Diagnostics
Diagnostic

American plum line pattern ilarvirus

Specific scope

This standard describes a diagnostic protocol for *American plum line pattern ilarvirus*.

Specific approval and amendment

Approved in 2005-09.

Introduction

American plum line pattern virus (APLPV) is the least extensively documented *Ilarvirus* reported to infect stone fruits. The virus infects stone fruits, in particular, Japanese plum, peach and flowering cherry, causing generally clear-cut symptoms. Sap and grafting transmission of the virus was reported early (Kirkpatrick *et al*., 1964; Paulsen & Fulton, 1968; Fulton, 1984). Detection by ELISA was described by Fulton (1982). Scott & Zimmerman (2001) reported the full genomic sequence of a Northern American isolate of APLPV and its detection by molecular hybridization and RT-PCR. Additional information on APLPV detection was also reported by Alayasa *et al*. (2003) and Al Rwahnih *et al*. (2004). Recently, simultaneous detection by one-step RT-PCR of eight stone-fruit viruses, including APLPV, was set up by Sánchez-Navarro *et al*., 2005).

APLPV is a positive-sense RNA virus with a tripartite genome. It has four types of quasi-isometric particles, 26, 28, 31 and 33 nm in diameter. The virus is known to be transmitted only by propagating material. Several APLPV isolates of Mediterranean origin have been sequenced and comparative analysis revealed low genetic diversity among their coat and movement proteins, as well as with the proteins of American isolates (Myrta *et al*., 2002; Herranz *et al*., 2003).

Identity

Name: *American plum line pattern ilarvirus*
Synonyms: plum line pattern virus, peach line pattern virosis virus, Plum American line pattern virus, prunus virus 10
Acronym: APLPV

[1]The figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Taxonomic position: Viruses, *Bromoviridae, Ilarvirus*
EPPO code: APLPV0
Phytosanitary categorization: EPPO A1 list n°28, EU Annex: I/A1 – as Plum line pattern virus (American)

Detection

Stone-fruit trees infected by APLPV generally show striking symptoms which vary seasonally, so visual inspection has practical importance. However, similar symptoms on *Prunus* spp. may also be caused by other ilarviruses, e.g. *Apple mosaic ilarvirus* (ApMV) and *Prunus necrotic ringspot ilarvirus* (PNRSV). Moreover, some host cultivars do not show overt symptoms. So laboratory tests are required for unequivocal identification of the virus.

Disease symptoms

On Japanese plum, there is a regular sequence of pattern types, starting with chlorotic ring (Web Fig. 1a) and oak-leaf type pattern (Web Fig. 1b) and finally yellow vein banding. In the early summer, the yellow pattern fades to a creamy-white one (Web Fig. 1c). In other cases, leaf borders are first chlorotic and then turn golden (Web Fig. 2). The symptoms do not disappear during the hot season, but new leaves emerging during this period are symptomless.

On peach, in spring and early summer, there are fine irregular, pale-green, wavy bands on each side of the main veins of the leaves. These either form a symmetrical pattern or are broken and turned back to form figures of various shapes. Some leaves develop a network of fine lines, or a golden net pattern, fine confluent rings, vein banding, or an oak-leaf pattern. Symptoms usually disappear in summer.

On *P. serrulata*, whitish, yellowish or pinkish discoloured areas of various forms occur, sometimes large rings but more

often oak-leaf pattern. Leaf borders are faintly chlorotic to pronounced golden or white.

Identification

Sampling

In spring, leaves are a better virus source than flowers and cortical tissues, whereas in summer, mature fruits are better than leaves. Dormant buds represent a reliable tissue source for testing in winter. If typical symptoms are present in leaves, symptomatic leaves should be collected. If the tree is symptomless, leaves should be collected from different parts of the canopy. When sampling is done in spring, the location of leaves on one-year branches seems not to have any effect in virus detection. During the hot season, basal mature leaves are a slightly better source than those of central and apical positions. Leaf samples, as for the other stone fruit ilarviruses, can be stored at 4°C for not more than 7 days before processing.

Sample preparation

Sample preparation is intended for serological or molecular testing. Approximately 0.5 g of plant material is weighed, cut into small peaces and placed in a plastic bag or mortar for processing. Processing is done manually or with different equipment: for details, see EPPO Standard PM 7/32 (1).

Elisa
Approximately 20 volumes of extraction buffer are added and the sample is homogenized. The composition of extraction buffer used is in Appendix 1b. The extracted sample is ready to be loaded.

Molecular testing
Approximately 10 volumes of extraction buffer (Appendix 1d) are added and the sample is homogenized. 50 µL of 20% SDS is added to 1 mL of the homogenate and incubated at 65°C for 20 min. Then 0.25 mL of 5 M potassium acetate added and incubated at 0°C (ice bath) for 20 min. The samples are centrifuged at 12 000 rev min-1 for 15 min, the supernatant is allowed to precipitate with ethanol and resuspended in 40 µL of sterile H_2O. For dot-blot hybridization, 5 µL of each acid nucleic preparation is denatured by adding 3 µL of 20X SSC and 37% formaldehyde (10 min at 65°C). Finally, aliquots of the samples are dotted onto positively charged nylon membranes and fixed by UV cross-linking. Membranes can be kept for several years in a dry place, before being developed by molecular hybridization. For RT-PCR, 0.5 µL of total nucleic acids is used.

Testing on woody indicators

The virus can be detected by grafting onto suitable woody indicators. Glasshouse testing is recommended on GF305 at 20°C for about 3 months. Field testing on Shiro plum requires about 2 years (ISHS, 1998). GF305 displays generally faint chlorotic patterns (Web Fig. 3), whereas Shiro gives a reaction ranging from a thin reticulation to the classical line pattern. Several European plum cultivars (e.g. 'President', 'Regina Claudia verde', 'Blue Free') may also be good indicators (Web Fig. 4) for APLPV in glasshouse conditions (Alayasa *et al*., 2003). Testing on woody indicators is extremely slow and would only be appropriate if such testing was also being done for other viruses. In any case, final identification requires serological or molecular tests.

Testing on herbaceous indicators

APLPV can be sap-transmitted to numerous herbaceous species, but many of them remain latently infected. Herbaceous plants should be observed for 2–3 weeks in a temperature-controlled glasshouse at 20°C. The indicators to be used are *Nicotiana occidentalis*, *Chenopodium amaranticolor*, *Vigna unguiculata*. *N. occidentalis* develop chlorotic blotching and necrotic ringspots (Web Fig. 5); *C. amaranticolor*, chlorotic spots, leaf deformation and apical stunting (Web Fig. 6); and *Vigna unguiculata*, leaf deformation. This method alone is not reliable for virus detection, but it can be used as a complementary test or when similar testing is being done for several viruses. In any case, final identification requires serological or molecular tests.

Serological tests

APLPV is not serologically related to any of the other ilarviruses that affect stone-fruit trees. DAS-ELISA (Double Antibody Sandwich ELISA) is performed according to Clark & Adams (1977), using the protocol described in Appendix 2 and materials described in Appendix 1a. ELISA is a reliable and rapid method, requiring relatively simple equipment and only short training. However, a commercially available ELISA kit has some reaction to healthy plant tissue. To overcome this problem, see details in Appendix 1b.

Molecular tests

Dot blot hybridization
Dot blot hybridization is performed according to Pallás *et al*. (1998), using the detailed protocol described in Appendix 3 and materials described in Appendix 1c and 1d. The synthesis of the digoxigenin-labelled riboprobe is performed according to Más *et al*. (1993) and Pallás *et al*. (1998), using the detailed protocol described in Appendix 3. Three different riboprobes have been developed so far to detect APLPV (Scott & Zimmerman, 2001; Alayasa *et al*., 2003; Sánchez-Navarro *et al*., 2005). Only in one case (riboprobe pCP-APLPV; Sanchez-Navarro *et al*., 2005) has the sensitivity limit been analysed by using serial dilutions of infected tissue. None of the three riboprobes cross-hybridized with other ilarviruses (ApMV, *Prune dwarf ilarvirus*, PNRSV). Molecular hybridization is more reliable than ELISA, especially in testing during the nonoptimal

detection season (Al Rwahnih *et al*., 2004), and therefore it can be considered as a satisfactory alternative for routine diagnosis.

RT-PCR

RT-PCR is performed according to Sánchez-Navarro *et al*. (2005) or Scott & Zimmerman (2001) using the detailed protocol described in Appendix 3 and material described in Appendix 1c. The amplified product consists of 563 bp or 123 bp, respectively. Primers described by Sánchez-Navarro *et al*., 2005) amplify up to 8 different APLPV isolates from different geographical origin, validating their wide-range use. The detection limit of APLPV is slightly affected in the simultaneous RT-PCR for eight viruses of stone fruit trees, by comparison with the single test (Sánchez-Navarro *et al*., 2005).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information on this organism can be obtained from:

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Acknowledgements

This protocol was originally drafted by A. Myrta, Istituto Agronomico Mediterraneo, Valenzano (IT) and V. Pallás, Universidad Politecnica de Valencia (ES).

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Appendix 1 Materials

Materials for detection of APLPV in plant tissues by serological tests

Standard APLPV-infected and healthy controls are commercially available.

ELISA buffers

For ELISA buffers, see EPPO Standard PM 7/32 (1). If some unspecific reaction is obtained to healthy plant tissue using conventional extraction buffer, it should be extracted with the following buffer, at a ratio of 1 : 100 (weight:volume): bovine serum albumin (BSA) 2 g, polyvinylpyrrolidone (PVP) MW 24-40 000 20 g, sodium azide 0.2 g, PBS-Tween 1 × 1 L, pH 7.2–7.4.

Materials for detection of APLPV in plant tissues by molecular tests

APLPV probes are available for nonprofit institutions at Instituto de Biologia Molecular y Celular de Plantas, Universidad Politecnica de Valencia-CSIC, Avenida de los Naranjos s/n 46022 Valencia (SP).

One step RT-PCR

Oligonucleotide primer sequences (Sánchez-Navarro *et al*., 2005):

VP 340 (sense) 5′-3′ GGTCGTCAAGGGAGAGGC (nt1490-1508)

VP 339 (antisense) 5′-3′ GGCCCCTAAGGGTCATTTC (nt 2034-53)

Oligonucleotide primer sequences (Scott & Zimmerman, 2001):
(sense) 5′-3′ GATATTGCTGCCTCACAAGTGG (nt1663-1684)
(antisense) 5′-3′ CCTCGAGAAATTTCTCGAGATGG (nt1562-1584)

Molecular hybridization

pAPLPV-385 dig-riboprobe: (126-510 nt RNA 3) as described by Alayasa *et al*. (2003).

pCP-APLPV: (1490-2053 nt RNA 3) as described by Sánchez-Navarro *et al*., 2005).

Buffers for molecular tests

Extraction buffer: 100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 7.0, 500 mM NaCl, 10 mM β-mercaptoethanol.
Pre-hybridization solution: 50% formamide, SSC 5X, 0.1% N-Lauroylsarcosine, 0.02% SDS and blocking 10X.
Buffer 1: 0.1 M Maleic acid, 0.15 M NaCl, ajust pH 7.5 with NaOH.
Blocking buffer: Buffer 1, blocking agent 1 X (ROCHE 1 096 176).
Buffer 3: 1 M Tris-HCl pH 9.5, 0.1 M NaCl.

Appendix 2 Detailed protocols for serological tests

DAS-ELISA (Clark & Adams, 1977) to be performed as described in EPPO Standard PM 7/32 (1). Positive controls (infected plant material, preferably hosts of the same species as the test plants where available) and negative controls (healthy plant material and buffer) should be included, if possible. The ELISA value of the sample should be at least twice than that of the negative control.

Appendix 3 Detailed protocols for molecular tests

Synthesis of digoxygenin-labelled riboprobes

Linearize 1 µg of pAPLPV385 (Alayasa *et al*., 2003) *or* pCP-APLPV (Sánchez-Navarro *et al*., 2005) both containing a partial sequence of the RNA 3 with *Sac*I and *Nco*I, or make blunt end with T4 DNA polymerase, purify with phenol:chloroform, precipitate with ethanol, and resuspend in sterile water. Synthesize dig-riboprobe with T7 RNA polymerase (Alayasa *et al*., 2003) and SP6 RNA polymerase (Sánchez-Navarro *et al*., 2005), as described by Más *et al*. (1993).

Transcription reaction (20 µL): 1 µg of linearized plasmid, 2 µL of 10x transcription buffer, 40 U of T7/SP6 RNA polymerase (ROCHE 881 767), 20 U RNase inhibitor (Amersham pharmacia biotech E 2310Y), 2 µL 10X DIG RNA Labeling Mix (Roche 1 277 073). Incubate 2 h at 37°C.

Molecular hybridization

Incubate the membranes at 68°C at least 1 h in the prehybridization solution (Appendix 1). Prepare the hybridization solution using 50–100 ng of riboprobe previously denatured (10 min at 65°C) per mL of prehybridization solution and incubate the membrane at least 4–6 h (up to overnight). After hybridization, wash first 2 × 5 min in SSC 2X containing 0.1% SDS at room temperature and then 2 × 15 min in SSC 0.1X containing 0.1% SDS at 68°C. The rest of the steps are carried out at room temperature. Wash 2 × 5 min with Buffer 1 containing 0.3% Tween 20 (Sigma P-1379). Block the membrane with the blocking buffer for 30 min and incubate with Anti-Digoxigenin-AP Fab fragments (Roche 1 093 274) diluted 1 : 10 000 in blocking buffer. Wash membrane 2 × 15 min in Buffer 1 containing 0.3% Tween 20 and then with Buffer 3 during 5 min. Place the membranes in a plastic bag with a chemiluminiscent substrate (CSPD diluted 1 : 100 in Buffer 3, Roche 1 655 884) during 5 min in darkness. Eliminate CSPD excess without totally drying the membrane. Expose the films for 10–60 min.

Amplification by RT-PCR

APLPV detection VP 340-VP 339 primers described by Sánchez-Navarro *et al*. (2005):
VP 340 (sense) 5′-3′ GGTCGTCAAGGGAGAGGC
VP 339 (antisense) 5′-3′ GGCCCCTAAGGGTCATTTC
One-step RT-PCR reaction: 0.4 µL SuperScript III one-step RT-PCR (Platinum Taq DNA polymerase kit, Invitrogen), 5 µL 2X SuperScript III buffer, 0.1 µL primer VP 340 (0.75 pmol), 0.1 µL primer VP 339 (0.75 pmol), 0.5 µL total nucleic acids, 3.9 µL of H_2O.

Conditions for one-step RT-PCR: 50°C for 30 min; 2 min at 94°C; 40 cycles of denaturation at 94°C for 15 s, 50 °C for 30 s, 68°C for 1 min; finally 68°C for 7 min.

APLPV detection by primers described by Scott and Zimmerman (2001):
(sense) 5′-3′ GATATTGCTGCCTCACAAGTGG
(antisense) 5′-3′ CCTCGAGAAATTTCTCGAGATGG
Conditions for RT-PCR: 1 cycle of 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, 60°C for 30 s, 72°C for 2 min

Electrophoresis of PCR products

To be performed as in EPPO Standard PM 7/32 (1).

Web Fig. 1. Symptoms caused by APLPV on Japanese plum: a) chlorotic rings in early spring, b) yellow oak-leaf pattern in summer and c) creamy-white line pattern in late summer.

a) b) c)

Web Fig. 2. Chlorotic to golden leaf borders of Japanese plum caused by APLPV.

Web Fig. 3. Chlorotic pattern caused by APLPV on GF305 in greenhouse.

Web Fig. 4. Chlorotic rings and vein clearing caused by APLPV on European plum cv. President in greenhouse.

Web Fig. 5. Chlorotic-necrotic blotching and ringspots on *N. occidentalis.*

Web Fig. 6. Chlorotic mottling, leaf deformation and apical stunting of *C. amaranticolor*.

All pictures were kindly provided by: A. Myrta, IAM Bari (IT).