

ISPM 27

Diagnostic protocols for regulated pests

DP 2: *Plum pox virus*

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1. Pest Information

Sharka (plum pox) is one of the most serious viral diseases of stone fruit. The disease was first reported in *Prunus 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, 2018).

Sharka, caused by *Plum pox virus* (PPV), affects plants of the genus *Prunus* (family Rosaceae). It is particularly detrimental in *Prunus armeniaca*, *P. domestica*, *P. persica* and *P. salicina* because it reduces quality and causes early fruit drop. It is estimated that the costs of managing sharka worldwide since the 1970s exceed 10 billion euros (Cambra *et al.*, 2006b).

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 *et al.*, 2014). 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. Transmission via seed and pollen have not been confirmed (Pasquini and Barba, 2006; Ilardi and Tavazza, 2015). PPV can be transmitted mechanically, under experimental conditions, to numerous *Prunus* species and to several herbaceous species such as *Arabidopsis thaliana*, *Chenopodium foetidum*, *Nicotiana benthamiana*, *Nicotiana clevelandii*, *Nicotiana glutinosa* and *Pisum sativum* (Barba *et al.*, 2011).

Plum pox virus isolates can currently be classified into nine monophyletic 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). The strains have specific genome sequences and may vary in their symptomatology, pathogenicity, host range, epidemiology and aphid transmissibility. 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. These two strains also differ in their pathogenicity, with M isolates generally causing 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 *Prunus avium* and *Prunus 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 show an epidemiological behaviour similar to the D strain. A second type of recombinant strain has been reported in Turkey and defined as a 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 putative strain (Tat), neither C nor CR, has also been proposed (Chirkov *et al.*, 2016).

Further information about PPV, including illustrations of disease symptoms, can be found in Barba *et al.* (2011), CABI (2018), EPPO (2004, 2006, 2018b), García *et al.* (2014) and PaDIL (2018).

2. Taxonomic Information

Name: *Plum pox virus* (acronym PPV)

Synonym: *Sharka virus*

Taxonomic position: *Potyviridae*, *Potyvirus*

Common names: Plum pox, sharka

3. Detection and Identification

Detection of PPV can be achieved using a biological, serological or molecular method, while identification requires use of either a serological or molecular method. A test using a serological or molecular method is the minimum requirement to detect and identify PPV, especially during routine diagnosis if the pest is known to be 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 carried out to confirm the identification. Where the initial identification was made using a molecular method, the confirmation should preferably be made using a method with a higher analytical sensitivity or, if possible, using a molecular method targeting a different genome region or sequence analysis. Further tests may also be carried out, including the use of serological methods that target protein elements or methods used 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.

This diagnostic protocol describes well-established methods for the detection and identification of PPV. Some new and advanced techniques have been used to detect PPV such as loop-mediated isothermal amplification (Varga and James, 2006b) and next-generation sequencing (Rodamilans *et al.*, 2014). However, since next-generation sequencing and loop-mediated isothermal amplification (LAMP)¹ have not yet been fully validated as tools for routine detection of PPV, with published protocols described, these techniques have not been included in this diagnostic protocol.

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. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Host range

Under natural conditions, PPV readily infects fruit trees of the genus *Prunus* (family Rosaceae) used as commercial varieties or rootstocks. Major hosts include *Prunus armeniaca*, *P. cerasifera*, *P. davidiana*, *P. domestica*, *P. mahaleb*, *P. marianna*, *P. mume*, *P. persica*, *P. salicina* and interspecific hybrids between these species. There are cherry-adapted strains of PPV (C and CR) that naturally infect *P. avium* and *P. cerasus* (James *et al.*, 2013). Occasionally, *Prunus dulcis* may be infected by PPV (Llácer and Cambra, 2006). 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* (James and Thompson, 2006). Under experimental conditions, PPV can be transmitted mechanically to numerous *Prunus* spp. and several herbaceous plants (see section 1).

3.2 Symptoms

Plum pox virus 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*. *P. cerasifera* ‘GF 31’ shows rusty-brown corking and cracking of the bark.

¹ When using LAMP on a regular basis in an area which has a patent system such as Japan (Patent Nos. 3,313,358, 3,974,441 and 4,139,424), the United States of America (US6,410,278, US6,974,670 and US7,494,790), the European Union (Nos. 1,020,534, 1,873,260, 2,045,337 and 2,287,338), China (ZL008818262), the Republic of Korea (Patent No. 10-0612551), Australia (No. 779160), and the Russian Federation (No. 2,252,964), it is necessary for users to receive a license from Eiken Chemical Co., Ltd. before use.

Flower symptoms may include discoloration (pinkish streaks) on flower petals and flower breaking symptoms (Barba *et al.*, 2011). These flower symptoms can occur on 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* typically show pale rings or spots. The alcohol or spirits produced from diseased fruits are unmarketable owing to an undesirable flavour.

Symptom development and severity depend strongly on the host plant and climatic conditions. The virus may be latent for several years in cold climates.

Symptoms on various hosts can be seen, for example, on the EPPO (European and Mediterranean Plant Protection Organization) Global database website (<https://gd.eppo.int/taxon/PPV000>).

3.3 Biological detection

The main indicator plants used for PPV indexing are seedlings of *P. cerasifera* ‘GF 31’, *P. persica* ‘GF 305’, *P. persica* × *P. davidiana* ‘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).

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.

Herbaceous plants can also be used for biological detection of PPV (Barba *et al.*, 2011). PPV can be transmitted mechanically to several herbaceous species (see section 1).

3.4 Sampling for serological and molecular tests

General guidance on sampling methodologies is provided in ISPM 31 (*Methodologies for sampling of consignments*). 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. *Prunus* plant material is often shipped as dormant cuttings. In this case, only buds or phloem tissue (bark scrapings) can be used directly for testing.

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 from 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 carried out during months with the highest temperatures. Tests on samples collected in the autumn are less reliable than tests carried out 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. In summer, buds from dormant cuttings can be tested using reverse transcription-polymerase chain reaction (RT-PCR) or real-time PCR, these also being the preferred techniques for testing mature leaves. 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 used for testing.

3.5 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 (or as recommended by the ELISA kit manufacturer) 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 suitable buffer. Plant material should be homogenized thoroughly and used fresh.

3.5.1 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.

The monoclonal antibody (MAb) 5B-IVIA has been shown to detect most if not all strains of PPV (Cambra *et al.*, 2006a). MAb 5B-IVIA will detect isolates of strain CR, but the extracts for analysis must be adjusted to pH 6.0 for enhanced MAb 5B-IVIA recognition (Glasa *et al.*, 2013; Chirkov *et al.*, 2013). The putative cherry-adapted strain (Tat) can also be detected by MAb 5B-IVIA (Chirkov *et al.*, 2016). However, there has been no report of the detection of PPV-An using MAb 5B-IVIA (Palmisano *et al.*, 2012).

In a DIAGPRO ring test (Harju *et al.*, 2000) conducted by 17 laboratories using a panel of ten samples, including both PPV-infected (PPV-D, PPV-M and PPV-D+M) and healthy samples from France and Spain, DASI-ELISA using MAb 5B-IVIA 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 RT-PCR (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 MAb 5B-IVIA 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 MAb 5B-IVIA was a true positive. Antibodies may exhibit variation between batches, therefore verification of performance should be carried out before routine use.

The 5B-IVIA monoclonal antibody detects all tested PPV strains specifically, sensitively and reliably (Cambra *et al.*, 1994; Cambra *et al.*, 2006a; Glasa *et al.*, 2013; Chirkov *et al.*, 2013; Chirkov *et al.*, 2016). Several commercial kits using polyclonal antibodies are available along with some validation data (Gougherty *et al.*, 2015; EPPO, 2018a). However, these have been shown to be less specific and to lack homogeneity among different batches (Cambra *et al.*, 2006a) and should therefore be validated before use. The use of additional methods is recommended in situations where polyclonal antibodies have been used in a test and the NPPO requires additional confidence in the identification of PPV.

3.5.2 Double-antibody sandwich enzyme-linked immunosorbent assay

The conventional or biotin–streptavidin system of double-antibody sandwich (DAS)-ELISA utilizes kits based on the specific monoclonal antibody 5B-IVIA or on polyclonal antibodies that have been demonstrated to detect most strains of PPV, including the most widely distributed strains D, M and Rec, without cross-reacting with other viruses or healthy plant material (Cambra *et al.*, 2006a; Capote *et al.*, 2009). The test should be carried out according to the manufacturer's instructions.

3.6 Molecular detection and identification

Molecular methods such as RT-PCR may be more expensive 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 and less prone to contamination (with the target DNA) than conventional PCR.

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 for analysis (Olmos *et al.*, 2005; Osman and Rowhani, 2006; Capote *et al.*, 2009). It is recommended that spotted or tissue-printed samples be tested using real-time RT-PCR rather than conventional PCR because of its higher sensitivity.

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.

3.6.1 RNA purification, immunocapture and cDNA synthesis

3.6.1.1 RNA purification

RNA purification should be carried out using appropriately validated protocols or using an RNA purification kit according to the manufacturer's instructions. The extracted RNA should be stored at -70°C (preferably) or at -20°C until its use as a template and for less than one year. Storage should be in small quantities to avoid degradation of RNA due to repeated freeze–thaw cycles.

3.6.1.2 Immunocapture

Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody mixture is prepared and used to coat the microtubes used for the reverse transcription reaction. See section 3.6.2 for details of the procedure.

3.6.1.3 cDNA synthesis

Because the preservation of RNA during storage is problematic, it is recommended that complementary (c)DNA be synthesized, as this can be preserved for long periods and with fewer temperature requirements compared with RNA.

3.6.2 Reverse transcription-polymerase chain reaction

The primers used in the RT-PCR methods described below have been well validated and are considered as references for general PPV detection even though there may be other broad-spectrum primers available (Olmos *et al.*, 2006). No false positive results were observed in the studies describing the development and validation of these methods (Wetzel *et al.*, 1991; Levy and Hadidi, 1994). Another benefit of the Wetzel *et al.* (1991) primers is that they also allow identification of the two most common strains of PPV, when combined with analysis of the 243 base pair (bp) product using restriction fragment length polymorphism.

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

P1 antisense (5'-ACC GAG ACC ACT ACA CTC CC-3')

P2 sense (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 and P2, or the 3'NCR primer pair), 250 µM dNTPs, 1 unit *Avian myeloblastosis virus* (AMV) reverse transcriptase², 0.5 units Taq DNA polymerase, 2.5 µl 10× Taq polymerase buffer, 1.5 mM MgCl₂, 0.3% Triton X-100 and 5 µl RNA template. The reaction is performed with the following thermocycling parameters: 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 60 s 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 bp amplicon and the 3'NCR primers produce a 220 bp amplicon.

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.

3.6.3 Immunocapture reverse transcription-polymerase chain reaction

The immunocapture phase may be performed according to Wetzel *et al.* (1992), using plant sap extracted as in section 3.5 and using individual tubes or plastic bags to avoid contamination. Any suitably validated antibody may be used. This test has been validated only for isolates of the widely distributed strains D and M.

A dilution (1 µg/ml) is prepared of polyclonal antibodies or a PPV-specific monoclonal antibody (e.g. 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 3.5) 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 3.6.2 using the primers of Wetzel *et al.*

² 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.

(1991), and added directly to the coated PCR tubes. The amplification is performed as described in section 3.6.2.

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.

3.6.4 Co-operational reverse transcription-polymerase chain reaction

The RT-PCR primers used in this Co-RT-PCR are the primers of Wetzel *et al.* (1991; P1 and P2) and Olmos *et al.* (2002; P10 and P20):

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 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, 2.5 µl 10× reaction buffer, 3 mM MgCl₂, 5% dimethyl sulphoxide, 0.3% Triton X-100 and 5 µl RNA template. The RT-PCR is performed with the following thermocycling parameters: 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. The amplified cDNA is denatured at 95 °C for 5 min and immediately placed 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 prehybridization, 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 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-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide) in 10 ml detection buffer. After incubation with the substrate, the reaction is stopped by washing with water.

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).

3.6.5 Real-time reverse transcription-polymerase chain reaction

Real-time RT-PCR can be performed using either TaqMan or SYBR Green I². 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):

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 MgSO₄), 200 nM each of forward and reverse primers, 100 nM TaqMan probe, 4.8 mM MgSO₄, 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 with the following thermocycling parameters: 15 min at 52 °C, 5 min at 95 °C, and 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 D isolates from the United States of America, and isolates of 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 method 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 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 with the following thermocycling parameters: 30 min at 48 °C, 10 min at 95 °C, and 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 was evaluated by Olmos *et al.* (2005) using three isolates each of PPV-D and PPV-M, and was 1 000 times more sensitive than DAS-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%), IC-RT-PCR (91.5%) or DAS-ELISA using the 5B-IVIA monoclonal antibody (86.6%) (Capote *et al.*, 2009).

Varga and James (2005) described a SYBR Green I² 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 (Menzel *et al.*, 2002) may be included to ensure the validity of the test results:

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 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 MgCl₂, 1× Karsai buffer (Karsai *et al.*, 2002), 1:42 000 SYBR Green I² 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 with the following thermocycling parameters: 2 min at 95 °C, and 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 with 0.1 °C/s melt rates and a smooth curve setting averaging 1 point. Under 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

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.

4. Identification of Strains

This section describes additional steps for the identification of PPV strains (using DASi-ELISA, RT-PCR, Co-RT-PCR and real-time RT-PCR) (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 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). Techniques for the identification of strains such as An and T are not provided as methods for their identification have not been validated and published or as yet too few isolates have been characterized.

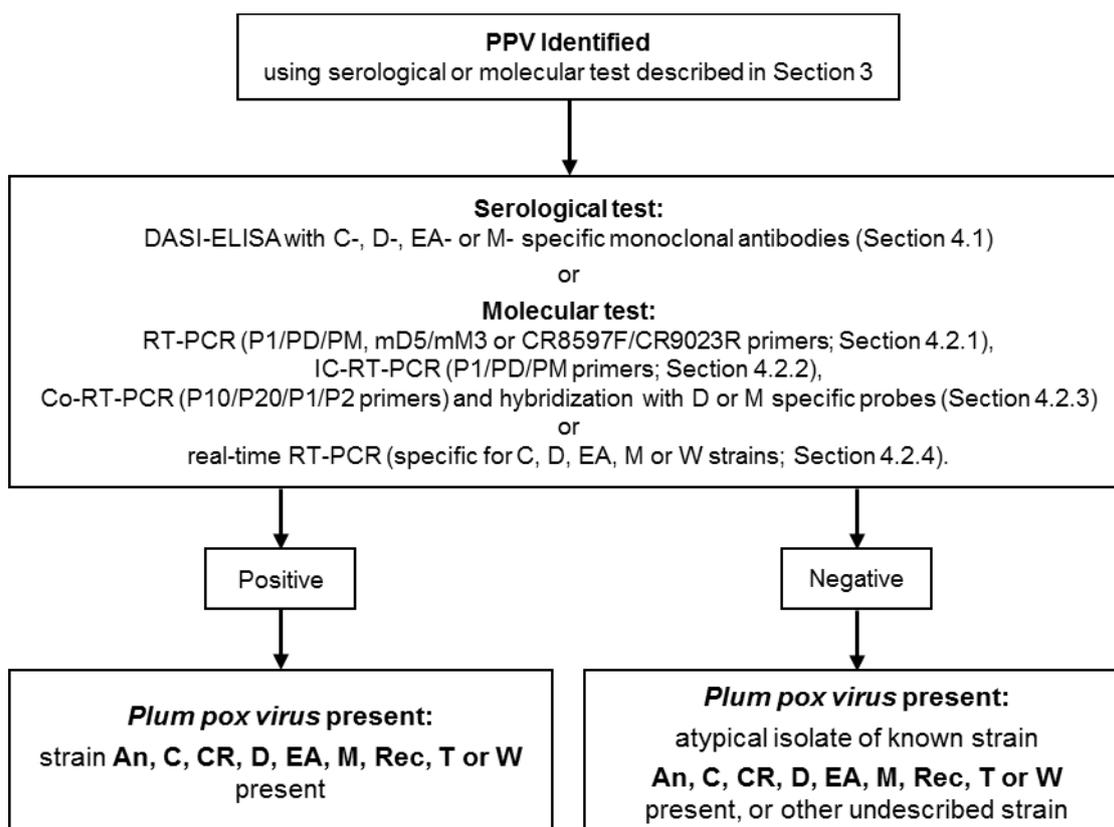


Figure 1. Steps in the methods for the identification of strains of *Plum pox virus*.

Further tests may be conducted 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 carried out where atypical or undescribed strains are present.

4.1 Serological identification of strains

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.

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.

Serological identification of PPV isolates from EA and C groups may be carried out by DASI-ELISA using the EA- or the C-specific monoclonal antibodies described by Myrta *et al.* (1998, 2000). However, these methods 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')

PM (5'-CTT CAA CAA CGC CTG TGC GT -3')

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 MgCl₂, 0.3% Triton X-100, 2% formamide and 5 µl RNA template. The RT-PCR is performed with the following thermocycling parameters: 45 min at 42 °C, 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 60 s 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.

The real-time RT-PCR with SYBR Green I² by Varga and James (2005), described in detail in section 3.6.5, is also suitable for the identification of D and M strains of PPV.

PPV-Rec is identified using the mD5 and 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')

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 MgCl₂, 0.3% Triton X-100 and 5 µl extracted RNA (see section 3.6). Reverse transcription is carried out with random hexanucleotide primers, at 42 °C for 45 min (Glasa *et al.*, 2002). PCR is carried out using an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 30 s and 72 °C for 60 s, and final elongation at 72 °C for 7 min (Šubr *et al.*, 2004). The PCR product of 605 bp is analysed by gel electrophoresis.

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

CR8597F (5'-ATG ATG TGA CGT TAG TGG AC-3')

CR9023R (5'-TCG TGT GTT AGA CAG GTC AAC-3')

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)² using random hexamer primers and AMV reverse transcriptase. An aliquot of cDNA is then added to the PCR reaction mixture containing EmeraldAmp GT PCR Master Mix (TaKaRa Bio Inc.)². The PCR is performed with the following thermocycling parameters: 60 s at 98 °C, 35 cycles of 98 °C for 30 s, 55 °C for 30 s and 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).

4.2.2 Immunocapture reverse transcription-polymerase chain reaction

The immunocapture phase should be performed as described in section 3.6.3. The PCR reaction mixture is added directly to the coated PCR tubes. Identification of PPV-D and PPV-M is carried out 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 carried out as described in section 3.6.4 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-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 I² chemistry according to the method of Varga and James (2005) (see section 3.6.5) 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)², 1× MultiScribe and RNase Inhibitor Mix (Applied Biosystems)² and 5 µl RNA template (see section 3.6). The RT-PCR is performed with the following thermocycling parameters: 30 min at 48 °C, 10 min at 95 °C, and 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.

PPV-C, PPV-EA and PPV-W are specifically identified using SYBR Green I² chemistry according to the method of Varga and James (2006a). 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 (Menzel *et al.*, 2002) may be included to ensure the validity of the test results:

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 mixture is composed as follows: 2.5 µl of a 1:10 (v/v) water dilution of extracted RNA (see section 3.6) 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 MgCl₂; 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 Tris-ethylenediaminetetraacetic acid (TE), pH 7.5) SYBR Green I² in 16.1 µl water. The reaction is performed with the following thermocycling parameters: 10 min at 50 °C, 2 min at 95 °C, and 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 with 0.1 °C/s melt rates and a smooth curve setting averaging 1 point. Under the conditions of Varga and James (2006a), 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

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

5. Controls for Molecular Tests

For the test result 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.

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. Total plant or viral 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.

Internal control. For RT-PCR, mRNA of the mitochondrial gene *NADH dehydrogenase 5* (*nad5*, Menzel *et al.*, 2002) 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.

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.

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 PPV infected host tissue, or healthy plant or insect tissues that have been spiked with PPV.

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

Negative extraction control. This control is used to monitor both 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 in random order when large numbers of positive samples are expected.

In the case of IC-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.

6. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where the virus is found in an area for the first time, the following additional material should be kept for at least one year in a manner that ensures traceability:

- The original sample (labelled appropriately) should be kept frozen, if possible, at -80°C or freeze-dried and kept at room temperature.
- If relevant, RNA extracts 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.
- If relevant, RT-PCR amplification products should be kept at -20°C .

7. Contact Points for Further Information

Further information on this protocol can be obtained from:

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Science and Technology Beltsville Laboratory, Building 580 BARC-East, Powder Mill Road, Beltsville, MD 20705, United States of America (Vessela Mavrodieva; email: vessela.a.mavrodieva@aphis.usda.gov; tel.: +1 3013139208; fax: +1 3023139232).

Equipe de Virologie Institut National de la Recherche Agronomique (INRA), Centre de Bordeaux, UMR GD2P, IBVM, BP 81, F-33883 Villenave d'Ornon Cedex, France (Thierry Candresse; email: tc@bordeaux.inra.fr; tel.: +33 557122389; fax: +33 557122384).

Faculty of Horticultural Science, Department of Plant Pathology, Corvinus University, Villányi út 29-43, H-1118 Budapest, Hungary (Laszlo Palkovics, email: laszlo.palkovics@uni-corvinus.hu; tel.: +36 14825438; fax: +36 14825023).

Institute of Virology, Slovak Academy of Sciences, Dúbravská, 84505 Bratislava, Slovakia (Miroslav Glasa; email: virumig@savba.sk; tel.: +421 259302447; fax: +421 254774284).

Instituto Valenciano de Investigaciones Agrarias (IVIA), Plant Protection and Biotechnology Centre, Carretera Moncada-Náquera km 5, 46113 Moncada (Valencia), Spain (Antonio Olmos; email: aolmos@ivia.es; tel.: +34 963424000; fax: +34 963424001).

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

Sidney Laboratory, Canadian Food Inspection Agency (CFIA), British Columbia, V8L 1H3 Sidney, Canada (Delano James; email: Delano.James@inspection.gc.ca; tel.: +1 250 3636650; fax: +1 250 3636661).

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).

8. Acknowledgements

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9. References

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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Publication history

This is not an official part of the standard

2006-4 CPM-1 added topic *Viruses and phytoplasmas*.

2008-09 Standards Committee (SC) approved for consultation via email.

2010-06 Consultation.

2011-10 SC e-decision recommended draft to CPM.

2012-03 CPM-7 adopted Annex 2 to ISPM 27.

ISPM 27. Annex 2. *Plum pox virus* (2012). Rome, IPPC, FAO.

2015-07 IPPC Secretariat incorporated editorial amendments and reformatted standards following revoking of standards procedure from CPM-10 (2015).

2016-11 SC added topic *Revision of DP 2: Plum pox virus* to the work programme.

2017-02 Technical Panel on Diagnostic Protocols (TPDP) discussed and revised.

2017-06 SC approved for consultation (2017_eSC_Nov_03).

2017-07 First consultation.

2017-11 Draft revised by the Lead based on consultation comments.

2018-02 TPDP approved draft to submit to SC for approval for adoption.

2018-04 SC approved draft to be submitted to the 45-day DP notification period (2018_eSC_May_09).

2018-07 DP notification period (no objections received).

2018-08 SC adopted DP on behalf of CPM.

ISPM 27. Annex 2. *Plum pox virus* (2018). Rome, IPPC, FAO.

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