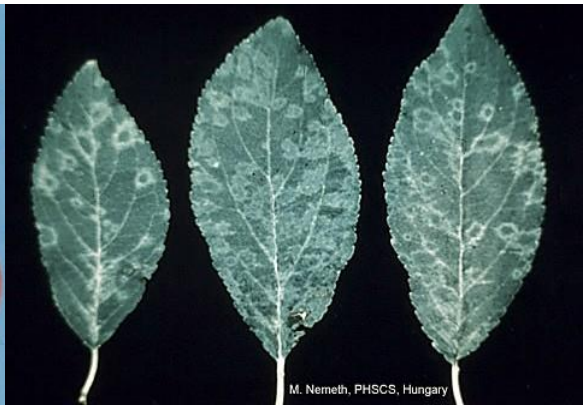
National Diagnostic Protocol

Plum pox virus (PPV)



NDP 2 V4

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/initiatives/national-diagnostic-protocols/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for Plum pox virus is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this project should be sent to: sphd@agriculture.gov.au

Contents

1 INTRODUCTION

This diagnostic protocol provides technical information for the identification of plum pox virus (PPV). PPV causes fruit deformity of stonefruit, with rings on fruit and leaves.

1.1 Host range

The major hosts of PPV are in the genus *Prunus*, and include apricots (*P. armeniaca*), nectarines (*Prunus persica* var. *nucipersica)*, peaches (*P. persica*) and plums (*P. domestica* and *P. salicina*) sweet cherries (*Prunus avium*) and sourcherries (*Prunus cerasus*) (Sochor et al 2012). Almond (*P. dulcis*) can express mild symptoms (Festic, 1978). There are host range differences amongst the PPV strains (Sihelská et al 2017).

Important wild or ornamental *Prunus* species that can naturally host PPV are *P. besseyi, P. cerasifera, P. insititia, P. cistena*, *P. glandulosa, P. tomentosa*, *P. laurocerasus*, *P. spinosa* and *P. triloba* (James and Thompson, 2006). It is also accepted that many other cultivated or weedy annual plant species can potentially carry PPV inoculum, but natural transmission between such herbaceous plants and *Prunus* has not been demonstrated. Wild woody and herbaceous hosts are also widespread and are potential reservoirs of the disease.

Appendix 8.1 provides a list of common and botanic names of alternate hosts of PPV.

1.2 Vectors

The virus is transmitted either by grafting or non-persistently by two main aphid vectors *Aphis spiraecola* and *Myzus persicae*. *Aphis craccivora, A. fabae, Brachycaudus cardui, B. helychrysi, B. persicae, Hyalopterus pruni, Myzus varians, Phorodon humuli* (Kunze & Krczal, 1971; Leclant, 1973). *Aphis gossypii, Rhopalosiphum padi* and *A. hederae* have been recently reported as vectors (Avinent *et al.,*1994; Labonne *et al.,* 1994). *Metopolophium dirhodum* (rose-grain aphid) and *Toxoptera citricida* transmitted PPV under experimental conditions (Gildow et al. 2004)

2 TAXONOMIC INFORMATION

Species *Plum pox virus*
Genus *Potyvirus*
Family *Potyviridae*
Order *Patatavirales*
Class *Stelpaviricete*
Phylum *Pisuviricota*
Kingdom *Orthornavirae*
Realm *Riboviria*

Common names: Sharka, plum pox (English); variole duprunier, sharka (French); Scharka-Krankheit (German); and vaiolatura delle drupacee (Italian).

2.1 Virus strains

Nine strains of PPV have been distinguished based on symptoms on inoculated herbaceous indicator plants, and according to their serological and molecular properties (Kerlan & Dunez, 1979; James & Varga, 2005; Candresse & Cambra, 2006; James & Glasa, 2006). Differentiation of strains can be determined by antigenic virus properties, electrophoretic mobility of the coat protein, antigenic properties of the N and C regions of coat protein, divergence in the RNA sequence and the presence or absence of a specific restriction site in the C-terminal region of the coat protein. The nine currently recognized monophyletic strains or serotypes of PPV have been identified as Dideron (PPV-D), Marcus (PPV-M), Ancestor Marcus (PPV-Am) (James et al., 2013), El Amar (PPV-EA) (Wetzel *et al.*, 1991a), Cherry (PPV-C), Wimona or W3174 (PPV-W) (James & Varga, 2005), Turkish (PPV-T), Cherry Russia (PPP-CR), as well as a widespread group of recombinants (of PPV-M and PPV-D) known as Recombinant (PPV-Rec). A "necrogenic" isolate of PPV-M which was previously referred to as PPV-SP, and further characterised by Adamolle *et al.* (1994), is not currently recognized as a separate strain from PPV-M. More recently a new recombinant group of PPV isolates have been characterised from Turkey and it has been proposed to be a novel strain referred to as PPV-T (Serçe et al., 2009). In addition, newly characterised sour cherry isolates of PPV from the Volga basin in Russia, have also recently been proposed as a novel cherry strain of PPV referred to as PPV-CR (Cherry Russia) (Glasa et al., 2013).The protocols described in this document will detect all currently known strains of PPV, including PPV-Rec, PPV-T and PPV-CR.

3 DETECTION

3.1 Symptoms

Symptom expression of PPV can depend on the strain involved, *Prunus* cultivar infected and weather conditions. Symptoms vary between different *Prunus* species. For example, in peach, symptoms are best observed on the flower petals and include discoloration and flower breaking (Barba *et al.,* 2011), and depending on the conditions, may be evident for only short periods of the growing season. In plums, symptoms occur on the leaves and are more persistent. Some *Prunus* cultivars may remain symptomless. A useful website for images is A useful website for images is https://www.sharco.eu/content/download/3316/35819/version/1/file/Leaflet.pdf (Sharka containment in view of EU-Expansion). It's important to note that some of the symptomology associated with PPV are similar to those caused by other *Prunus* viruses such as *Amercian plum line pattern virus*.

Other good websites for descriptions of symptoms and photos are:

http://www.inspection.gc.ca/english/plaveg/pestrava/ppv/ppve.shtml

https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/crop-growing/priority-pest-disease/plum-pox-virus

3.1.1 Apricot (Prunus armeniaca) symptoms

Fig 1: Mild to moderately PPV infected apricot 'Castle Brite' fruit show spots which are slightly darker than the surrounding skin colour (left) in Chile. These darker spots may also be raised in severely infected fruit (right) (© M. Cambra)

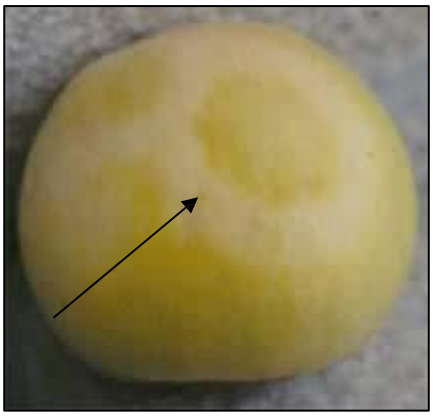

Fig 2 (Left) and 3 (Right): Light rings on yellow fruits may be apparent on apricots near maturity. Valencia, Spain (© J.W. Travis).

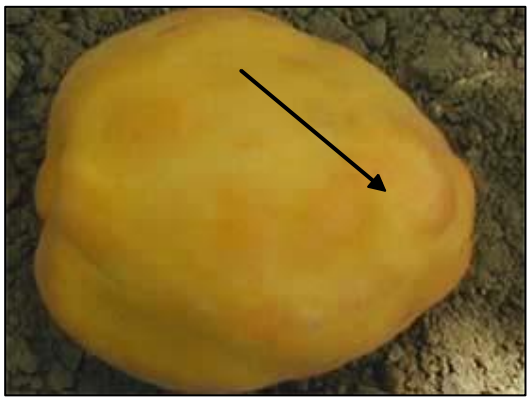

Fig 4: Apricot fruit infected with PPV showing a misshapen bumpy appearance. Montpellier, INRA, France (© J.W. Travis)

Fig 5: Light ring spots on pit and deformities in the flesh of an apricot fruit 'Castle Brite' in Chile (© M. Cambra).

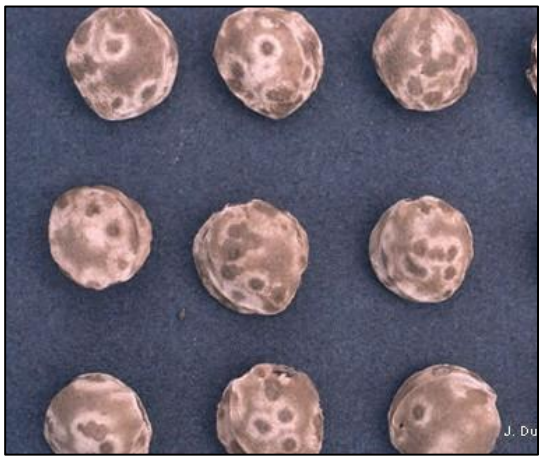

Fig 6: Misshapen and deformed apricot fruits infected with PPV (© M. Nemeth, Hungary).

Fig 7: Ring spots on apricot fruits' pit infected with PPV (© J. Dunez, France).

Fig 8: Apricot deformities caused by PPV extending into the flesh and light ring spots which can occur on the pit (© M. Barba, Italy).

Fig 9: Ring spots on a pit of an apricot. Europe (© R. Scorza)

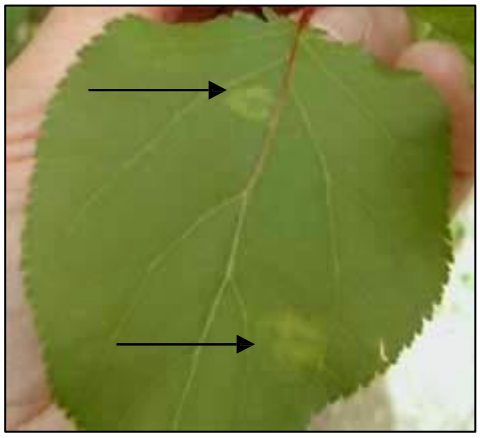

Fig 10: Light green ring spots on apricot leaf. Southern France © (J.W. Travis).

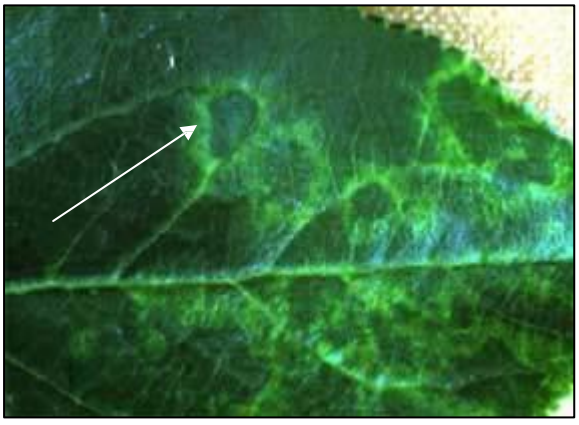

Fig 11: Light green ring spots on an apricot leaf. Murcia, Spain (© M. Cambra).

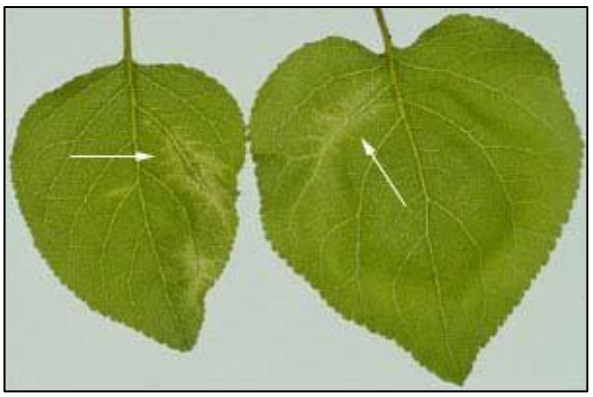

Fig 12: Apricot leaves showing a netting discolouration associated with the veins. France (© P. Gentit)

Fig 13: One or several ring spots may appear on an apricot leaf. Valencia, Spain (© J.W. Travis)

3.1.2 Plum (Prunus domestica) symptoms

Fig 14: Yellow ring spot symptoms on a red plum variety infected with PPV. Southern France (© P. Gentit).

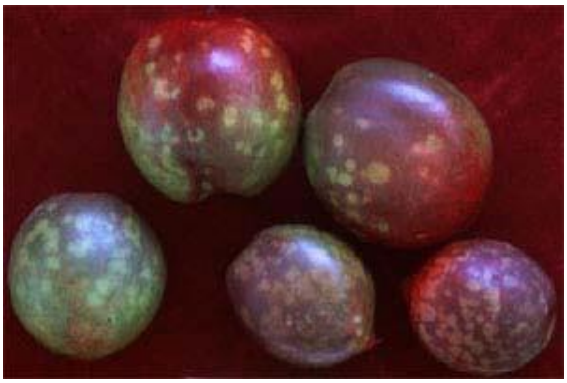

Fig 15: Speckling pattern that may be evident on some varieties of red plums. Pictured 'Arm King' plum. INRA, Montpellier, France (© J. Quiot).

Fig 16: Infected Japanese plum fruits 'Red Beaut' showing severe deformity. Sevilla, Spain (© M. Cambra; Cambra *et al.*, 2008).

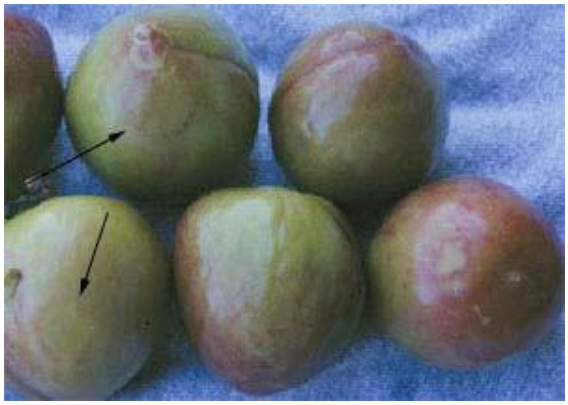

Fig 17: Immature plums showing irregular surface and red ring spots. Valencia, Spain (© F.E. Gildow).

Fig 18: Sunken lesions on plums infected with PPV (© M. Nemeth, Hungary)

Fig 19: Yellow plums showing red ring spots near harvest. Valencia, Spain (© F.E. Gildow)

Fig 20: Severely infected plums showing necrotic areas and a bumpy appearance. Valencia, Spain (© J.W. Travis)

Fig 21: Premature fruit fall on sensitive plum cultivars in Central and Eastern Europe (© M.Nemeth, PHSCS, Hungary). Levy *et al.* (2001): http://www.apsnet.org/online/feature/PlumPox/Top.html

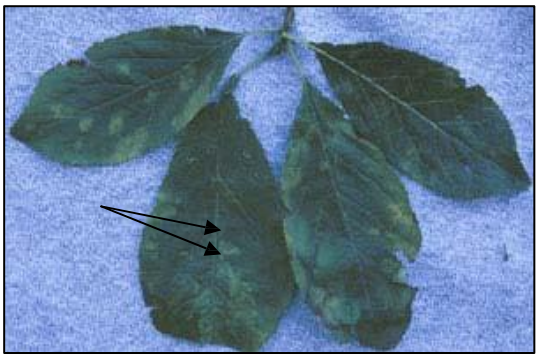

Fig 22: Blotches of light green to yellow on plum leaves. Valencia, Spain (© F.E. Gildow).

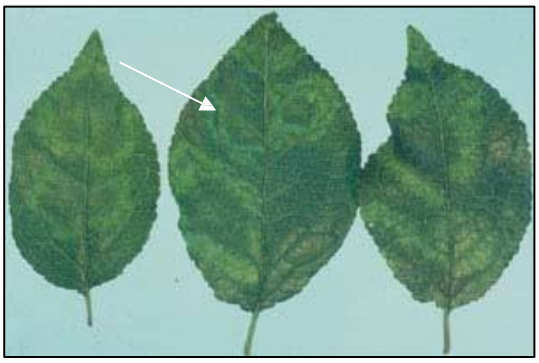

Fig 23: Some plum varieties may have very large ring spots on their leaves. Quetsche, Southern France (© P. Gentit)

Fig 24: Speckling appearance on leaves is another symptom of a PPV infection. Montpellier, INRA, France (© P. Quiot).

Fig 25: Light green ring spots on plum leaves. Europe (© R. Scorza)

Fig 26: Necrotic areas on plum leaves often fall out giving a shot hole appearance. Valencia, Spain (© F.E. Gildow).

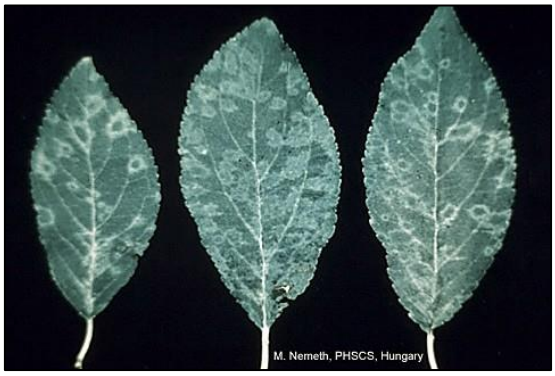

Fig 27: Light green ring spots on plum leaves (© M. Nemeth, Hungary)

3.1.3 Peach (Prunus persica) symptoms.

Fig 28: Early symptom of the virus on peach flowers. Note the dark pink streaks. This is the most reliable symptom to look for on peaches. This picture shows infected "Babygold 5" flowers. Southern France (© P. Gentit)

Fig 29: PPV ring spots. Pictured PPV-D infecting 'Encore' peach fruits from Adams County, Pennsylvania, USA (© F.E. Gildow)

Fig 30: 'Encore' peaches express PPV symptoms more clearly than other varieties. Pennsylvania, USA (© R. Welliver).

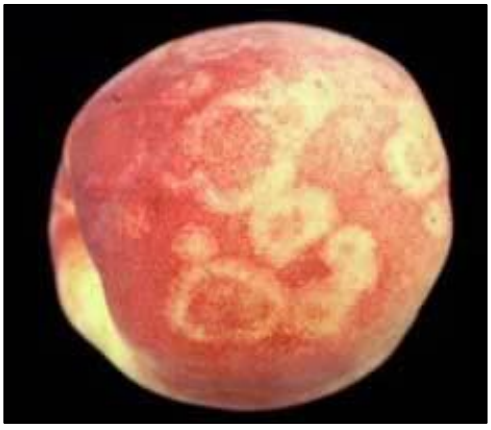

Fig 31: Obvious yellow rings on a red-skinned 'Encore' peach. Pennsylvania, USA (© K.D. Hickey).

Fig 32: Light green veins of a peach leaf. Montpellier, INRA, France (© J.W. Travis).

Fig 33: Peach leaves showing necrotic areas and yellow blotching patterns. Pennsylvania, USA (© J.W. Travis)

Fig 34: Veinal yellowing and deformity of peach leaves caused by PPV. Symptoms are similar to those caused by insect damage. Pennsylvania, USA (© J.W. Travis).

3.1.4 Cherry (Prunus avium) symptoms

Fig 35: Cherry leaves showing mottling and necrotic spots (© P. Gentit, France).

3.2 Sampling

The distribution of the virus in a tree can be irregular. However, the detection from flowers, young leaves, old leaves, fruit, dormant wood, and roots is possible. The time of year when the sample is taken is critical and can greatly affect the test results. Optimal sampling time is during spring, or early summer (18-28 °C) but can vary from season to season depending on the weather conditions.

3.2.1 Sampling the correct tissue from a tree

When sampling leaf material for the presence of PPV, leaves from the middle of the branch should be sampled from various points around the tree. Optimal tissue for peach samples is petals if possible.

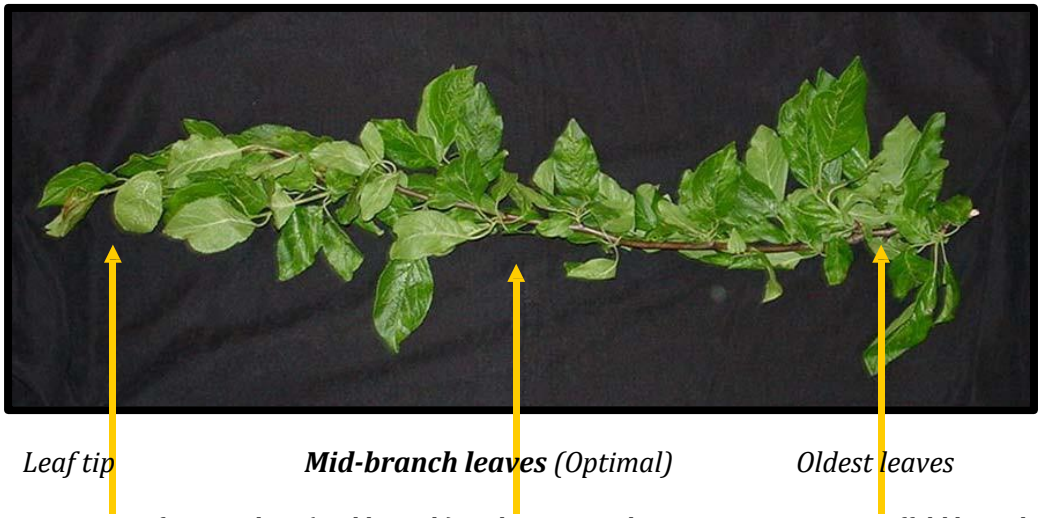

Fig 36: Best section for sampling (mid branch) is shown in a characteristic *Prunus* scaffold branch.

4 IDENTIFICATION

The diagnostic procedure described in this chapter follows the diagnostic scheme depicted in Figure 37. In general, when pursuing a high standard of unequivocal diagnostics, the diagnostician must consider four core methods: electron microscopy, mechanical transmission, serological and molecular tests. Some variation to this scheme can be given depending on the scope of the screening, whether it is for quarantine purposes only, for general surveillance or during incursion responses. However its application can be also extended to quality control in nursery production industries or production of virus-free plants under a national certification programme.

Symptom expression in the field and the results obtained from mechanical inoculation assays on herbaceous indicators or woody indexing indicators, gives an indication of the infectivity and transmissibility through sap of the virus under investigation. However, this information is not sufficient for a definitive diagnosis. Mixture of viruses with similar morphology and symptomatology may be a cause of confusion. For example, the pseudopox caused by the mixed infection of *Prunus necrotic ringspot virus* and *Apple chlorotic leaf spot virus* (Lebas et al., 2004).

Therefore, positive or negative samples derived from mechanical inoculation assays require additional serological or molecular validation. Another useful and rapid method for the identification of PPV-like particles is the observation of leaf-dip preparations by electron microscopy. However, observing the virus structures under the electron microscope can only be used as a general PPV detection method. The observation of crude sap preparation using Immunosorbent Electron Microscopy Technique and decoration may lead to a more definitive diagnostic result, but still requires validation by ELISA and/or RT-PCR.

In general, the specificity and high throughput of the serological methods such as ELISA can be exploited to specifically target PPV during surveys in production areas, commercial nurseries or for quarantine purposes, at the border and post entry quarantine. On the other hand, the RT-PCR technique is very sensitive and specific and allows the detection of minimal amounts of target RNA, which facilitates the validation of results from all other previously applied techniques. These results require further validation by sequencing the DNA amplicon. The obtained sequence is required to be aligned with sequences held in the NCBI-GenBank database using the Basic Local Alignment Search Tool (BLAST).

Note that not all laboratories will have high throughput RT-PCR capability and although the overall cost of RT-PCR can be higher, the RT-PCR is still recommended for the screening of large numbers of samples with ELISA used a secondary confirmation tool. This is in part due to the increase in sensitivity from RT-PCR when compared to ELISA, which has not been validated in Australian laboratories.

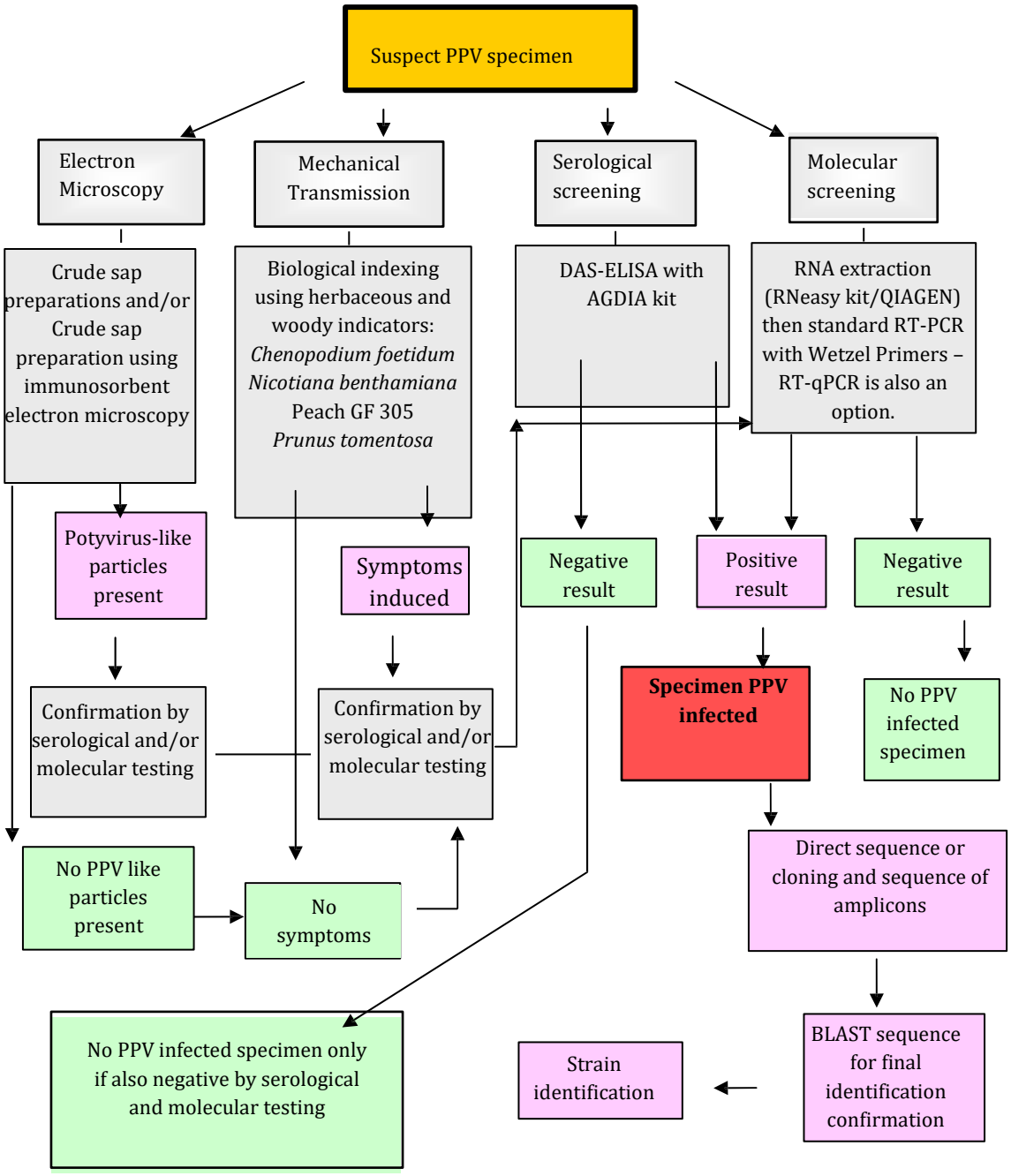

Fig 37: Diagnostic flow chart for detection and identification of PPV (Adapted from PPV EPPO 2004; https://gd.eppo.int/taxon/PPV000/documents).

4.1 Morphological methods

4.1.1 Direct observation of Plum pox virus by electron microscopy

Transmission electron microscope (TEM) examination of grids prepared from small sections of homogenised leaf tissue can be negatively stained with 1% uranyl acetate (UA) for rapid detection of PPV. The grids are examined at 40,000 x magnification for flexuous filamentous potyvirus particles of 660-770 nm in length. Two detailed protocols are provided for crude sap preparation (Section 3.3.1) and crude sap preparation using immunosorbent electron microscopy (Section 3.3.2).

Crude sap preparation - TEM

Preparation for transmission electron microscope examination using uranyl acetate stain. Method adapted from Milne et al. (1996).

Equipment:

- 1.5 ml microcentrifuge tubes
- Sterile micropestle for 1.5 ml microcentrifuge tubes (e.g. Eppendorf part: 0030 120.937)
- Pipettes and tips
- Formvar-carbon reinforced copper grids, square 400 mesh (e.g. SPI supplies catalogue number 3440C-CF), store at room temperature
- Forceps (very fine tips)
- Filter paper (cut in small triangular pieces)
- Small containers (e.g. weight boat)
- Beakers
- Sharp blade or razor blade (one per sample)

Reagents:

- Deionised water
- Polyvinylpyrrolidone (PVP; MW 24,000-45,000)
- 0.5 M Phosphate Buffer pH 7 (stock solution, store at room temperature)
    - Prepare two stock solutions: 100 ml deionized water
    - 0.5 M KH_2PO_4 6.8 g
    - 0.5 M K_2HPO_4 8.71 g
    - Mix 39 ml of 0.5M KH_2PO_4 with 61 ml of 0.5 M K_2HPO_4
    - Working solution: 0.1 M phosphate buffer pH 7, prepare fresh
- 1% (w/v) aqueous uranyl acetate 50 ml
    - Uranyl acetate 0.5 g
    - Dissolve in deionised water
    - Store in the dark at room temperature

HAZARD: Uranyl acetate has a slight radioactivity emission.

A 0.5% (w/v) uranyl acetate stain may also be used if needing to clarify the background.

Method:

1. Prepare 0.1 M phosphate buffer (about 100 µl per sample) containing 2% (w/v) PVP [TEM extraction buffer]. The buffer may be kept up to one month in the fridge. Phosphate buffer may be modified depending on the tissue used.

2. Add 100 µl of TEM extraction buffer in a 1.5 ml microcentrifuge tube (1 tube per sample).
3. Cut a small piece of plant material (~5 mm^2), preferably from the edge between symptomatic and asymptomatic tissue, using a clean blade and immediately transfer it into the 1.5 ml microcentrifuge tube containing TEM extraction buffer. Use a clean blade for each sample. If touching plant material with gloves, change gloves immediately.

NB: if using dry tissue, leave to soak in TEM extraction buffer for 5-10 min on ice before grinding.

4. Grind using the sterile micropestle. The crude sap should be a clear green colour. If dark green (too concentrated), add more TEM extraction buffer to dilute the sap.
5. Incubate crude sap preparation on ice for about 5 min, then briefly centrifuge at maximum speed for one min.
6. Hold a grid (bright side upward) by the edge using forceps.
7. Place a drop (~4 µl) of crude sap preparation on the grid and leave for about 5 min. The grid may also be incubated at room temperature for up to one hour in a sealable box containing damp paper.
8. Rinse with ~30 drops of deionised water by holding the grid slightly tilted on the side.
9. Add ~5 drops of 1% (w/v) uranyl acetate stain while holding the grid slightly tilted on the side. If touching uranyl acetate stain with gloves, change gloves immediately.
10. Leave the grid to dry on the bench for 10-15 min, alternatively it can be put in a 37^{o}C incubator for 5 min.
11. Grid observation
    - Use liquid nitrogen in the EM cold finger all the time (where applicable)
    - Usually start observation at 25-30K
    - Need to see at least 3-4 particles before being confident that there is a virus present

Crude sap preparation – ISEM

For immunosorbent electron microscopy technique and uranyl acetate stain for transmission electron microscope examination. Method adapted from Matthews (1993).

Immunosorbent electron microscopy (ISEM) is used when particles in the crude sap preparation are low in concentration. In ISEM, the virus is first trapped with an antibody on the grid with the purpose of increasing the number of virus particles. This is followed by a second application of the same antibody.

Alternatively, if the virus particles in a crude sap preparation are known to be in high concentration, a decoration technique may be sufficient. Decoration of a crude sap preparation is used to confirm and/or identify the virus presence.

Equipment:

- 1.5 ml microcentrifuge tubes
- Sterile micropestle for 1.5 ml microcentrifuge tubes (e.g. Eppendorf part: 0030 120.937)
- Pipettes and tips
- Formvar-carbon reinforced copper grids, square 400 mesh (e.g. SPI supplies catalogue number 3440C-CF), store at room temperature
- Forceps (very fine tips)
- Filter paper (cut in small pieces)
- Small containers (e.g. weight boat)

- Beakers
- Sharp blade or razor blade (one per sample)

Reagents:

- Deionised water
- Polyvinylpyrrolidone (PVP; MW 24,000-45,000)
- 0.5 M Phosphate Buffer pH 7 (stock solution, store at room temperature)
    - Prepare two stock solutions: 100 ml deionized water
    - 0.5 M KH_2PO_4 6.8 g
    - 0.5 M K_2HPO_4 8.71 g
    - Mix 39 ml of 0.5M KH_2PO_4 with 61 ml of 0.5 M K_2HPO_4
    - Working solution: 0.1 M phosphate buffer pH 7, prepare fresh
- 1% (w/v) aqueous uranyl acetate 50 ml
    - Uranyl acetate 0.5 g
    - Dissolve in deionised water
    - Store in the dark at room temperature

HAZARD: Uranyl acetate has a slight radioactivity emission.

A 0.5% (w/v) uranyl acetate stain may also be used if needing to clarify the background.

- Antisera specific to the targeted viruses

Method:

1. Dilute the antisera in 0.1 M phosphate buffer (without PVP).
2. Hold a grid (bright side upward) by the edge using forceps.
3. Add a drop (~4 µl) of the diluted antisera on a grid and incubate in a closed humid box at room temperature for 5 min.

   NB: If using an antisera for the first time for decoration technique, several dilutions may be used starting 1/1, 1/10, 1/50 (v/v).

4. Wash with ~15 drops of 0.1 M phosphate buffer (without PVP) by holding the grid slightly tilted on the side.
5. Transfer 100 µl of 0.1 M phosphate buffer containing 2% (w/v) PVP [TEM extraction buffer] per 1.5 ml microcentrifuge tube (1 tube per sample).
6. Cut a small piece of plant material (~5 mm^2), preferably from the edge between symptomatic and asymptomatic tissue, using a clean blade for each sample and immediately transfer it into the 1.5 ml microcentrifuge tube containing TEM extraction buffer. Change gloves immediately if they contact the plant material.

   NB: if using dry tissue, leave to soak in TEM extraction buffer for 5-10 min on ice before grinding.

7. Grind using the sterile micropestle. The crude sap should be of a clear green colour. If dark green (too concentrated), add more TEM extraction buffer to dilute the sap.
8. Incubate crude sap preparation on ice for about 5 min, then briefly centrifuge at maximum speed for one min.
9. Place a drop (~4 µl) of crude sap preparation on a grid and incubate about 5 min in a closed humid box.

10. Wash with ~15 drops of 0.1 M phosphate buffer (without PVP) by holding the grid slightly tilted on the side.
11. Place a drop of the antisera and incubate in a closed humid box at room temperature for 15 min.
12. Rinse with ~30 drops of deionised water by holding the grid slightly tilted on the side.
13. Add ~5 drops of 1% (w/v) uranyl acetate stain while holding the grid slightly tilted on the side. If touching uranyl acetate stain with gloves, change gloves immediately.
14. Leave it to dry on the bench for 10-15 min alternatively it can be put in a 37°C incubator for 5 min.
15. Grid observation
    - Use liquid nitrogen in the EM cold finger all the time
    - Usually start observation at 25-30K
    - Need to see at least 3-4 particles before being confident that there is a virus in a sample

Tips for Grid preparation

For woody species (e.g. plum, apricot etc.), it is recommended to add 2% (w/v) polyvinylpyrrolidone (PVP; MW 24,000-45,000) or polyethylene glycol (PEG; MW 6,000). The 0.1 M phosphate buffer with 2% PVP may be stored in the fridge for up to one month.

Problem when observing the grid under the microscope	**Suggestions**
Too dark	③ Too much plant material on grid ③ Too much stain
Dark lumps	③ Too much stain
Crystalline structures	③ Stain precipitation or phosphate buffer ③ Grid was not rinsed enough with deionised water

4.2 Serological Tests – ELISA

4.2.1 Equipment

- 20, 100 and 1000ul pipettes and tips
- Microcentrifuge and microcentrifuge tubes (2.0mL)
- Container with ice
- Mortar and pestle (or similar macerating device)
- ELISA plates
- ELISA plate reader.

4.2.2 Reagents

- Coating Antibody (provided by supplier)
- Detecting Conjugate (provided by supplier)
- Controls: Positive and negative (provided by supplier – Permit required).
- 5X PBS Buffer
- PBS-Tween-Buffer

- Coating Buffer
- ELISA extraction Buffer
- Conjugation Buffer
- Substrate Buffer

4.2.3 ELISA Buffers

Coating buffer

Dissolve in distilled water to 1000 ml:

Sodium carbonate (anhydrous)	1.59 g
Sodium hydrogen carbonate	2.93 g

Adjust pH to 9.6 and store at 4° C.

PBS-Tween Buffer (Wash Buffer)

Dissolve in distilled water to 1000 ml:

Sodium chloride	8.0 g
Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic (anhydrous)	0.2 g
Potassium chloride	0.2 g
Tween-20	0.5 g

Adjust pH to 7.4. Store at 4°C.

ECI Buffer (use as Sample Buffer and as Conjugate Buffer)

Add to 1000 ml 1X PBST:

Bovine serum albumin (BSA)	2.0 g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g

Adjust pH to 7.4. Store at 4° C.

PNP Buffer

Dissolve in 800 ml distilled water:

Magnesium chloride hexahydrate	0.1 g
Sodium azide	0.2 g
Diethanolamine	97.0 ml

4.2.4 ELISA Protocol and Interpretation of Results

For PPV detection using double-antibody sandwich (DAS)-ELISA using the BIOREBA (Reinach, Switzerland), AGRITEST (Italy) or AGDIA (USA) Kits, including interpretation of results, follow the manufacturer's instructions. For further information:

http://www.bioreba.ch/saas/web/bioreba/web.aspx?PageID=58&search=PPV

http://www.agritest.it/ppv-universal-elisa-kit.html

https://orders.agdia.com/agdia-set-ppv-alkphos-sra-31505

Molecular methods

4.2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a rapid, specific and sensitive test that can be used to detect and diagnose PPV from extracted nucleic acids. The primers recommended are those described by Wetzel *et al.* (1991b), which have become standard primers used worldwide for the detection of PPV. These primers detect all strains of PPV and a restriction digestion test can be used to differentiate PPV strains.

RNA extraction from plant tissue using QIAGEN RNeasy™ Plant Mini Kit

Equipment

- Autoclave
- Sterile 1.5 ml microcentrifuge tubes
- 0-2 µl, 2-20 µl, 20-200 µl, and 200-1000 µl Pipettes and sterile tips
- Sterile micropestle (e.g. Eppendorf part: 0030 120.937) (if necessary)
- Balance
- Microcentrifuge (at room temperature)
- Freezer/Refrigerator
- Sterile scalpel blades (if necessary)
- Vortex
- Variable speed electric drill (optional)
- MagNa Lyser machine (optional)
- Dewar liquid nitrogen dispenser and liquid nitrogen (if appropriate for protocol)
- Water bath or Dry heat block at 56°C ± 2.5°C or 70°C ± 2.5°C (depending on the protocol used)

Reagents

- Ethanol 100% (room temperature)
- QIAGEN RNeasy™ Plant Mini Kit reagents
- BioRad Quantum Prep® Freeze 'N Squeeze DNA gel Extraction Spin Columns.
- PVP-40 (if using the modified protocol)
- 20% (w/v) Sarkosyl (if using the modified protocol by MacKenzie *et al.,* 1997)
- 2 M sodium acetate, pH 5 (if using the modified protocol by MacKenzie *et al.,* 1997) For 100 ml: Sodium acetate 16.406 g
- Adjust pH to 5 using acetic acid

Method

The kit provides two extraction buffers, the RLT and the RLC, which contain guanidine isothiocyanate (GITC) and guanidine hydrochloride, respectively. In most cases, the buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of GITC. For woody plants samples and/or *Prunus* suspected to be PPV infected it is recommended to use the RLT buffer.

If using the QIAGEN RNeasy Plant Mini Kit, add 10 µl of β-Mercaptoethanol to 1 ml extraction RLT buffer. Follow the manufacturer's instructions from this point forward.

RT-PCR procedures

Method

Reverse Transcription-polymerase chain reaction can be performed either in a one or two step reaction. This document describes the one-step RT-PCR reaction with Superscript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Invitrogen), which has been validated within the laboratory and is the preferred method for detection. An internal control (e.g. NAD5) is recommended to ensure a successful RNA extraction.

RT-PCR PPV specific and internal control

Primers	Name	Sequence (5'-3')
Forward	P2 (Wetzel)	CAG ACT ACA GCC TCG CCA GA
Reverse	P1 (Wetzel)	ACC GAG ACC ACT ACA CTC CC

Primers	Name	Sequence (5'-3')
Forward	Nad2.1a	GGA CTC CTG ACG TAT ACG AAG GAT C
Reverse	Nad2.2b	AGC AAT GAG ATT CCC CAA TAT CAT

*Following the procedure by Thompson *et al.,* 2003.

PCR Reagent mix	Volume reaction (25µl)	Cycling parameters
Sterile distilled H_2O	8µl	48°C 45 min x 1
2X Reaction Buffer	12.5 µl	94°C 3 min x 1
Taq DNA Polymerase	0.5 µl	94°C 45 sec
F-Primer (10µm)	1.0 µl	62°C 30 sec x 35
R-Primer (10µm)	1.0 µl	72°C 45 min
RNA	2.0 µl	72°C 7 min
		4°C 1 min x 1

PCR controls	Description
Positive	Known PPV-infected sample (a positive control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland; or alternatively use the target product cloned into a plasmid) or acquired from Agriculture Victoria.
Negative	Healthy plant tissue (a negative control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland).
No template control	Water

Electrophoresis

Electrophoresis	Description	Buffer	Predicted size amplicon (bp)
Agarose gel	2%	1X TAE	243 bp for primers P1 & P2; 188bp NAD5 (Thompson *et al.,* 2003)

For better resolution of the PCR products it is recommended to perform a 2% agarose-TAE gel electrophoresis at 110 volts for 30 min (depending on gel size) and using a 100 bp marker. It is also recommended to load 10 µl or the complete volume of the RT-PCR reaction in each electrophoretic cell if further testing such as restriction enzyme is planned. Stain the gel with SYBR™ DNA Gel Stain (Invitrogen). Visualise the RT-PCR products using an UV trans-illuminator, photograph the gel and record the results (Fig 38).

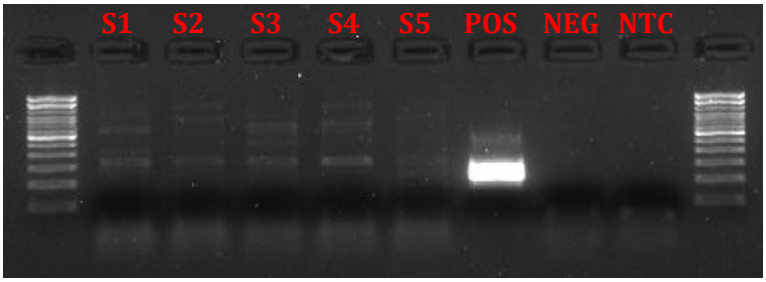

Figure 38: Gel electrophoresis of PPV positive (POS) (243bp), negative (NEG), no template control (NTC) and 5 plum samples (S1-S5) (Source: Agriculture Victoria).

Band excision and further testing

Excise the band of the expected PCR product with a sterile blade and place it into a Quantum Prep BioRad gel extraction column to purify the amplicon. Freeze for 5 min and centrifuge full speed at room temperature for 3 min. The amplicon will be ready for immediate cloning (see Sambrook *et al.,* 1989 for standard cloning procedures), direct sequencing, and/or restriction digestion. Freeze the remaining product at -20°C for up to 6 weeks. The DNA will progressively degrade.

PCR Product clean-up and Sequencing

Following the manufacturer's instructions (QIAquick PCR Purification Kit, Qiagen or similar), the PCR product can be cleaned directly from the RT-PCR tube or excised from the gel. The product along with the appropriate primers can then be sent to a sequencing facility (e.g. Micromon, Monash University) if it cannot be completed on site. The resulting sequences can then be compared to PPV isolates on Genbank, including the type species (NC_001445.1).

4.2.1 Real time-polymerase chain reaction (RT-qPCR)

Real time-polymerase chain reaction (RT-qPCR) (Schneider et al. 2004 https://www.ippc.int/en/publications/dp-2-2012-plum-pox-virus/) can be performed using any validated RT-qPCR system such as QuantStudio 3 (Thermofisher), Corbett Rotor gene (Qiagen) etc. For this protocol the Applied Biosystems AgPath-ID One-Step qRT-PCR was used, however any other RT-qPCR kit can be used provided it has been validated (NT-DPIR have validated using SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline Pty Ltd.) on a Rotor Gene Q (Corbett)). The RT-qPCR from Schneider et al., 2004 (IPPC 2018) has been validated within the laboratory against an isolate of PPV.

Primer	Name	Sequence (5'-3')
Reverse	PPV-qF	CCAATAAAGCCATTGTTGGATC
Forward	PPV-qR	TGAATTCCATACCTTGGCATGT
Probe	PPV-qP	[6FAM]-CTTCAGCCACGTTACTGAAATGTGCCA-[TAMRA]

PCR Reagent mix	Volume reaction (25µl)	Cycling parameters
Sterile distilled H2O	5.0 µl	48°C 30 min x 1
2 X Reaction Buffer	12.5 µl	94°C 5min
10 µM PPV-qF primer	1.0 µl	
10 µM PPV-qR primer	1.0 µl	94°C 10 sec x 40
10 µM PPV-qP Probe	0.5 µl	60°C 45 sec
25 X Enzyme	1.0 µl	
RNA template	4.0 µl	

PCR controls	Description
Positive	Known PPV-infected sample (a positive control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland; or alternatively use the target product cloned into a plasmid) or acquired from Agriculture Victoria.
Negative	Healthy plant tissue (a negative control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland).
No template control	Water

Run the RT-qPCR (Figures 39 & 40) at the above cycling conditions and set a cut off Ct value of 37. For results where Ct values are obtained between 32-37, the sample is initially regarded as suspect positive, below Ct 32 a sample is regarded as positive, and above Ct 37 is regarded as negative. Follow up all results with conventional RT-PCR. Information is based upon validation of the Schneider *et al.,* 2004 PPV RT-qPCR (IPPC 2018) undertaken within the laboratory.

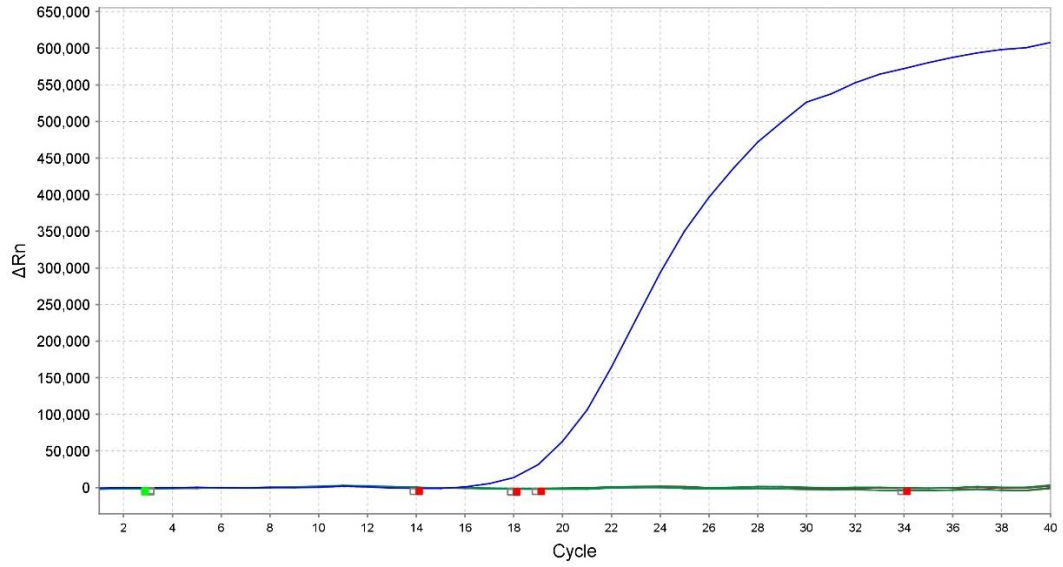

Figure 39: Results of the Schneider *et al.,* 2004 PPV RT-qPCR with positive control, negative control, NTC and two potyvirus speices, *Potato virus Y* and *Papaya ringspot virus* (Source: Agriculture Victoria).

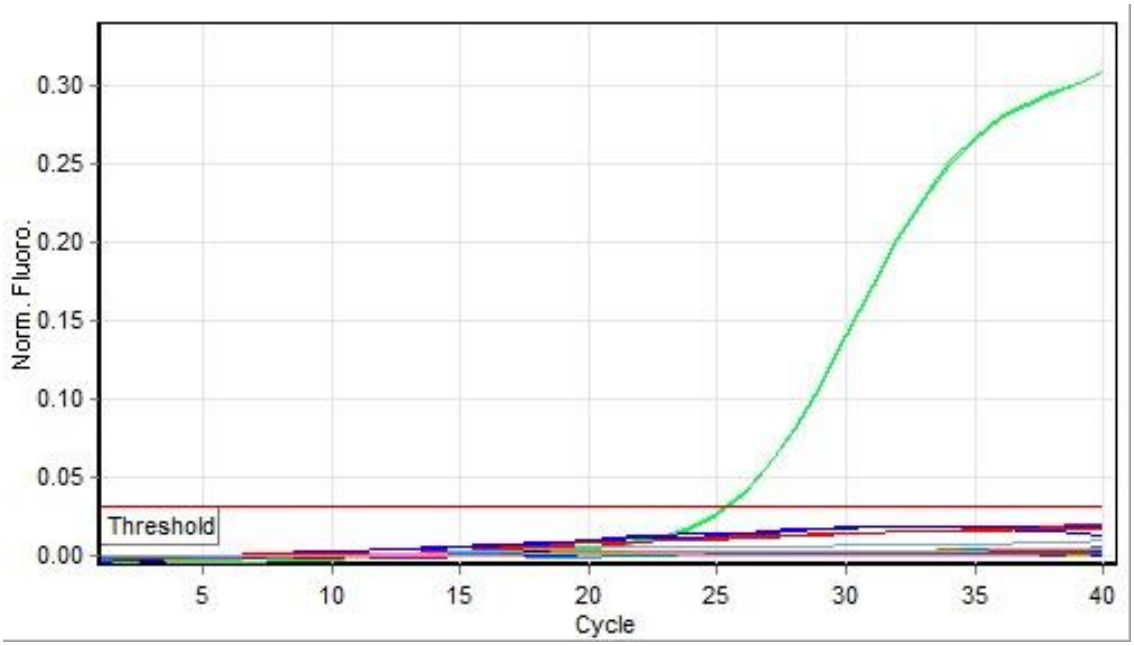

Figure 40: Results of the Schneider *et al.,* 2004 PPV RT-qPCR with positive control, negative controls and NTC (Source: NT-DPIR).

4.3 Biological methods

4.3.1 Herbaceous Indexing

Equipment

- mortar and pestle
- wash bottle
- tags
- pen

Reagents

- 0.5X PBS
- 0.68 M sodium chloride
- 40 mM sodium phosphate dibasic anhydrous
- 7 mM potassium phosphate
- 3 mM potassium chloride
- Carborundum powder (Saint-Gobain Industrial Ceramics Pty)

Indicator plants (x2)

- *Chenopodium foetidum*
- *Nicotiana benthamiana* (preferred indicator)

Method

1. Choose two leaves from each indicator and punch a small hole in the end of the leaf.
2. Lightly cover both leaves with fine carborundum powder.
3. Grind leaf sample in mortar and pestle with 0.5X PBS buffer
4. Dip fingers in homogenised sample and gently rub liquid 8 times onto leaves sprinkled with carborundum powder.
5. Wash off all traces of carborundum powder as it burns the leaves.
6. Check indicators every week for 6 weeks for symptoms such as:
    - *Chenopodium foetidum* – local lesions
    - *Nicotiana benthamiana* – mosaic symptoms (PPV-M), delayed mosaic (PPV-D).

4.3.2 Woody Indexing

The recommended woody indicator plant is *Prunus tomentose* or Peach GF 305. An indexing test requires at least 5 replicates per sample. This is not a rapid test to be implemented as a routine diagnostic test but can be used to maintain the virus in an alternate host for further characterisation. The woody indexing can be used to assist with strain differentiation. The PPV strains can induce differential leaf symptoms on these indicators. PPV-D produces chlorotic, vein–associated patterns and after several weeks some of the chlorotic areas become necrotic. PPV-EA produces mild chlorotic patterns with some necrotic flecking. PPV-M produces strong chlorotic, vein-associated patterns followed quickly by necrotic flecking and vein-associated necrotic patterns. See Nemeth (1986) for the method of indexing and Damsteegt *et al.* (1997) for images of symptoms.

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8 APPENDICES

8.1 Alternate *Rosaceae* hosts of PPV

Alternate *Rosaceae* hosts of PPV often used in Australia and New Zealand as ornamental plants. All listed *Prunus* sp. and more can be found at CABI: https://www.cabi.org/isc/datasheet/42203

Botanic Name	**Common Name**
Prunus cerasifera	Cherry Plum, Myrobalan, Purple-leafed plum
Prunus glandulosa	Dwarf flowering almond, Almond cherry
Prunus insititia	Bully-bloom, Bullies, Bolas, Bullace, Wild Damson
Prunus japonica	Japanese single bush cherry
Prunus mahaleb	Mahaleb cherry
Prunus maritima	Beach plum
Prunus salicina	Japanese plum
Prunus sibirica	Siberian apricot
Prunus spinosa	Blackthorn, Sloe
Prunus tormentosa	Nanking cherry, Hansen bush cherry

Alternative weed hosts of PPV, further information can be found at CABI: https://www.cabi.org/isc/datasheet/42203

Botanic Name	**Common Name**
Taraxacum officinale	Common Dandelion
Sonchus sp.	Sow thistles
Cirsium arvense	Creeping thistle
Convolvulus arvensis	Field bindweed
Solanum nigrum	Black nightshade
Clematis sp.	Clematis
Trifolium sp.	White clover, Dutch clover
Cichorium sp.	Chicory
Rorippa sylvestris	Yellow fieldcress
Ajuga genevensis	Blue bugle

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

9.1 Introduction

Plum Pox Virus (PPV) is a *Potyvirus* which is known to infect hosts of the genus *Prunus* including apricots (*P. armeniaca*), peaches (*P. persica*) and plums (*P. domestica* and *P. salicina*) (Festic, 1978; Eichmeier *et al*., 2016). Depending on the host, symptoms are more common on the flower petals (peaches) and leaves (plums), some *Prunus* cultivars are however symptomless (Llácer & Cambra, 2006). The main vectors of PPV are the aphid species *Aphis spiraecola* and *Myzus persicae* (Gildow *et al*., 2004).

Testing for PPV includes common laboratory methods such as RT-PCR, ELISA and morphological identification, while an Agdia Immunostrip can be used in both field and laboratory situations (Table 1). In situations where volumes of material/samples are very high (+300), it is recommended that the laboratory undertake ELISA initially as although this will not point to a specific strain of PPV, it will allow for a more targeted approach if RT-PCR is required for strain identification. One method which aids in targeting surveillance/sampling in the field is the Agdia Immunostrip, which identifies several isolates of PPV, with a result obtained within minutes of undertaking the test.

Table 1: Methodology required for the identification of PPV.

Method	**Identification level**	**Identification Confidence**	**Deployment (Field/Lab)**	**Required Time**	**Throughput (No. of samples)**
Morphological (4.1)	To Genus	Low (<90%)	Laboratory	<1 d - >1 d	Low (1-10)
RT-PCR (4.3)	To Species/Strain	High (99%+)	Laboratory	<1 w	High (100s)
ELISA (4.2)	To Species	Medium (90-99%)	Laboratory	<1 week	Very High (1000s)
Agdia Immunostrip (9.3)	To Species	Medium (90-99%)	Field/Laboratory	<1 h	Medium (10-25)
Agdia AmplifyRP® Acceler8®	To Species	Medium (90-99%)	Field/Laboratory	<1 h	Medium (10-25)
Herbaceous Indexing (4.4)	To Genus	High (99%+)	Laboratory	>2 weeks	Low (1-10)

9.2 Sampling

Sampling for PPV should be undertaken during spring or early summer when temperatures are mild to warm (18-28 °C) (Glasa *et al*., 2003). Material sampled should include mid-branch leaves (Fig. 1), the leaf tip and those closest to the trunk (oldest) are not optimal sample types. Any leaves with virus like symptoms (Fig. 2) should be targeted from all *Prunus* species, for peaches, the petals are the preferred material to sample if possible. For bulk sampling of large orchards, 5 leaves per tree should be picked and bulked with 10 trees, these will then be subsampled within the lab. For small orchards, 10 leaves per tree can be taken as a single sample. A similar approach can be taken for the petals of peach trees.

Samples should be placed into zip-lock bags with appropriate labelling and stored in a cool dry place until being sent/transported to the laboratory for analysis. If temperatures are high (+28°C), an esky with freezer blocks should be on hand to prevent the samples from deterioration whilst out in the field and during transport.

Fig 1: Best section for sampling (mid branch) is shown in a characteristic *Prunus* scaffold branch.

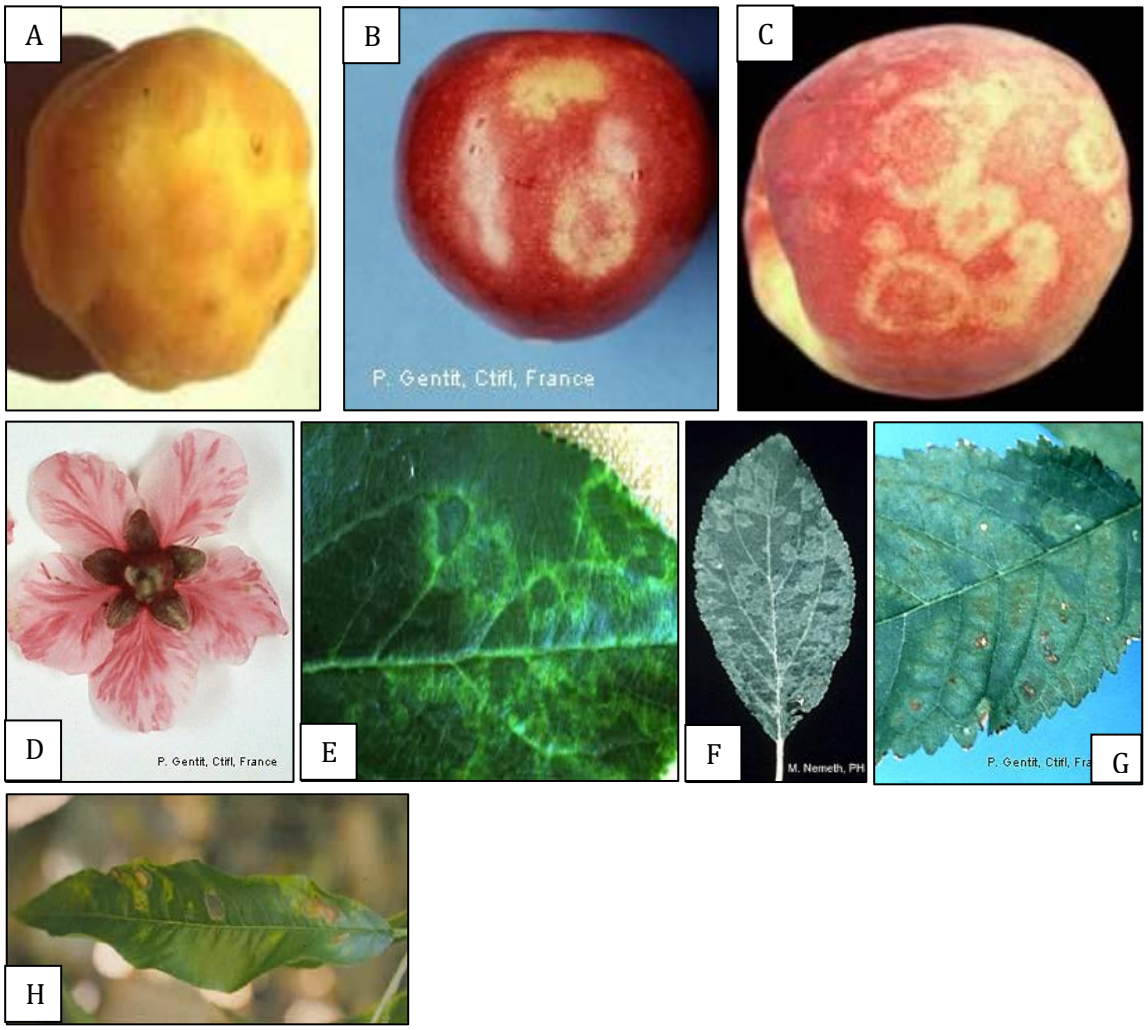

Fig 2: Apricot showing mild PPV symptoms (**A**, © M. Cambra); yellow rings on plum (**B**, © P. Gentit) and peach (**C**, © K. D. Hickey); early virus symptoms on peach flowers (**D**, © P. Gentit); ring spots on an apricot (**E**, © M. Cambra) and plum leaf (**F**, © M. Nemeth); mottling and necrotic spots on cherry leaves (**G**, © P. Gentit) and yellow blotching and necrotic spots on peach leaves (**H**, © J. W. Travis).

9.3 In Field Tests

In field testing may help surveillance identify suspect plants, this will also help with diagnostics within the laboratory as it will minimise the number of samples for testing. Training may be required for field staff to do this testing if they are not familiar with the techniques.

9.3.1 Agdia ImmunoStrip

The ImmunoStrip® for Plum Pox Virus (PPV) (Fig 3a) is available from Agdia. Following the user guide which accompanies the test kit, the user can identify the following isolates of PPV including; PPV-C, PPV-M, PPV-D, PPV-EA, PPV-Rec, PPV-W, PPV-T and PPV-CR.

For testing of Apricot tissue with the PPV ImmunoStrip®, it is recommended that a 1:50 dilution of the sample be used rather than the standard 1:10 sample dilution. Be sure to check the ImmunoStrip immediately at the 20min incubation and do not let the ImmunoStrip incubate longer than 20min.

9.3.2 PPV AmplifyRP® Acceler8

The PPV AmplifyRP® Acceler8, also from Agdia®, allows for the user to utilise the ELISA buffer to screen for PPV. The AmplifyRP® test is similar to the Immunostrip® in its output, however, is relatively quick in returning a positive/negative result (Fig 3b). This method can also be utilised within the laboratory.

More information can be found on the Agdia website (https://orders.agdia.com/agdia-immunostrip-for-ppv-isk-31505 and https://orders.agdia.com/amplifyrp-acceler8-for-ppv-acs-31505).

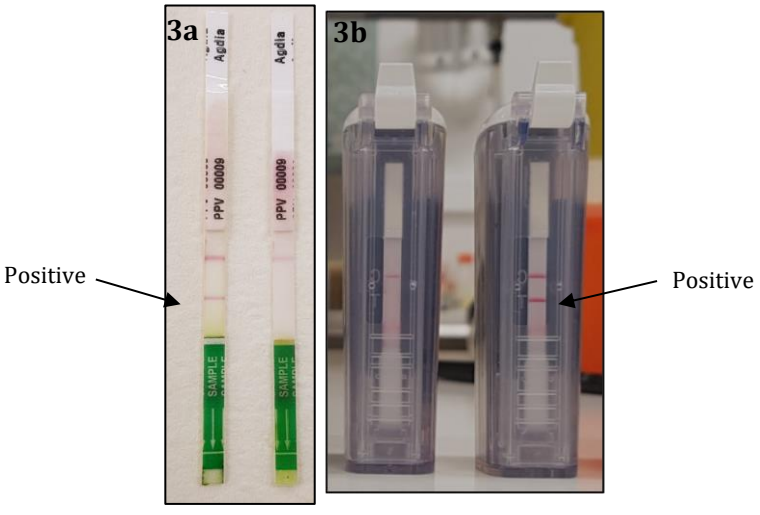

Fig 3: Agdia PPV Immunostrip® (**a**) and PPV AmplifyRP® Acceler8® (**b**) with positive and negative results (Source: Agriculture Victoria).

9.4 Laboratory Tests

Laboratory methods for the detection of PPV include; Reverse transcription polymerase chain reaction (RT-PCR) and Herbaceous/woody indexing. Both methods including the instructions to complete can be located in the NDP for Plum Pox Virus in sections 4.3.1 and 4.4.1 and 4.4.2 respectively.

9.5 Acknowledgements

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9.6 References

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