

[161]CR9023R (5′-TCG TGT GTT AGA CAG GTC AAC-3′).

[162]A two-step RT-PCR protocol is used for specific detection of PPV-CR isolates (Glasa *et al*., 2013). Complementary (c)DNA is synthesized from total RNA extracts (NucleoSpin® RNA Plant Kit, Macherey-Nagel[1]) using random hexamer primers and AMV reverse transcriptase. An aliquot of cDNA is then added to the PCR reaction mix containing EmeraldAmp GT PCR Master Mix (TaKaRa Bio Inc.[1]). The PCR is performed under the following thermocycling conditions: 1 min at 98 °C, 35 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The PCR products are analysed by gel electrophoresis. The CR-specific primers amplify a product 427 bp in size, targeting the 5′ terminal CP coding region. The specificity of the CR primers was validated using isolates of PPV strains D, M, Rec, T, W, EA and C (Glasa *et al*., 2013).

[163]*4.2.2 Immunocapture reverse transcription-polymerase chain reaction*

[164]The immunocapture phase should be performed as described in section 3.3.2. The PCR reaction mixture is added directly to the coated PCR tubes. Identification of PPV-D and PPV-M is done as described in section 4.2.1.

[165]*4.2.3 Co-operational reverse transcription-polymerase chain reaction*

[166]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):

[167]PPV-D Specific Probe: 5′-CTT CAA CGA CAC CCG TAC GGG CA-DIG-3′

[168]PPV-M Specific Probe: 5′-AAC GCC TGT GCG TGC ACG T-DIG-3′.

[169]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).

[170]*4.2.4 Real-time reverse transcription-polymerase chain reaction*

[171]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).

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

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

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

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

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

[177]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)[2], 1× MultiScribe™ and RNase Inhibitor Mix (Applied Biosystems)[2] and 5 µl RNA template (see section 3.3). 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, and 60 s at 60 °C. The PCR products are analysed in real time according to the manufacturer's instructions. Capote *et al*. (2006) evaluated the method using 12 isolates each of PPV-D and PPV-M, and 14 samples co-infected with both strains.

[178]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:

[179]P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

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

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

[182]The following internal control primers may be included to ensure the validity of the test results:

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

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

[185]The 25 µl RT-PCR reaction mixture is composed as follows: 2.5 µl of a 1:10 (v/v) water dilution of extracted RNA (see section 3.3) and 22.5 µl master mix. The master mix has the following composition: 2.5 µl Karsai buffer (Karsai *et al.*, 2002); 0.5 µl each of 5 µM primers PPV-U, PPV-RR,, Nad5R and Nad5F; 0.5 µl 10 mM dNTPs; 1 µl 50 mM $MgCl_2$; 0.2 µl RNaseOUT™ (40 units/µl; Invitrogen)[2]; 0.1 µl Superscript™ III reverse transcriptase (200 units/µl; Invitrogen)[2]; 0.1 µl Platinum® Taq DNA high fidelity polymerase (5 units/µl, Invitrogen)[2]; and 1 µl of 1:5 000 (in TE, pH 7.5) SYBR® Green I (Sigma)[2] 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 melt rates, with a smooth curve setting averaging 1 point. Following the conditions of Varga and James (2006), the melting temperatures for each product are:

[186]C strain (74 bp fragment): 79.84 °C

[187]EA strain (74 bp fragment): 81.27 °C

[188]W strain (74 bp fragment): 80.68 °C.

[189]Varga and James (2006) evaluated their method using one isolate each of PPV-C, PPV-D, PPV-EA and PPV-W.

[190]*4.2.5 Controls for molecular tests*

[191]For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.

[192]**Positive nucleic acid control.** This control is used to monitor the efficiency of the test method (apart from the extraction) and, in RT-PCR, the amplification. Pre-prepared (stored) RNA or PPV-infected plant material printed on a membrane may be used. The stored RNA or PPV preparations should be verified periodically to determine the quality of the control with increased storage time.

[193]**Internal control.** For the real-time RT-PCR, mRNA of the mitochondrial gene *NADH dehydrogenase* 5 (*nad5*) could be incorporated into the RT-PCR protocol as an internal control to eliminate the possibility of RT-PCR false negatives due to nucleic acid extraction failure or degradation or the presence of RT-PCR inhibitors.

[194]**Negative amplification control (no template control).** This control is necessary for conventional and real-time RT-PCR to rule out false positives due to contamination (with the target DNA) during the preparation of the reaction mixture. RNase-free PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

[195]**Positive extraction control.** This control is used to ensure that the target nucleic acid extracted is of sufficient quantity and quality for RT-PCR and that the target virus is detectable. Nucleic acid is extracted from infected host tissue, or healthy plant or insect tissues that have been spiked with PPV.

[196]For RT-PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples.

[197]**Negative extraction control.** This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises nucleic acid that is extracted

from uninfected host tissue and subsequently amplified. It is recommended that multiple controls be included when large numbers of positive samples are expected.

[198]In the case of immunocapture RT-PCR where no nucleic extraction is performed, plant sap from a known PPV positive should be used as a positive control, and plant sap from a healthy plant should be used as a negative control. A negative amplification control may also be included. The latter control is used to rule out false positives due to contamination during the preparation of the reaction mixture. RNase-free PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage for use as a negative amplification control.

[199]5. Records

[200]The records required to be kept are listed in ISPM 27 (*Diagnostic protocols for regulated pests*).

[201]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:

- [202]The original sample (labelled appropriately for traceability) should be kept frozen at −80 °C or freeze-dried and kept at room temperature.
- [203]If relevant, RNA extractions should be kept at −80 °C and spotted plant extracts or printed tissue sections (paper on paper or nylon membranes) should be kept at room temperature.
- [204]If relevant, RT-PCR amplification products should be kept at −20 °C.

[205]6. Contact Points for Further Information

[206]United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Registrations Identifications Permits and Plant Safeguarding (RIPPS), Molecular Diagnostic Laboratory, BARC Building 580, Powder Mill Road, Beltsville, Maryland 20705, United States of America (Ms Laurene Levy, e-mail: Laurene.Levy@aphis.usda.gov; tel.: +1 3015045700; fax: +1 3015046124).

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[211]Istituto di Virologia Vegetale del CNR, sezione di Bari, via Amendola 165/A, I-70126 Bari, Italy (Mr Donato Boscia, e-mail: d.boscia@ba.ivv.cnr.it; tel.: +39 0805443067; fax: +39 0805442911).

[212]Sidney Laboratory, Canadian Food Inspection Agency (CFIA), British Columbia, V8L 1H3 Sidney, Canada (Mr Delano James, e-mail: Delano.James@inspection.gc.ca; tel.: +1 250 3636650; fax: +1 250 3636661).

[213]A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on

Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), who will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

[214]7. Acknowledgements

[215]This diagnostic protocol was drafted by Mr M. Cambra, Mr A. Olmos and N. Capote, IVIA (see preceding section); Mr N.L. Africander, Department of Agriculture, Forestry and Fisheries, Private Bag X 5015, Stellenbosch, 75999, South Africa; Ms L. Levy, USDA, United States of America (see preceding section); Mr S.L. Lenardon, Instituto de Fitopatologia y Fisiologia Vegetal - Instituto Nacional de Tecnologia Agropecuaria (IFFIVE-INTA), Cno. 60 Cuadras Km 51/2, Córdoba X5020ICA, Argentina; Mr 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.

[216]8. References

[217]The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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