Diagnostics
Diagnostic

Xylophilus ampelinus

Specific scope

This standard describes a diagnostic protocol for *Xylophilus ampelinus*[1].

Specific approval and amendment

Approved in 2009–09

Introduction

Xylophilus ampelinus is the plant pathogenic bacterium causing 'bacterial blight' of grapevine. The disease was originally described in Greece (Crete) and was named *Xanthomonas ampelina* (Panagopoulos, 1969). It was transferred to the new genus *Xylophilus* (Willems *et al.*, 1987) on the basis of DNA and RNA studies. The bacterium infects only grapevine (*Vitis vinifera* and *Vitis* spp. used as rootstock). It is a systemic pathogen infecting the xylem tissues. It over-winters in plant tissue. Cuttings used either as rooting or grafting material represent the primary source of inoculum and the main pathway of long distance dissemination. In a heavily infected vineyard, up to 50% of the canes can be latently infected, representing a major risk of long distance dissemination of the pathogen should they be used as propagating material. Short distance dissemination occurs through contaminated tools and machinery, and by direct contamination from plant to plant. The disease appears sporadically and its expression is closely related to climatic and cultural conditions. Symptoms can disappear for several years and return years later under more favourable conditions and may be easily confused with other disorders (see section disease symptoms). The disease has been reported in Greece, France, Italy, Moldova, Slovenia, Spain and South Africa, under the following names: 'tsilik marasi' in Greece, 'maladie d'Oléron' in France, 'mal nero' in Italy, 'necrosis bacteriana' in Spain, and 'vlamsiekte' in South Africa. Further information on its host range, geographic distribution and biology can be found in the EPPO data sheet on *Xylophilus ampelinus* (EPPO/CABI, 1997 and http://www.eppo.org). A flow diagram describing the diagnostic procedure for *Xylophilus ampelinus* is presented in Fig. 1.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Identity

Name: *Xylophilus ampelinus* (Panagopoulos) Willems *et al.*, 1987.
Synonyms: *Xanthomonas ampelina* Panagopoulos, 1969.
Taxonomic position: Bacteria, Eubacteria, Proteobacteria, *Betaproteobacteria, Burkholderiales.*
EPPO code: XANTAM
Phytosanitary categorization: EPPO A2 list no 133, EU Annex II/A2.

Detection

The bacterial distribution in the plant is irregular, varying both during the year and between years. This means that detection of the bacterium in healthy looking plants is uncertain. Formal confirmation of preliminary positive results from presumptive tests can be difficult.

Symptoms

In the field, symptoms can appear on all aerial parts of the plant. Buds in infected shoots either fail to sprout or give stunted growth in the spring. Cracks appear along infected shoots, because of the force exerted by the hyperplasia of the cambial tissues, resulting in canker formation. These cracks mainly appear in the lowest parts of the shoots. Infection spreads along the branches which show a brown discoloration of tissues and may eventually die. Young shoots on infected spurs, develop pale yellowish-green areas on the lower internodes. Spurs easily crack at the canker sites. These expand upwards to become darker, crack and develop into cankers. When these cankers split, the xylem tissues are revealed. Later in summer, cankers are often seen on one side of petioles causing a characteristic one-sided necrosis of the leaf.

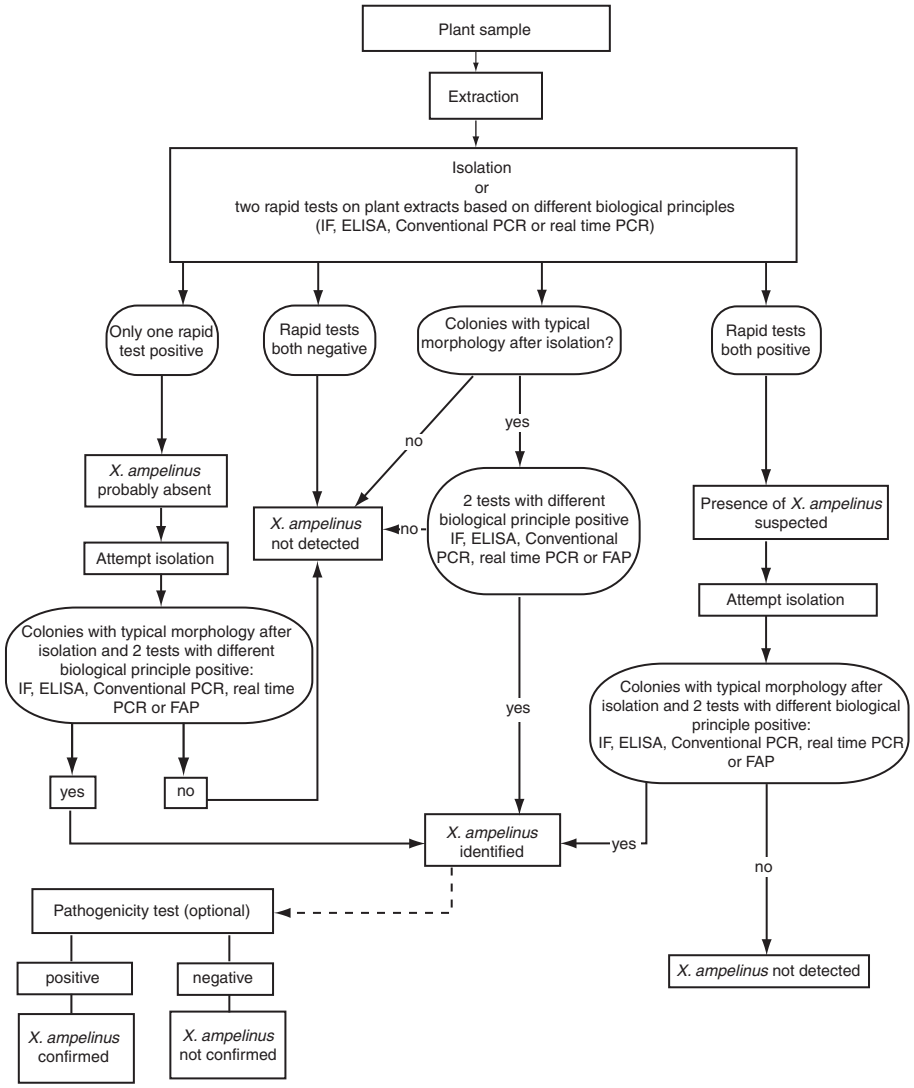

Fig. 1 Flow diagram for the detection and identification of *X. ampelinus*.

They may also appear on main and secondary flower and fruit stalks. Depending on the age of the infected shoots, the bacterium may survive and develop into the cane. Such canes could either show cankers (usually on the lower inter-nodes), or have no visible symptoms, being latently infected. Almost all plant parts with disease symptoms exhibit a brown discoloration of the xylem tissues in longitudinal sections. Late and irregular lignification is observed in the canes of the affected plants of some cultivars. The general aspect of affected grapevines may change, affected plants being less erect than healthy ones.

On leaves, necrotic spots surrounded by a discoloured halo can be observed when contamination occurs via drops of contaminated sap falling down onto the young leaf or from other external contamination. Eventually the central dried part of the spot drops out and a 'shot hole' symptom appears. However, when contamination reaches the leaf via the petiole, necrotic sectors surrounded by a halo occur.

The symptoms described (Fig. 2 and http://www.eppo.org), especially on new vegetation and leaves, are typical of the disease, but not specific. Bacterial blight can affect both the cultivar and rootstock.

Confusion may occur with other diseases or disorders. Cankers on shoots and leaf spots similar to those caused by *X. ampelinus* could be induced in cases of heavy infections by the fungi *Sphaceloma ampelinum* (without brown discoloration of the xylem vessels) and *Phomopsis viticola*. Failure of spurs to sprout and dead branches could also be caused by the wood fungi *Togninia minima* (anamorph: *Phaeoacremonium aleophilum*), *Phaeomoniella clamydospora, Fomitiporia mediterranea, Eutypa lata, Botryosphaeria* spp. or *Verticillium* spp. In these cases no cankers are evident or they are different but the brown discoloration of

Fig. 2 Symptoms of *X. ampelinus* on shoots.

the xylem tissues is present. Canker-like symptoms could also be caused by hailstorms.

Extraction

Extraction procedures for different plant material are presented in Appendix 1.

Extraction buffers may be different according to the test to be conducted subsequently. Where different tests are to be used on a single extract, extraction should be done with sterile laboratory grade water and aliquots should be supplemented with appropriate concentrated buffers (to a 1× final concentration in plant sample, avoiding a too high dilution) according to the test to be done.

Screening tests

Direct isolation, immunofluorescence, ELISA, PCR and real-time PCR can be used as screening tests and are described hereafter and in Appendices 2–4. Cross reactions leading to false positive responses could occur with saprophytes when performing these tests.

Isolation on non-selective media

Isolation from symptomatic plant material

Optimal period for sampling may vary between regions. A loopfull from the crude and diluted (1/10, 1/100) extract is plated under sterile conditions onto the surface of at least two (per dilution) freshly prepared and appropriately labelled Petri dishes of YPGA and/or of NA media (see Appendix 5). The plates should be incubated at 25°C. As *X. ampelinus* is a slow growing bacterium which can easily be inhibited by saprophytes, plates should be checked daily from the third day so that *X. ampelinus* like colonies can be subcultured before possible inhibition. *Xylophilus ampelinus* colonies may reach 2 mm in diameter after 7–12 days at 25°C on YPGA, pale yellow in colour, circular, entire, shiny, slightly convex and slightly translucent. Colonies are usually much smaller on NA and other media. A brown pigment can diffuse into YPGA. The production of this pigment is very typical of *Xylophilus* strains, but not all strains produce it, and production may vary according to the media and incubation conditions. Colonies looking like the positive control should be subcultured and purified onto new YPGA or NA media and incubated under similar conditions. Purified isolates are then ready for further tests. Note that if high numbers of fast growing saprophytic bacteria are present, the probability of successful isolation of the target bacterium can be dramatically reduced because of overgrowth or inhibitory effects.

Isolation from asymptomatic plant material

Direct isolation is only reliable and advisable from symptomatic plant material due to the poor growing capabilities of *X. ampelinus* on artificial media, the low level of target populations expected and possible competition with other bacteria present in samples. Isolation from other material can nevertheless be attempted using the procedure described above, plating diluted and undiluted extract on more plates to maximise the possibilities of isolating the target organism. However, unsuccessful isolation of the bacterium would not necessarily mean that *X. ampelinus* is absent.

Identification

Xylophilus ampelinus is the only species belonging to the genus *Xylophilus*. *Xylophilus ampelinus* is a slow growing bacterium on artificial media. Colonies are rarely visible before 5 days of incubation and a maximum of 2 mm in diameter after incubation for 7–12 days at 25°C. When isolated, confusions are unlikely to happen with colonies of other bacteria (see section Isolation from symptomatic plant material).

Biochemical tests

Tests are performed according to Lelliott & Stead (1987). Biochemical characteristics of *X. ampelinus* strains are given in Table 1.

Serological tests

Immunofluorescence

For identification, the test should be performed on a pure culture (approximately 10^6 cells per mL, no other dilution is needed) in phosphate buffer as described in PM 7/97 (*Bulletin*

Table 1 Biochemical characteristics for *X. ampelinus* (Dreo *et al.*, 2005)

Characteristics	Results
Gram's reaction	–
Catalase test	+
Kovac's oxidase	–
Urease production	+
H_2S production from cysteine	–
Utilisation as carbon sources	
Citrate	+
Fumarate	+
Malate	+
DL-tartrate	+
Acid production from	
L-arabinose	+
D-galactose	+
Glucose	–
Sucrose	–
Lactose	–
Utilization of asparagine as the sole source of carbon and nitrogen	+

–, negative result; +, positive result.

OEPP/EPPO Bulletin **39**, 413–416). The test is positive for a suspect culture if the size and form of the stained cells of the culture is equivalent to that of the positive control strain.

ELISA

For identification, the test should be performed as described in Appendix 2 using a pure culture (approximately 10^6 cells per mL) in specific ELISA extraction buffer. The test result is positive for an identification of a suspect culture if its OD is in the range of those obtained with positive controls.

Molecular tests

PCR and real-time PCR

One of the proposed primer sets (Manceau *et al.*, 2005) as described in Appendix 3, can be used for identification of pure cultures (approximately 10^6 cells per mL) in distilled sterile water. No DNA extraction is required when pure cultures are used. The test is positive for a suspect culture if the DNA fragment amplified is similar to the one obtained with the positive control.

A real-time PCR-based method (Dreo *et al.*, 2007), as described in Appendix 4, can be used for identification of pure cultures.

Fatty acid profiling

This is based on use of the MIDI system (Stead, 1991; Janse, 1991; Stead, 1992; Stead *et al.*, 1992, Dickstein *et al*., 2001). See also http://www.midi-inc.com/pdf/MIS_Technote_101.pdf for full details. Fatty acid profiling can be used for identification of pure cultures (Appendix 6).

The fatty acid profiling of *X. ampelinus* is fairly simple. Major acids are 8:0 3 OH (2%), 14:0 (3%), 16:0 (24%), 16:1 w7c (41%) and 18:1 w7c (28%) (Dreo *et al.*, 2005). The most taxonomically important of these is 8:0 3 OH, an uncommon hydroxy fatty acid in bacteria. Among plant pathogenic bacteria it is currently known only from the genus *Xylophilus* in which it appears to be the sole hydroxy fatty acid (Stead, 1992; Dickstein *et al.*, 2001).

Pathogenicity test

This test, only available for bacterial cultures isolated from fresh culture, is used as a confirmation of the diagnosis of *X. ampelinus* (when necessary). Two methods are described. Other methods are available but not presented here, and are intended to evaluate the virulence of strains (Ridé *et al.*, 1983; López *et al.*,1985). Inoculation by spraying is not recommended, except when facilities are adapted to the aerial containment of quarantine organisms.

Methods for inoculations are presented in Appendix 7.

Reference strains

NCPPB 2217 ^{T} (= CFBP 1192 = CFBP 3674 = LMG 5949 = ATCC 33914).

Reporting and Documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (EPPO, 2006).

Further information

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Appendix 1 – Extraction procedures

1. Extraction from symptomatic plant material (shoots, leaves)

When a sample consists of more than one leaf or shoot, the laboratory should select the most appropriate material to be tested. Each leaf or shoot should be processed individually.

1.1. Extraction from shoots

To try to overcome the problems resulting from the heterogeneity of bacterial distribution in tissues, the following method which gives very good results in obtaining pure cultures on agar plates is recommended. It is used for shoots of approximately 5 cm long.

Shoots are washed with tap water and the bark is quickly surface disinfected with 70% ethanol. Ends of shoots are removed with a disinfected tool. The necrotic tissues are carefully removed with a disinfected scalpel. The shoot is quickly dipped into 70% ethanol and flamed immediately (disinfection by flaming is needed only for isolation). The whole shoot is put into a sterile plastic bag and crushed with a hammer. Approximately 6 mL of sterile demineralised water are added per gram of tissue, and then left 40–60 min under gentle agitation at room temperature or for 16 h at 5°C for diffusion of the bacteria [if a PCR is performed subsequently it is recommended to add a polyvinylpolypyrrolidone (PVPP) suspension (0.1 g PVPP per mL of sterile demineralised water)]. 200 µL of PVPP suspension are added to 1800 µL of soaking liquid. Then the sample is incubated about 30 min at room temperature under gentle agitation. The soaking liquid is filtered through a cellulose paper (Whatman no 3) or centrifuged at 100 *g* for 5 min, the pellet being discarded.

Alternative method

Remove bark and necrotic tissues as above. Pieces containing mainly xylem vessels at the limit of necrotic tissues or cankers are taken with a sterile scalpel blade. Small sections of affected vascular tissue (0.5–1 cm long) are transferred into a Petri dish with 3–5 mL of sterile distilled water or sterile phosphate buffered saline (0.01 M, see Appendix 5), then comminuted and left for 10–15 min for diffusion of bacteria.

1.2. Extraction from leaves

If leaves are dirty, they are washed carefully in tap water, quickly surface disinfected using 70% ethanol and immediately rinsed in sterile distilled water. Pieces of mid-rib and petiole tissues taken at the margin of the spots or necrosis are removed with a disinfected scalpel blade and transferred to a small volume of sterile distilled water or sterile phosphate-buffered saline (0.01 M). Pieces are cut aseptically into smaller pieces, and then left 10–15 min for diffusion of bacteria.

Extracts from leaves and shoots should be analyzed immediately and the remaining extracts should be kept refrigerated in sterile adequately labelled single use tubes for further use if necessary. For medium and long-term storage (more than 24 h),

sterile double distilled glycerol is added to the remaining extract (20–30% v/v) and it is kept at a temperature below −18°C.

2. Extraction from asymptomatic shoots or canes

2.1 General remarks

As very low populations of bacteria occur in asymptomatic plant material and available tests have a relatively low sensitivity, each test sample should not constitute more than 3 shoots. Biological enrichment of the bacteria, according to Serfontein *et al.* (1997) may be performed prior to extraction but the presence of fungi may cause difficulties.

2.2. Extraction from shoots or lignified canes

Samples are processed as described in the section 1.1 taking approximately 5 cm long surface disinfected shoots, at random.

Alternative method

For cane analysis, the extract is flushed out from vessels by vacuum suction. Ends of canes (1–2 cm removed) are cut to create fresh wounds, basal ends of canes are placed in 10–15 mL sterile phosphate buffer and a vacuum is applied at the distal end until approximately 2 mL is extracted and collected. Alternatively, suction using a 50 mL syringe can be used to collect extract from the canes in 10–15 mL buffer. This extract is used directly for further tests.

The extract is used immediately and if necessary the remaining extract is kept refrigerated in sterile adequately labelled disposable tubes for further use. For medium and long term storage (more than 24 h), double distilled glycerol (20–30% glycerol v/v) is added and then kept at a temperature below −18°C.

2.3. Extraction from sap bleeding

Sap bleeding needs no further processing before analysis. Extract is used immediately or if needed the samples should be kept refrigerated for up to 24 h after collection, or sterile double distilled glycerol to 20–30% (v/v) is added for longer storage at a temperature below −18°C.

Appendix 2 – ELISA

For extraction of the bacteria from plant samples or colonies, use a buffer recommended by the kit supplier.

A kit for DAS-ELISA using specific monoclonal antibodies (mAb) and a polyclonal antiserum is commercially available from different suppliers. Perform the test as described by the supplier or in the absence of instruction of the suppliers follow the description below:

Prepare a 1/100 dilution of rabbit polyclonal immunoglobulins in carbonate buffer pH = 9.6 (see Appendix 5) for coating the plates.

Add 200 µL to each well, incubate at 37°C for 4 h (±15 min) and wash three times 5 min (±1 min) with PBS 0.05% Tween 20 (see Appendix 5).

Add 200 µL per well of the plant extract or internal washing (including healthy vine material as a plant negative control) or bacterial suspension.

Incubate for 16 h (±1 h) at 5°C or for 4 h (±15 min) at 37°C.

Wash as above.

Prepare 1:1000 dilution of the mAb and add 200 µL to each well.

Incubate for 2 h (±15 min) at 37°C.

Wash as above and prepare a 1:1000 dilution of anti-mouse immunoglobulins conjugated with alkaline phosphatase in conjugate buffer (see Appendix 5).

Add 200 µL to each well.

Incubate at 37°C for 2 h (±15 min).

Wash as above and prepare a 1 mg mL^{-1} alkaline phosphatase substrate (para nitrophenyl-phosphate) in substrate buffer (see Appendix 5).

Add 200 µL to each well, incubate at room temperature and read at 405 nm preferably when positive controls or positive samples give an OD value between 1.2 and 1.6 (see supplier recommendation).

Note that the test can also be done with volumes of only 100 µL at each step, but with a lower detection sensitivity.

Samples are positive when their OD is at least twice that obtained with the plant negative control sample.

Appendix 3 – Conventional PCR

1. General information

1.1. Protocol adapted from Manceau *et al.* (2005) based on a conventional PCR with DNA extraction and optional colorimetric detection of PCR products.
1.2. Amplicon location : ITS sequences within *rrn* operon
1.3. Amplicon size: 129 bp
1.4. Oligonucleotides:
Xa TS1 5′-TGC GTA GTT CAA CAC CAA AGT-3′
Xa TS2 5′-TAT GAC CCT CTT TCC ACC AGC-3′ or
Xa TS2 BIO 5′Biotine-TAT GAC CCT CTT TCC ACC AGC-3′
1.5. Taq DNA polymerase RedGoldStar® DNA polymerase, 5 U μL^{-1}, Eurogentec (Ougree, BE)
1.7. Amplification buffer 10× Eurogentec (provided with RedGoldStar® DNA polymerase)
1.8. $MgCl_2$ solution, 25 mM (provided by Eurogentec with RedGoldStar® DNA polymerase)
1.9. dNTPs, 20 mM
1.10. PCR-grade water.

2. Methods

2.1. Nucleic acid extraction and purification
2.1.1. The first steps of the extraction protocol are described in Appendix 1.

Bacterial suspensions are used directly for PCR, no treatment is required for DNA purification.

2.1.2. Nucleic acid cleanup procedure

1 mL of filtrate or supernatant is centrifuged at 13 000 *g* for 10 min at 4°C. The supernatant is discarded.

Option 1: the pellet is resuspended with 120 µL of lysis buffer (Edwards *et al.*, 1991) (see Appendix 5). The tubes are incubated at room temperature for 10 min. Then the mix is centrifuged for 10 min at 13 000 *g* at 4°C. A 100 µL aliquot of supernatant is added to 300 µL of 6 M sodium iodide solution (SALT) (see Appendix 5) and 5 µL of silica suspension (BIND).

Option 2: the pellet is resuspended with 300 µL of lysis buffer (see Appendix 5). The tubes are incubated at room temperature for 10 min. 900 µL of SALT solution (see Appendix 5) and 10 µL BIND solution (see Appendix 5) are added.

Then the tubes are inverted from time to time during 5 min of incubation. The silica is pelleted by centrifugation at 13 000 *g* for 20 s. The supernatant is discarded. The pellet is centrifuged again at 13 000 *g* for 20 s and the droplets of SALT buffer are discarded. Then the pellet is suspended in 1 mL of WASH solution (see Appendix 5). The silica is pelleted by centrifugation at 13 000 *g* for 20 s and the supernatant is discarded. The pellet is centrifuged again at 13 000 *g* for 20 s and the droplets of WASH solution are discarded.

The silica pellet is suspended in 50 µL of PCR grade water and then incubated up to 5 min at room temperature. Finally, the tubes are centrifuged at 13 000 *g* for 2 min. The supernatant is removed immediately and transferred to a new tube (SALT, BIND and WASH solution are equally provided in UltraClean™ 15 DNA Purification Kit by MO BIO Laboratories, Inc, Carlsbad, CA, US).

Direct PCR from ligneous plants can lead to difficulties due to inhibitors, and the use of GeneReleaser™ (BioVentures, Murfeesboro, TN, US) according to supplier's recommendations improves the sensitivity. In this case, before amplification, 5 µL of GeneReleaser™ are added into a PCR tube, and then 2.5 µL of sample are added without mixing. The following programme is run with the thermal cycler : 65°C for 30 s, 8°C for 30 s, 65°C for 90 s, 97°C for 180 s, 8°C for 60 s.

PCR mix is added into the tube without mixing.

2.2. Polymerase chain reaction

2.2.1. Total reaction volume of a single PCR: 25 µL per tube

2.2.2. 12.15 µL PCR grade water

2.2.3. 2.5 µL 10× buffer

2.2.5. 5 µL $MgCl_2$ (5 mM)

2.2.6. 1.125 µL dNTPs (0.90 mM)

2.2.7. 0.125 µL Taq DNA polymerase RedGoldStar® (0.625 U)

2.2.8. 0.80 µL Xa TS1 (0.64 µM)

2.2.9. 0.80 µL XaTS2 or XaTS2 BIO (0.64 µM)

2.2.10. 2.5 µL DNA solution

2.2.11. PCR cycling parameters

PCR is carried out an initial denaturation at 94°C for 2 min followed by 40 cycles (94°C for 60 s, 60°C for 45 s, 72°C for 60 s) and one final step at 72°C for 2 min before cooling at 4°C.

2.2.12. Amplification products are either detected after migration on agarose gel and ethidium bromide straining or by a colorimetric ELISA procedure. It can make interpretation easier and the sensitivity of the test is ten fold higher.

10 µL of internal probe Xa TS-Dig (2 µM) (5′digoxygenin-AAT CGG CTG TTC TTT A-3′) are added to PCR products (not critical as colorimetric detection is a post-amplification procedure, even though 10-fold lower detection limit of PCR products) and denaturated in thermal cycler at 95°C for 3 min to anneal the probe. The mixture is cooled at room temperature for 20 min. 50 µL of amplification product (in that case, the total reaction volume for PCR and GeneReleaser™ volume need to be doubled to obtain 50 µL in regard to the point 2.2.1 above) and probe are added to pre-coated plates with streptavidine (Roche) containing in each well 200 µL buffer 1 (see Appendix 5) supplemented with 0.1% BSA. The plates are incubated for 30 min at room temperature. The liquid contents of the plate are discarded with a multichannel pipette and then washed three times with washing buffer (see Appendix 5). 240 µL of anti-digoxygenin antibodies are added (Roche; antibodies solution diluted to 1/5000 in buffer 1) conjugated to alkaline phosphatase (150 U mL^{-1}) with a multichannel pipette. The plate is incubated for 30 min at room temperature, the contents of the plate is discarded and then washed as indicated above. 1 mg mL^{-1} alkaline phosphatase substrate is prepared and 200 µL are added to each well. The plate is incubated at least 37°C for 20 min. OD is read at 405 nm. Samples are positive when their OD exceeds the average of the values for the negative controls with twice the standard deviation value added.

2.2.13. It is highly recommended to carry out a tenfold dilution of the purified DNA solution before amplification in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

3. Procedural Information

3.1. Controls: every time a PCR-based test is performed, the following controls are considered essential:

- A **contamination ('negative') control** containing no target nucleic acid (NA)
- A **test ('positive') control** containing NA that will be amplified by the target assay. This can include genomic NA extracted from the target organism; total NA extracted from a host that contains target organism genomic NA or a synthetic control (e.g. a cloned PCR product)

Recommended

- An **extraction inhibition control** used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify

a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used.

- An **extraction contamination control** for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. uninfected plant material or clean extraction buffer) (essential).

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed

- If amplicons of 129 bp are produced, the test will be considered positive provided that all contamination controls are negative.
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 4 – Real-time PCR

1. General Information

1.1. The test (Dreo *et al.*, 2007) has been tested with pure bacterial cultures of *Xylophilus ampelinus* (22 strains from France, a strain from Slovenia, a reference strain from Greece, a strain from Spain, 3 strains from South Africa), spiked plant material and a limited number of field samples (positive and negative). A signal was obtained with all tested strains of *Xylophilus ampelinus.* No signal was obtained with three other grapevine pathogens (*X. fastidiosa, Agrobacterium vitis*, *Candidatus Phytoplasma solani*). No signal was obtained with *Candidatus* Phytoplasma ulmi or plant pathogenic bacteria infecting other hosts. Also, no cross-reactivity was observed with grapevine tissues of different varieties or with their microflora. Testing included DNA extracted from grapevines of different varieties without symptoms and bacteria isolated from such samples.

1.2. The nucleic acid source is pure cultures or plant material.

1.3. The targeted gene is Xamp 1.27A, 16S rDNA (Manceau *et al.*, 2000)

1.4. Amplicon size in base pairs (including primer sequences): 91 bp

1.5. Oligonucleotides:
Xamp 14F (5′-CCCGATGATAAATACCGAAAAC-TC-3′)
Xamp 104R (5′-TGTCTTCTGGTTGTTTTGGTTTT-AAT-3′) and probe with minor groove binder Xamp 14F/104 MGB (5′-FAM-AGCGCCTGACGCAT-MGB)
Amplification of COX (Weller *et al.*, 2000) can be used as DNA extraction control.

1.6. Enzyme: 2× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, US)

1.7. All buffer components in 2× TaqMan Universal Master Mix (Applied Biosystems)

1.8. Source/quality of water: Milli QUF system or molecular biology grade water

1.9. Real-time PCR system i.e. ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using the universal cycling conditions for all amplicons (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C).

2. Methods

2.1. Nucleic Acid Extraction and Purification
DNA is extracted using the DNeasy plant mini kit (Qiagen, Les Ullis, FR) according to the manufacturer's manual, with the following modifications: (i) DNA is extracted from 250 µL of plant extract (ii) no RNase treatment is used and (iii) DNA is eluted into 2 × 50 µL of TE buffer. A modified Chelex procedure (Botha *et al.*, 2001) was tested as an alternative DNA extraction method for leaves and shoots, omitting the first flushing step (Dreo *et al.*, 2007). Extracts are prepared by cutting selected tissue, leaf lesions, stem cankers or cane cankers, (approximately 0.5 g fresh weight) and placing it in sterile 10 M PBS (Appendix 5), vortexing and incubating at room temperature for several minutes. The supernatant was subsequently separated from plant tissue by pipetting and mixed with 10% v/v of glycerol and stored at −80°C until required for further testing.

Storage temperature and conditions of DNA/RNA: <−15°C

2.2. Polymerase chain reaction – PCR

2.2.1. Total reaction volume of a single PCR reaction in 10 µL

2.2.2. 3 µL of PCR grade water

2.2.3. 5 µL of PCR buffer

No additives or special enzymes should be added. Components $MgCl_2$, dNTPs and polymerase as in 2× TaqMan Universal Master Mix (Applied Biosystems).

2.2.4. 0.9 µM forward primer

2.2.5. 0.9 µM reverse primer

2.2.6. 0.25 µM of probe

2.2.7. 2 µL DNA

2.2.8. Cycling conditions 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C

It is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

3. Essential Procedural Information

3.1. Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A **contamination ('negative') control** containing no target nucleic acid (NA)
- An **assay ('positive') control** containing NA that will be amplified by the target assay. This can include genomic NA extracted from the target organism, total NA extracted from a host that contains target organism genomic NA or a synthetic control (e.g. a cloned PCR product)

Recommended:

- An **extraction contamination control** for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. uninfected plant material or clean extraction buffer)
- An **extraction inhibition control** used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used.

3.2. Interpretation of results: in order to assign results acquired from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces a Ct below 40 (undiluted samples) and provided that the contamination controls are negative
- A sample will be considered negative, if it produces no signal below Ct 40 provided that the assay and extraction inhibition controls are positive

Appendix 5 – Preparation of media and buffers

Buffer 1 (for colorimetric detection)

100 mM maleic acid
150 mM NaCl
pH adjusted to 7.5 with NaOH

Carbonate buffer pH = 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	add 1.0 L

pH adjusted to 9.6 with HCl. Sterilised by autoclaving.

Conjugate buffer

Bovine serum albumin	0.02 g
PBS-T	10 mL

Adjust to pH 7.4
The buffer will be not autoclaved.
Prepare just before use.

Lysis buffer (Edwards buffer)

Tris–HCl	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5%
PVP360	2%

Sterilized by autoclaving.

Phosphate-buffered saline (0.01 M) (PBS 0.01 M)

NaCl	8.0 g
Na_2HPO_4, 12 H_2O	2.7 g
NaH_2PO_4, 2 H_2O	0.4 g
Distilled water	add 1.0 L

pH adjusted to 7.2 if necessary. Sterilized by autoclaving.

Phosphate-buffered saline – Tween (PBS-T)

Tween 20	0.5 mL
PBS (0.01 M)	1.0 L

pH adjusted to 7.2 if necessary. Sterilized by autoclaving.

Silica suspension (BIND)

15 g of silica (Sigma, S5631) is suspended in 100 mL of ultra pure water in a 100 mL graduated cylinder. The solution is well mixed then left for sedimentation to occur for 24 h at room temperature. 85 mL of supernatant are aspirated and 100 mL of ultra pure water are added. The silica is resuspended then left for sedimentation to occur for 5 h. 90 mL of supernatant are aspirated. pH is adjusted to 2.0 with HCl. The suspension is aliquoted in small volumes and sterilized by autoclaving.

The suspension is stored at 5°C.

6 M sodium iodide (NaI) solution (SALT)

0.75 g Na_2SO_3 (Sigma, S4672) are dissolved in 40 mL distilled water.

45 g NaI (Sigma, 383112) are added and stirred until dissolved.

The solution is filtered through paper and stored in the dark at 5°C.

If a precipitate is observed, the solution is discarded.

Substrate buffer (for alkaline phosphatase)

Diethanolamine	97.0 mL
Distilled water	to make up to 1.0 L

pH is adjusted to 9.8 with concentrate HCl solution (HCl solution volume is deduced from distilled water volume)
Sterilized by autoclaving.
Just before use add para nitrophenyl phosphate (pNPP) to reach a final concentration of 1 mg mL^{-1}.

Washing buffer (for nested PCR protocol)

Tris–HCl	100 mM
NaCl	20 mM

pH is adjusted to 8.8.

Washing buffer (for colorimetricdetection)

Buffer 1	
Tween 20	0.3%

Wash solution (WASH)

Tris–HCl pH 7.4	20 mM
NaCl	100 mM
EDTA	1 mM

An equal volume of absolute ethanol is added.
The solution is stored at 5°C (±4).

Yeast peptone glucose agar (YPGA)

Yeast extract	5.0 g
Oxoid proteose peptone	5.0 g
D(+) glucose	10.0 g
Oxoid agar N°3	12.0 g
Demineralised water	1.0 L

pH is adjusted to 6.5–7.0. Sterilized by autoclaving.

Appendix 6 – Fatty acid profiling

Grow the suspect isolates and the reference culture of *X. ampelinus* for 48 h at 28°C on Trypticase Soy Agar (TSA). Remove approximately 50 mg of culture to the base of a screw capped test tube. Add 1 mL of sodium hydroxide methanol solution to each tube (NaOH 45 g, methanol 150 mL, distilled water 150 mL). Cap tightly and mix before placing in a boiling water bath for 5 min. Mix again and replace for a further 25 min. Methylate the fatty acids by adding 2 mL HCl–methanol solution (6.0 M HCl 325 mL, methanol 275 mL). Mix and place in a water bath at 80°C for 10 min. Cool immediately. Extract the FAMEs by adding 1.25 mL methyl-tert butyl ether–hexane (1:1 v/v). Rotate tubes for 10 min. Remove lower aqueous layer with a Pasteur pipette. Add 3 mL base wash (NaOH 10.8 g, 900 mL distilled water) and rotate before pipetting off top two thirds of the organic layer into a GC vial. Seal with a Teflon liner and carry out gas chromatography e.g. as for the MIDI system.

Appendix 7 – Pathogenicity tests

In vivo inoculations can be made according to Panagopoulos (1969) on young green shoots from potted plants (for example, use the susceptible variety Sultana) kept in adapted quarantine growing facilities. Five to ten test plants should be inoculated per suspected bacterial strain. Immediately before inoculation prepare a suspension of suspected *X. ampelinus* isolated from a fresh culture on YPGA medium approximately 10^8 cells per mL in sterile distilled water. Put one or two drops of this bacterial suspension onto a 2–4 cm long longitudinal scalpel wound cut deep enough to reach xylem vessels or on a fresh leaf scar. Immediately cover the wound with moist cotton wool and wrap with aluminium foil. Do the same with a known pathogenic strain of *X. ampelinus* and with sterile distilled water on 2 sets of at least 2 plants. Keep the inoculated plants in appropriate conditions (target daytime temperature 27°C, and 20°C overnight, 14–18 h of light per day, mild air moisture and enough watering, fertilization and light so that vine can grow normally). Symptoms should appear after 3–4 weeks in the inoculated plants and in the positive control but not in those inoculated with sterile distilled water. When typical symptoms appear from a test plant inoculated with suspected *X. ampelinus* strains, re-isolation of the bacterium from symptomatic plants is not necessary except if formal evidence of the contamination of the sample is requested.

Because *in vivo* inoculation are not always successful, an alternative option is to inoculate *in vitro* plants. Rooted plantlets of a susceptible grapevine cultivar (Syrah: low susceptibility; Grenache noir, Alicante Bouschet or Tinto de Madrid: medium susceptibility; Juan Ibanez, Rebula: high susceptibility) can also be used for inoculations according to Arregui *et al.*, 1988 and Peros *et al.*, 1995. Five to ten *in-vitro* plants should be inoculated per suspected bacterial strain. Prepare in sterile distilled water immediately before inoculation a suspension of suspected *X. ampelinus* isolate from a fresh culture on YPGA, approximately 10^9 cells per mL. Put one drop of this bacterial suspension onto freshly decapitated plants. Cut the plant at the top and let the drop enter and keep the inoculated plantlets at appropriate conditions (27°C during the day and 20°C during night are recommended). Do the same with a suspension of known pathogenic *X. ampelinus* strain and distilled sterile water. Symptoms consisting of abnormal tissue proliferation, necrosis or cankers should appear after 3 weeks in the inoculated plants and in the positive controls but not in those inoculated with water. In many cases plantlets inoculated with *X. ampelinus* will die. Re-isolation of the bacterium from symptomatic plants should be done only when formal evidence of contamination of the sample is needed.