Food and Agriculture Organization of the United Nations

International Plant Protection Convention
Protecting the world's plant resources from pests

ISPM 27
ANNEX 2

ENG

INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DIAGNOSTIC PROTOCOLS

DP 2:
Plum pox virus

Produced by the Secretariat of the
International Plant Protection Convention (IPPC)

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ISPM 27
Diagnostic protocols for regulated pests

DP 2: *Plum pox virus*

Adopted 2012; published 2016

CONTENTS

1. Pest Information

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).

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 and Middle East. It has been found with a restricted distribution in South and North America and Asia (EPPO, 2006; CABI, 2011).

PPV 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.

PPV isolates can be classified currently into seven types or strains: D (Dideron), M (Marcus), C (Cherry), EA (El Amar), W (Winona), 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 *P. armeniaca* and *P. domestica* but differ in their ability to infect *P. persica* cultivars. The strains vary in the 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 recently. These isolates form a distinct type that has been defined as PPV-C. An atypical PPV was isolated from *P. domestica* 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).

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

2. Taxonomic Information

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

3. Detection and Identification

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*).

PPV 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.

General guidance on sampling methodologies is described 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, 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 (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.

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 occur 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 techniques 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.

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.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Biological detection

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 3 weeks, are pruned to a few centimetres above the top graft (Gentit, 2006). The grafted plants should be inspected for symptoms for at least 6 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).

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.

3.2 Serological detection and identification

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

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.

3.2.1 Double-antibody sandwich indirect enzyme-linked immunosorbent assay

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.

5B-IVIA is currently the only monoclonal antibody demonstrated to detect all strains of PPV 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 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/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*., 2006c; 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 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.

3.2.2 Double-antibody sandwich enzyme-linked immunosorbent assay

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.

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). Therefore the use of additional methods is recommended in situations where polyclonal antibodies have been used in an assay and the NPPO requires additional confidence in the identification of PPV.

3.3 Molecular detection and identification

Molecular methods using reverse transcription-polymerase chain reaction (RT-PCR) 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.

With the exception of immunocapture (IC)-RT-PCR (for which RNA isolation is not required), RNA extraction should be done 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 to use spotted or tissue-printed samples in conventional PCR because of the lower sensitivity compared with real-time RT-PCR.

Each method 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-1; Co-RT-PCR, 1 fg RNA template ml-1; and real-time RT-PCR, 2 fg RNA template ml-1.

3.3.1 Reverse transcription-polymerase chain reaction

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′).

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 $MgCl_2$, 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 ºC, 40 cycles of 30 s at 94 °C, 30 s at either 60 °C (P1/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 and 3′NCR primers produce a 243 base pair (bp) and 220 bp amplicon, respectively.

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 10 fg of viral RNA, corresponding to 2 000 viral particles (Wetzel *et al.*, 1991). The method of Levy and Hadidi (1994) was evaluated using PPV isolates from Egypt, France, Germany, Greece, Hungary, Italy, Spain and Romania.

3.3.2 Immunocapture reverse transcription-polymerase chain reaction

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.

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 PCR tubes and incubate at 37 °C for 3 h. Wash the tubes twice with 150 µl of sterile PBS-Tween (washing buffer). Rinse the tubes twice with RNase-free water. 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 PCR tubes. Incubate for 2 h on ice or at 37 °C. Wash the tubes three times with 150 µl of sterile PBS-Tween. 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 PCR tubes. Perform the amplification as described in section 3.3.1.

IC-RT-PCR generally 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 (Cambra *et al.*, 2006c; Olmos *et al.*, 2007). 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.

3.3.3 Co-operational reverse transcription-polymerase chain reaction

The RT-PCR primers used in this co-operational (Co)-RT-PCR assay are the primers of Olmos, Bertolini and Cambra (2002):

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

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

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

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

The 25 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 $MgCl_2$, 5% DMSO, 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.

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. 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 sterilized 1%blocking solution (1 g blocking reagent dissolved in 100 ml maleic acid buffer). Incubate the membrane at room temperature with anti-DIG-alkaline phosphatase conjugate antibodies at a working concentration of 1:5 000 (150 units $litre^{-1}$) in 1% blocking solution (w/v) for

30 min. Wash the membrane twice for 15 min with washing buffer, and equilibrate 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^{-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. After incubation with the substrate stop the reaction by washing with water.

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

3.3.4 Real-time reverse transcription-polymerase chain reaction

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′).

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 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 kit; Invitrogen)[1] 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.

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*.

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′).

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 TaqMan probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems)[2], 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems)[3] and 5 µl RNA template. The RT-PCR is performed under the following thermocycling conditions: 30 min at 48 °C,

[1] The use of the brand Invitrogen for the Superscript™ One-Step RT-PCR with Platinum® Taq kit in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[2] The use of the brand Applied Biosystems for the TaqMan Universal PCR Master Mix and the MultiScribe and RNase Inhibitor Mix in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[3] See footnote 2.

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.

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 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).

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′).

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′).

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 at 72 °C for 5 min, place on ice. Add 4 µl 5 × first strand buffer (Invitrogen)[4], 2 µl 0.1 M DTT, 1 µl 10 mM dNTPs, 0.5 µl RNaseOUT™ (40 units μl^{-1}) (Invitrogen)[5], 1 µl Superscript™ II (Invitrogen)[6] and 2.5 µl water. Incubate 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, 2mM $MgCl_2$, 1 × Karsai buffer (Karsai *et al.*, 2002), 1:42 000 SYBR Green I (Sigma)[7] and 0.1 µl Platinum® Taq DNA high fidelity polymerase (Invitrogen)[8]. The reaction mixture and 1 µl of 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 done by incubation at 60 °C to 95 °C at 0.1 °C s^{-1} with a smooth curve setting averaging 1 point. Following the conditions of Varga and James (2005), 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.

[4] The use of the brand Invitrogen for the first strand buffer, RNaseOUT™, Superscript™ II and Platinum® Taq DNA high fidelity polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[5] See footnote 4.

[6] See footnote 4.

[7] The use of the brand Sigma for SYBR Green I in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[8] See footnote 4.

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

4. Identification of Strains

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.

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 (Cambra *et al.*, 2006a; Candresse and Cambra, 2006; Capote *et al.*, 2006).

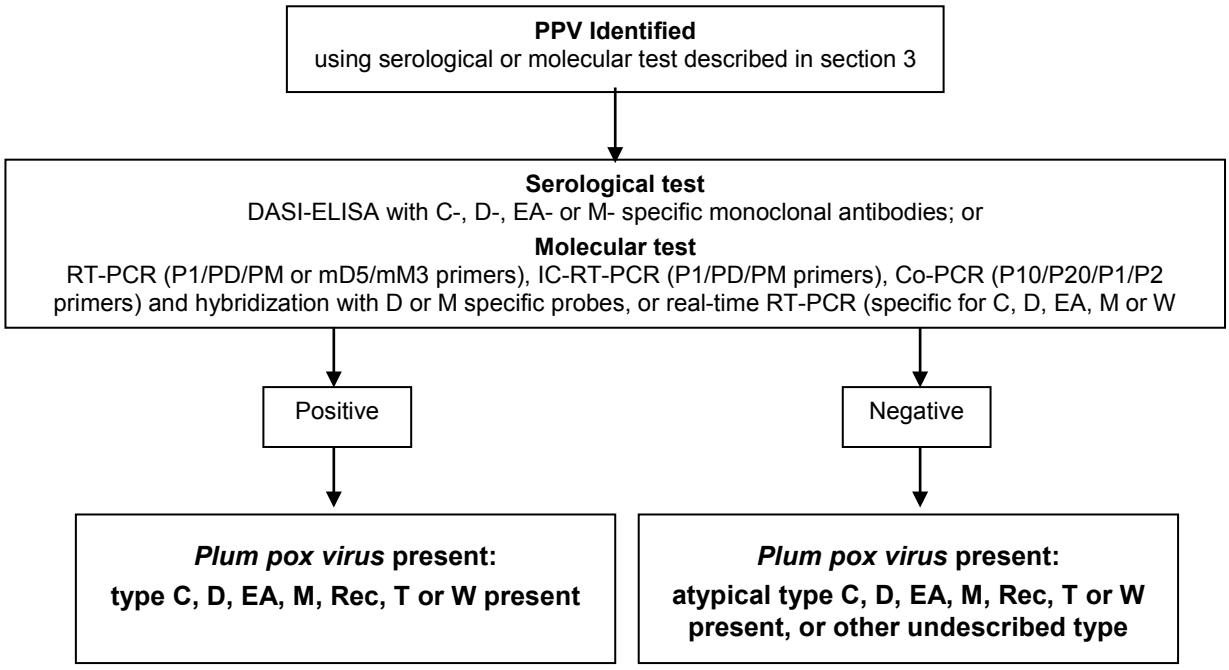

Figure 1: Methods for the identification of strains of *Plum pox virus*.

Further tests may be done in instances where the NPPO requires additional confidence in the identification of PPV type. 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 types are present.

4.1 Serological identification of strains

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.

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.*, 2006c; Olmos *et al.*, 2007). The 4D monoclonal antibody is PPV-D specific but does not react with all PPV-D isolates. In addition, the AL monoclonal antibody used for PPV-M detection 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 differentiate between M, Rec and T types detected using an M-specific monoclonal antibody.

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

4.2 Molecular identification of strains

4.2.1 Reverse transcription-polymerase chain reaction

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′).

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 $MgCl_2$, 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 and P1/PM primers produce a 198 bp amplicon. The method was evaluated using six isolates of PPV-D and four PPV-M isolates.

PPV-Rec is identified using the mD5/mM3 Rec-specific primers described by Šubr, Pittnerova and Glasa (2004):

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

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

The 25 µl reaction mixture is composed as follows (modified from Šubr, Pittnerova and Glasa, 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 $MgCl_2$, 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.

4.2.2 Immunocapture reverse transcription-polymerase chain reaction

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 detection is done as described in section 4.2.1.

4.2.3 Co-operational reverse transcription-polymerase chain reaction

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, Bertolini and Cambra, 2002):

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

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

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).

4.2.4 Real-time reverse transcription-polymerase chain reaction

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).

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′).

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)[9], 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems)[10] and 5 µl of 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. The method has been evaluated using 12 isolates each of PPV-D and PPV-M, and 14 samples co-infected with both types.

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′).

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′).

The 25 µl RT-PCR reaction 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 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 $MgCl_2$; 0.2 µl of RNaseOUT™ (40 units μl^{-1}; Invitrogen)[11] ; 0.1 µl of Superscript™ III (200 units μl^{-1}; Invitrogen)[12]; 0.1 µl of Platinum® Taq DNA high fidelity polymerase (5 units μl^{-1}, Invitrogen)[13]; and 1 µl of 1:5 000 (in TE, pH 7.5) SYBR Green I (Sigma)[14] in 16.1 µl water. The reaction is performed under the following thermocycling

[9] The use of the brand Applied Biosystems for the TaqMan Universal PCR Master Mix and the MultiScribe and RNase Inhibitor Mix in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[10] See footnote 9.

[11] The use of the brand Invitrogen for RNaseOUT™ , Superscript™ II and Platinum® Taq DNA high fidelity polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[12] See footnote11.

[13] See footnote 11.

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. Following the conditions of Varga and James (2006), 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.

This method was evaluated using one isolate each of PPV-C, PPV-D, PPV-EA and PPV-W.

5. Records

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

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 paper or nylon membranes should be kept at room temperature.
- If relevant, RT-PCR amplification products should be kept at −20 °C.

6. Contact Points for Further Information

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[14] The use of the brand Sigma for SYBR Green I in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

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8. References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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