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EPPO STANDARD ON DIAGNOSTICS

PM 7/20 (3) *Erwinia amylovora*

Specific scope: This Standard describes a diagnostic protocol for *Erwinia amylovora*.[1]

It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment: This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) and EUPHRESCO Pilot project (ERWINDECT) by a partnership of contractor laboratories. Test performance studies were performed with different laboratories in 2002, 2009 (Reisenzein et al., 2010), 2010 (López et al., 2010) and 2019 (Alič et al., 2020).

Approved as an EPPO Standard in 2003–09. Revised in 2012–09 and 2021–09.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Erwinia amylovora is the causal agent of fire blight, a disease that affects most species of the subfamily Maloideae of the family Rosaceae (Spiraeoideae). It was the first bacterium described as the causal agent of a plant disease (Burrill, 1883). *E. amylovora* is considered to be native to North America and was first detected outside North America in New Zealand in 1920. Fire blight was reported in England in 1957 and since then the bacterium has been detected in most areas of Europe where susceptible hosts are cultivated. *E. amylovora* is now present in more than 50 countries. It has not been recorded in South America and most African and Asian countries (except for countries surrounding the Mediterranean Sea), and it has been eradicated in Australia after a first report there (van der Zwet, 2004). It represents a threat to the pome fruit industry of all these countries (Bonn & van der Zwet, 2000). The most important host plants from both economic and epidemiological viewpoints are in the genera *Chaenomeles*, *Cotoneaster*, *Crataegus*, *Cydonia*, *Eriobotrya*, *Malus*, *Mespilus*, *Pyracantha*, *Pyrus*, *Sorbus* and *Stranvaesia* (Bradbury, 1986). The *E. amylovora* strains isolated from *Rubus* sp. in the United States are distinct from the strains on other hosts (Starr et al., 1951; Powney et al., 2011). Details on geographic distribution and host plants can be found in the EPPO Global Database (EPPO, 2021a).

Fire blight is probably the most serious bacterial disease affecting *Pyrus communis* (pear) and *Malus domestica* (apple) cultivars in many countries. Epidemics are sporadic and are dependent on several factors, including favourable environmental conditions, sufficient inoculum level present in the orchard and host susceptibility. The disease is easily dispersed by birds, insects, rain or wind (Thomson, 2000). The development of fire blight symptoms follows the seasonal growth development of the host plant. The disease begins in spring with the production of the primary inoculum from bacteria overwintering in cankers (Thomson, 2000) causing blossom infection driven by the activity of pollinating insects (van der Zwet & Keil, 1979) and other climatic factors (e.g. rain, wind, and hail), continues into summer with shoot and fruit infection, and ends in winter with the development of cankers. The pathogen appears quiescent through the dormant period of the host (van der Zwet & Beer, 1995), but the experience in Portugal is that it may remain active during winter (L. Cruz, personal communication).

Flow diagrams describing the diagnostic procedure for *E. amylovora* in symptomatic and asymptomatic material are presented in Figures 1 and 2.

2 | IDENTITY

Name: *Erwinia amylovora* (Burrill, 1882) Winslow et al., 1920

Other scientific names: *Micrococcus amylovorus* (Burrill, 1882), *Bacillus amylovorus* (Burrill, 1882) Trevisan, 1889, *Bacterium amylovorus* (Burrill, 1882) Chester, 1897, *Erwinia amylovora* f. sp. *rubi* (Starr, 1951), Cardona & Falson

Taxonomic position: Bacteria, Proteobacteria, γ Subdivision, Enterobacteriales, Enterobacteriaceae

EPPO Code: ERWIAM

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

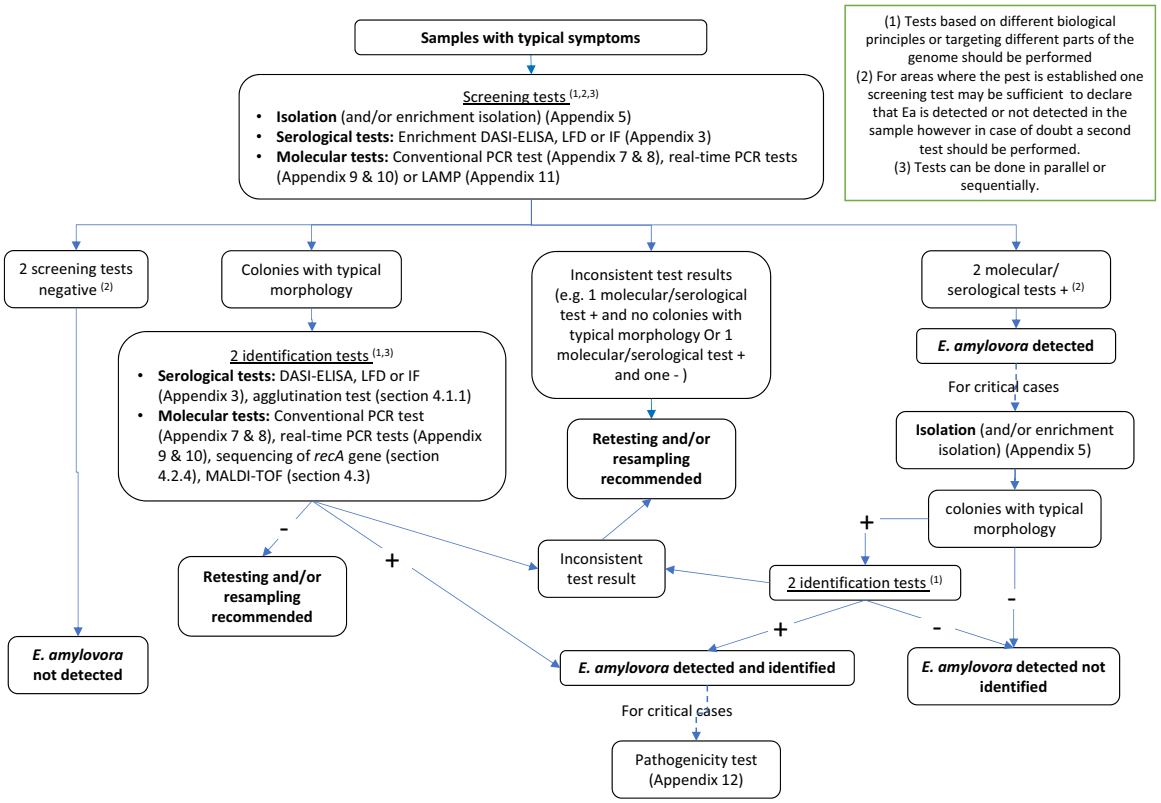

FIGURE 1 Flow diagram for diagnosis of *Erwinia amylovora* in symptomatic samples

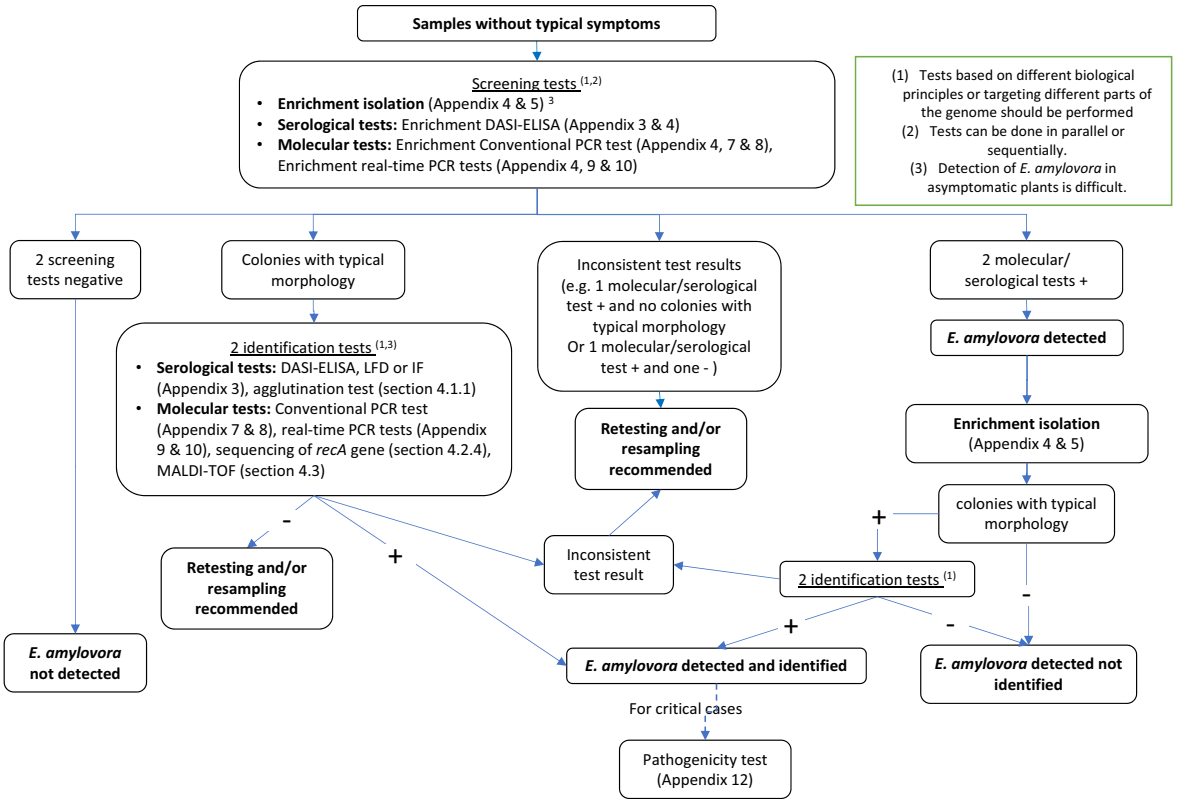

FIGURE 2 Flow diagram for analysis of *Erwinia amylovora* in asymptomatic samples

Phytosanitary categorization: EPPO A2 list no. 52, EU Protected Zone Quarantine pest (Annex III), EU Regulated Non-Quarantine Pest (Annex IV)

3 | DETECTION

3.1 | Disease symptoms

Symptoms of fire blight on the most common hosts such as *P. communis* (pear), *M. domestica* (apple), *Cydonia* spp. (quince), *Eriobotrya japonica* (loquat), *Cotoneaster* spp. (cotoneaster), *Pyracantha* spp. (pyracantha) and *Crataegus* spp. (hawthorn) are relatively similar and easily recognized (Figures 3–5). The name of the disease is descriptive of its major characteristic: the brownish to blackish necrotic appearance of twigs, flowers and leaves, as though they had been burned by fire. The typical symptoms are the brown to black colour of leaves on affected branches, the production of exudates, and the characteristic 'shepherd's crook' of terminal shoots. Depending on the affected plant part and phenological stage, the disease symptoms may include blossom blight, shoot or twig blight, leaf blight, fruit blight, limb or trunk blight, or collar or rootstock blight (van der Zwet & Keil, 1979; van der Zwet & Beer, 1995).

In apple and pear trees the first symptoms usually appear in early spring when the average temperature rises above 15°C, during humid weather. Infected blossoms become soaked with water, then wilt, shrivel and turn orange or brown to black. Peduncles may also appear water-soaked, become dark green, and finally brown or black, sometimes oozing droplets of sticky bacterial exudates. Infected leaves wilt and shrivel, and entire spurs turn brown in apples and dark brown to black in pears but remain attached to the tree for some time. On infection young fruitlets turn brown but also

FIGURE 3 Symptoms of fire blight on pear trees: (a) necrotic flowers, (b) necrosis on leaves and typical shepherd's crook, (c) mummified immature fruits with small ooze drops and (d) canker after removing bark showing necrotic inner tissues

FIGURE 4 Typical symptoms of fire blight on (a) pear branches, (b) apple shoot, (c) quince shoot and (d) loquat shoot

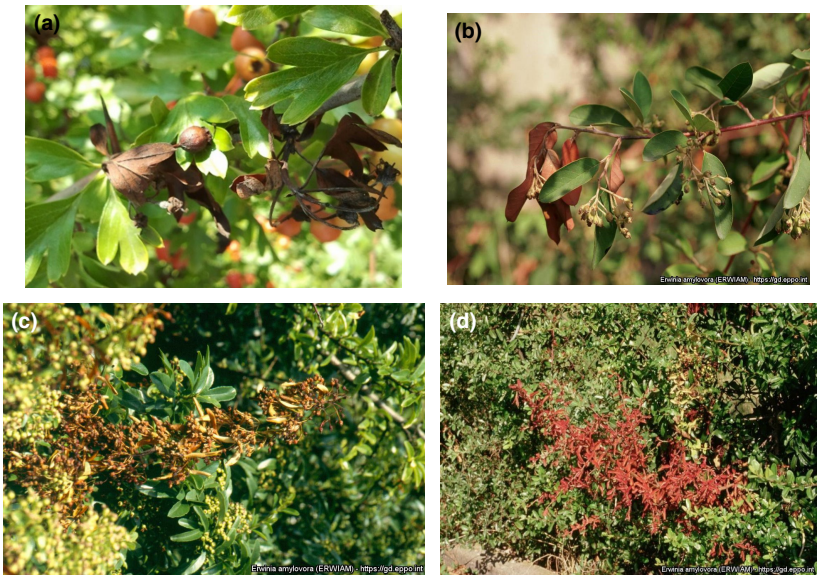

FIGURE 5 Typical symptoms of fire blight on (a) *Crataegus* sp. shoot, (b) *Cotoneaster* sp. shoot and (c, d) *Pyracantha* sp. branches

remain attached to the tree. Immature fruit lesions appear oily or water-soaked, become brown to black and often ooze droplets of bacterial exudate. Characteristic running reddish-brown streaks are often found in the subcortical tissues when the bark is peeled from infected limbs or twigs (van der Zwet & Keil, 1979). Brown to black slightly depressed cankers form in the bark of twigs, branches or the trunk of infected trees. These cankers later become defined by cracks near the margin of diseased and healthy tissue (Thomson, 2000). Additionally, the epidermis may roll up, resembling papyrus paper (L. Cruz, personal communication).

Confusion may occur between fire blight and blight- or blast-like symptoms – especially in blossoms and buds – caused by other pathogens including bacteria (Figure 6a,b) and fungi (Figure 6c,d), insect damage (Figure 6e) and physiological disorders.

Other bacteria that cause fire blight-like symptoms include *Erwinia pyrifoliae*, the causal agent of bacterial shoot blight of *Pyrus pyrifolia* (Asian pear) (Kim et al., 1999), *Erwinia piriflorinigrans* (Figure 6a), isolated from necrotic pear blossoms in Spain (López et al., 2011) and Iran (Moradi Amirabad & Khodakaramian, 2017; Moradi-Amirabad et al., 2020), *Erwinia uzenensis*, recently described in Japan (Matsuura et al., 2012), other *Erwinia* spp. reported in Japan that cause bacterial shoot blight (Tanii et al., 1981; Kim, Hildebrand, et al., 2001; Kim, Jock, et al., 2001; Palacio-Bielsa et al., 2012), and *Pseudomonas* species such as *Pseudomonas syringae* pv. *syringae*, the causal agent of blossom blast (Figure 6b).

3.2 | Detection from symptomatic samples

3.2.1 | Test sample requirements

Symptomatic samples can be processed individually or in small batches combining material from several samples (see Appendix 1). Precautions to avoid cross-contamination should be taken when collecting samples and during the extraction process. Samples with symptoms for diagnosis of fire blight should preferably be composed of flowers, shoots or twigs, leaves, fruitlets (with necrosis and/or with exudates), or the discoloured subcortical tissues (after peeling bark from cankers in branches, trunk or collar). Samples should be processed as soon as possible after collection but can be kept at 4–8°C for up to 1 week before analysis, if necessary.

3.2.2 | Screening tests

At least two tests, based on different biological principles or targeting different parts of the genome, should be performed. For areas where the pest is established one screening test may be sufficient to declare that *E. amylovora* is detected or not detected in the sample. However, in case of doubt a second test should be performed. In addition, if isolation of colonies with typical morphology and identification of *E. amylovora* is positive, a second screening test is not necessary.

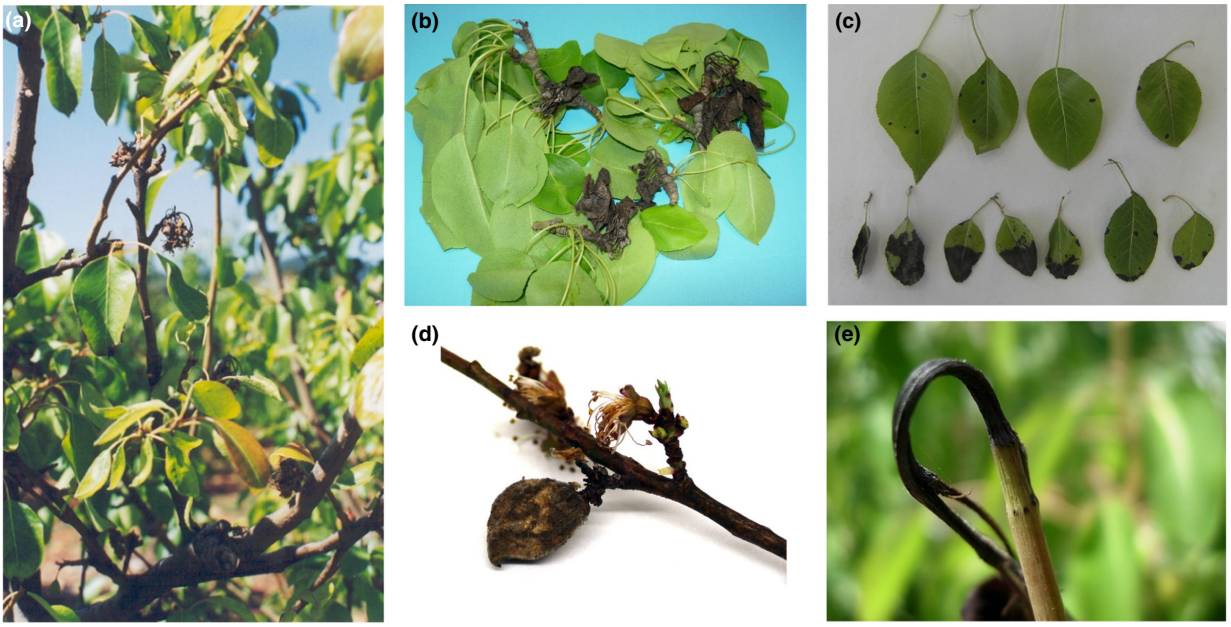

FIGURE 6 Confusing symptoms caused by (a) *Erwinia piriflorinigrans* in *Pyrus*, (b) *Pseudomonas syringae* pv. *syringae* in *Pyrus*, (c) *Stemphylium vesicarium* in *Pyrus*, (d) *Monilia laxa* in *Prunus persicae* (similar mumification caused by monilia may be observed) and (e) *Janus compressus* (Hymenoptera) (note oviposition punctures at the base of the crook)

3.2.2.1 | *Isolation*

Fresh sample extracts are necessary for successful isolation. Details on the extraction procedure from plant material are given in Appendix 1. Details of isolation are provided in Appendix 5. Isolating *E. amylovora* from symptomatic samples is relatively easy because the number of culturable bacteria in such samples is usually high. However, when phytosanitary treatments with bacteriostatic products are used, when symptoms are very advanced or when the environmental conditions after infection are not favourable for bacterial multiplication, the number of culturable *E. amylovora* cells can be very low. Isolation under these conditions can result in plates with few cells of the pathogen that can be overgrown with saprophytic and antagonistic bacteria. If this is suspected, the sample should be re-tested and/or enriched before isolation. The induction of the reversible viable but non-culturable state (VBNC) has been shown for *E. amylovora* in vitro using copper treatments and in fruits (Ordax et al., 2009), and it can be the cause of false-negative isolation results. Indeed, in this VBNC state, bacteria do not grow in the solid culture media but remain pathogenic. This state is reversible, and the bacteria can become culturable and pathogenic again (Ordax et al., 2006). If *E. amylovora* is found in the samples in the VBNC state, the results of isolation will be negative, but the bacteria will still be potentially pathogenic and can be detected by Enrichment-ELISA and PCR-based methods.

When plates are overcrowded by plant microbiota, the sample should be retested and enrichment (according to Appendix 4) performed before isolation (as described in Appendix 5). For direct isolation, plating on at least two different media in parallel (to be chosen depending on the sample) is recommended for maximum recovery of *E. amylovora*, in particular when samples are in poor condition. The efficiency of the different media depends on the number and composition of microbiota in the sample. Three media, King's B, NSA and CCT (Appendix 2), have been validated in a test performance study. Colonies of *E. amylovora* on CCT appear at about 48 h and are pale violet, circular, highly convex to domed, smooth and mucoid after 72 h, showing slower growth than on King's B or NSA. CCT medium inhibits most pseudomonads but not enterobacteria such as *Pantoea agglomerans*. Colonies of *E. amylovora* on King's B appear at approximately 24 h and are creamy white, circular, tending to spread and non-fluorescent under UV light at 366 nm after 48 h. This allows distinction from fluorescent pseudomonas. Colonies of *E. amylovora* on NSA medium appear at about 24 h and are whitish, circular, domed, smooth and mucoid after 48 h. NSA-negative colonies of *E. amylovora* have also been reported (Bereswill et al., 1997). Figure 7 shows the typical appearance of *E. amylovora* bacterial cultures in the three media after incubation at 25°C for 24, 48 and 72 h.

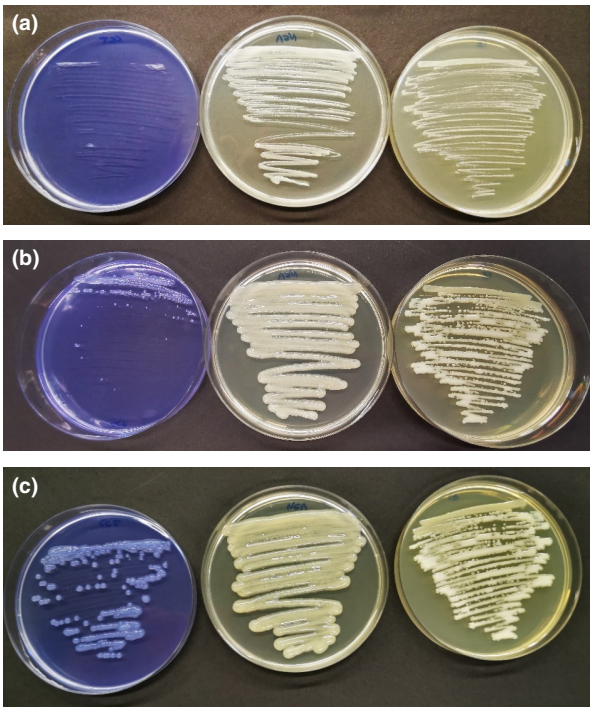

FIGURE 7 Typical colony morphology of *Erwinia amylovora* on CCT medium (left), NSA medium (middle) and King's B medium (right) after incubation at 25°C for (a) 24 h, (b) 48 h and (c) 72 h

Colonies that were in VBNC state may appear later. Very rarely, *E. amylovora* colonies may exhibit a pink colour because of the presence of other bacteria affecting bacterial iron acquisition. When re-streaked and purified, the *E. amylovora* colonies lose the pink colouration (Stockwell et al., 2008; personal communication, Tanja Dreo, NIB).

Pure cultures from individual suspect colonies of each sample should be obtained by plating on King's B medium and presumptive colonies of *E. amylovora* should be identified as indicated in the identification section.

The isolation is negative if no bacterial colonies with morphology similar to *E. amylovora* are observed after 96 h in any of the media (provided no inhibition is suspected due to competition or antagonism) and typical *E. amylovora* colonies are found in the positive controls. The isolation is positive if presumptive *E. amylovora* colonies are isolated in at least one of the media used and the identification is confirmed by one of the methods indicated.

On the mentioned media and under the same incubation conditions, *E. piriflorinigrans* colonies are similar to *E. amylovora* in terms of size and morphology but appear in general faster than *E. amylovora*. *E. pyrifoliae* colonies are more mucoid and fluid compared to the *E. amylovora* colonies under the same incubation conditions and on the same media (Figure 8). *Pseudomonas syringae* pv. *syringae* grows faster on NSA medium and colonies are fluorescent under King's B medium.

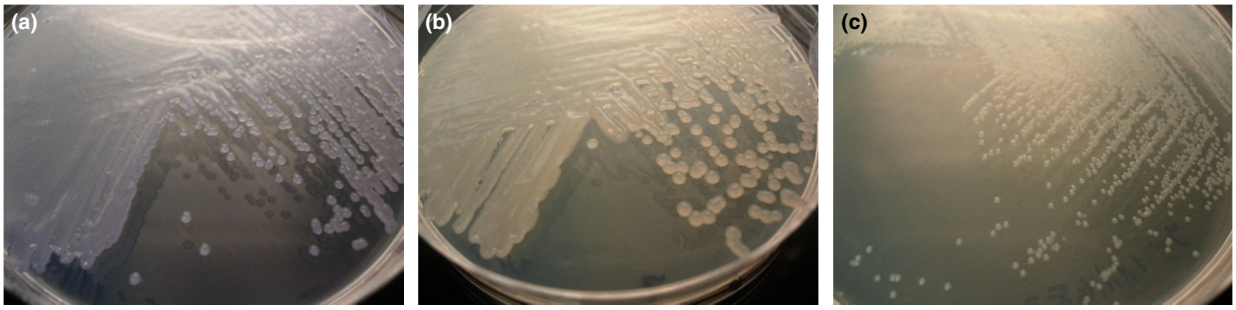

FIGURE 8 Typical colony morphology of *Erwinia piriflorinigrans* on (a) CCT medium, (b) NSA medium and (c) King's B medium after incubation at 25°C for 48 h

3.2.2.2 | *Other screening tests*

These tests facilitate the presumptive diagnosis of plants with fresh pronounced symptoms. Several tests are described in Appendices 3–11. Test performance studies were conducted, and the results are indicated.

3.2.2.2.1 | *Serological tests*. Indirect immunofluorescence (IF), enrichment DASI-ELISA and lateral flow devices are described for analyses of organs with symptoms. Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009) and those for performing ELISA are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010). Quality of the antibodies is critical for the performance of the tests. In test performance studies, several commercial antisera and monoclonal antibodies were compared for IF [polyclonal antiserum from Loewe Biochemica GmbH (Sauerlach, Germany) and monoclonal antibodies from Plant Print Diagnostics S.L. (Faura, Spain)]. For ELISA, a complete kit based on a combination of specific monoclonal antibodies, from Plant Print Diagnostics S.L., was also evaluated.

Two lateral flow devices commercialized by Bioreba, Reinach, Switzerland (Ea AgriStrip) and Abingdon Health, York, UK (Pocket Diagnostics) are available for the rapid analysis of symptomatic plant material (Braun-Kiewnick et al., 2011). Details of the tests are given in Appendix 3.

3.2.2.2.2 | *Molecular tests*. Many tests for conventional PCR have been developed for *E. amylovora* but some have shown a lack of analytical specificity, e.g. cross-reaction with *Erwinia piriflorinigrans* (Maes et al., 1996), or do not detect all strains (Bereswill et al., 1992; McManus & Jones, 1995 and Llop et al., 2000). It has also been observed that the nested PCR (Llop et al., 2000) generates several false-positive results which cannot be confirmed with any other test or subsequent testing or symptom observation (Tanja Dreo, personal communication, NIB). Two conventional PCR tests (Taylor et al. (2001), and an adaptation from Obradovic et al. (2007), two real-time PCR tests (Pirc et al., 2009 and Gottsberger, 2010) and one loop-mediated isothermal amplification (LAMP) test are recommended in this diagnostic protocol and are described in Appendices 7–11.

All tests were evaluated in test performance studies in 2009 (Reisenzein et al., 2010), 2010 (López et al., 2010) and/or 2019 (Ališ et al., 2020; Trontin et al., 2021), and are recommended for the analyses of organs with symptoms after a DNA extraction step. The DNA extraction protocols that were evaluated in a test performance study in 2009 and/or 2019 are indicated in Appendix 6.

3.3 | Detection from asymptomatic samples

3.3.1 | Test sample requirements

Warning: Detection of *E. amylovora* in asymptomatic plants is difficult.

Whenever possible, testing of asymptomatic plants should be performed in summer or early autumn to increase the likelihood of detecting *E. amylovora*. Asymptomatic samples may be processed individually or bulked (see Appendix 1). Precautions to avoid cross-contamination should be taken when collecting the samples and during the extraction process. Sampling and sample preparation can be performed following one of the methods described in Appendix 1 for asymptomatic samples.

Direct analysis of asymptomatic samples is usually negative for *E. amylovora* due to the low bacterial population. Consequently, an enrichment step is recommended (Appendix 4).

3.3.2 | Screening tests

Enrichment-isolation, enrichment-DASI ELISA and enrichment conventional PCR or enrichment real-time PCR can be used as screening tests and are described in Appendices 4–10. At least two screening tests should be performed.

3.4 | Confirmation of positive results of screening tests

In critical cases (EPPO, 2017) and for asymptomatic samples, if two of the screening tests are positive, an attempt should be made to isolate the pathogen directly from the extract of non-enriched samples (Appendices 1 and 5), or from the enriched samples (Appendices 4 and 5). As little is usually known about the microbiota present in the samples, at least two different media (CCT, King's B, NSA) described in Appendix 2 should be used to maximize the likelihood of successful direct isolation of *E. amylovora*. However, plating on CCT only is sufficient after enrichment of the samples in King's B or CCT. If necessary, the extract is conserved at approximately −20 or −80°C under glycerol (Appendix 1).

4 | IDENTIFICATION

Pure cultures of presumptive *E. amylovora* isolates should be identified with at least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) and, when necessary, a pathogenicity test. Two molecular tests may be used if they are based on different DNA sequence targets in the genome and provided that the specificity of the primers has been evaluated. Known *E. amylovora* reference strains should be included for each test performed (see the section on Reference material).

4.1 | Serological tests

Different sources of antibodies should be used for identification to reduce the risk of false positives.

4.1.1 | Agglutination test

Suspected *E. amylovora* colonies can be tested for agglutination by mixing them in a drop of PBS (Appendix 2) with a drop of *E. amylovora*-specific antiserum (not diluted, or five- or tenfold dilution) on a slide. Monoclonal antibodies can be used only if they agglutinate with the reference strains. Colonies grown on media promoting the production of polysaccharides (e.g. NSA) should be washed three times in saline solution.

4.1.2 | Immunofluorescence test

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). For identification, IF can be performed using specific monoclonal antibodies from Plant Print Diagnostics S.L. or antiserum from Loewe Biochemica GmbH.

4.1.3 | ELISA tests

Instructions for performing ELISA are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010). DASI-ELISA for isolate identification can be performed using the same specific monoclonal antibodies as used for the analysis of plant samples (kit from Plant Print Diagnostics S.L.). For DASI-ELISA, a suspension of approximately 10^8 cells/mL from suspected colonies is prepared in PBS (Appendix 2). The DASI-ELISA procedure (Appendix 3) can be followed without prior enrichment for isolate identification.

4.1.4 | Lateral flow immunoassays

A suspension of approximately 10^8 cells/mL prepared in PBS (Appendix 2) from suspected colonies should be used following the manufacturers' instructions. The two kits evaluated in a test performance study (Agri-strip and Pocket Diagnostic) and recommended for analyses of symptomatic plants can be used for identification of isolates.

4.2 | Molecular tests

Conventional and/or real-time PCR and LAMP are the recommended molecular tests for rapid identification, but other available techniques are also indicated.

4.2.1 | Conventional PCR

A suspension of approximately 10^6 cells/mL in molecular-grade water should be prepared from *E. amylovora*-like colonies. Appropriate PCR procedures should be applied, following Appendices 7 or 8, without DNA extraction, just after treatment at approximately 95–100°C for approximately 8–10 min.

4.2.2 | Real-time PCR

Two published real-time PCR tests, described in Appendices 9 and 10, are recommended. Colonies can be prepared as for conventional PCR (section 4.2.1).

4.2.3 | LAMP

One LAMP test described in Appendix 11 is recommended. Suspensions can be prepared as for conventional PCR (section 4.2.1).

4.2.4 | DNA sequencing methods

Comparisons of sequenced PCR products amplified from selected housekeeping genes allow differentiation of *E. amylovora* isolates from other members of the Enterobacteriaceae. For example, all isolates of *E. amylovora* tested so far are clonally related according to partial *recA* gene sequence (Waleron et al., 2002) using the method described by Parkinson et al. (2009). These data are also confirmed by comparative genome analysis (Mann et al., 2013; Zeng et al., 2018; Parcey et al., 2020). Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021b).

4.3 | Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

A matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry method for proteomic analysis has been described by Sauer et al. (2008) and Wensing et al. (2011). This allows rapid, reliable and robust identification of *E. amylovora* isolates from plant samples. For their routine identification all individual isolates should be included in duplicate by directly depositing harvested 3-day-old bacterial cells from nutrient agar (NA) plates onto a stainless plate, without any prior formic acid treatment. All spectra should be obtained in linear positive-ion mode with an *m/z* range of 2000–20 000 Da. Validation data of the MALDI-TOF as an identification test of *E. amylovora* isolates is already available.

4.4 | Pathogenicity tests and hypersensitivity

When necessary, suspected *E. amylovora* colonies from the isolation and/or enrichment plates may be inoculated in plants to confirm their pathogenicity, preferably on detached organs of a fire blight host (Appendix 12).

The hypersensitive reaction in tobacco leaves can give an indication of the presence of the *hrp* pathogenicity genes, but is also positive for many other plant pathogenic bacteria and can be difficult to interpret (Appendix 12).

4.5 | Other tests

4.5.1 | Biochemical tests

The genus *Erwinia* has been defined as Gram-negative bacteria, facultative anaerobes, motile by peritrichous flagella, rod-shaped, acid produced from glucose, fructose, galactose and sucrose. The phenotypic properties are described in Paulin (2000) and should be determined according to the methods of Jones and Geider (2001). The tests in Table 1, based mainly on results in API 50 CH strips (BioMérieux, France), allow differentiation of *E. amylovora* from *E. pyrifoliae*, causal agent of Asian pear blight on *Pyrus pyrifolia* (Kim et al., 1999; Rosello et al., 2006) and *E. piriflorinigrans* (López et al., 2011; Moradi Amirabad & Khodakaramian, 2017; Moradi-Amirabad et al., 2020). However, certain physiological and biochemical characteristics can vary for some strains.

TABLE 1 Differences between *Erwinia amylovora*, *Erwinia pyrifoliae* and *Erwinia piriflorinigrans*

Microbiological tests	*Erwinia amylovora*	*Erwinia pyrifoliae*	*Erwinia piriflorinigrans*
Gelatine hydrolysis	+	−	−
Inositol[a]	−	+	+
Sorbitol[a]	+	+	−
Esculin[a]	V	−	+
Melibiose[a]	+	−	+
d-Raffinose[a]	−	−	+
β-Gentibiose[a]	+	−	+

Abbreviation: V, variable

[a]Oxidation of substrates in API 50CH (BioMérieux) with a modified protocol from Roselló et al. (2006). More than 90% of strains gave the results indicated here.

4.5.1.1 | *Biochemical characterization by API system (BioMérieux, France)*

Biochemical identification of *E. amylovora* can be obtained by specific profile in API 20 E and API 50 CH strips. For API 20 E, the manufacturer's instructions should be followed for preparing the suspension and inoculating the strip. After incubation at 25–26°C, the strips should be read after 24 and 48 h (Table 2). For API 50 CH, a suspension of OD = 1.0 should be prepared in PBS (Appendix 2), and 1 mL added to 20 mL of Ayers' medium (Appendix 2). The manufacturer's instructions should be followed for inoculation of the strip. After incubation at 25–26°C in aerobiosis, the strip should be read after 72 h.

4.5.1.2 | *Automated Biolog identification system*

The new version (third-generation) Biolog GENIII 96 microplate allows rapid identification of isolated bacteria, both Gram-negative and Gram-positive, using the same microplate.

The microplate and the program are commercially available (Biolog, Omnilog, USA). The manufacturer's instructions should be followed for automatic identification of suspected strains of *E. amylovora*.

4.5.2 | Fatty acid profiling

Erwinia amylovora-like colonies should be grown on Trypticase Soy Broth Agar (TSA) for 24 h at 28°C, and

TABLE 2 Typical readings of *Erwinia amylovora* in API 20E tests after 48 h

Test	Reaction (48 h)
ONPG	Variable
ADH	– (or weak +)
LDC	–
ODC	–
CIT	–
SH2	–
URE	–
TDA	–
IND	–
VP	+ (or variable)
GEL	Variable
GLU	+
MAN	Variable
INO	Variable
SOR	Variable
RHA	–
SAC	+
MEL	– (or weak +)
AMY	–
ARA	+ (some −)

an appropriate fatty acid profiling (FAP) procedure applied. A positive FAP test is achieved if the profile of the presumptive culture is identical to that of the positive control (Sasser, 1990). Commercial software from the MIDI system (Newark, DE, USA) allows rapid identification of *E. amylovora*-like colonies. The manufacturer's instructions should be followed for automatic identification. Fatty acid composition can be affected by growth medium, physiological age of cells and chromatograph sensitivity, but in general *E. amylovora* strains have a similarity index between 0.6 and 0.9 in this system.

5 | REFERENCE MATERIAL

The following *E. amylovora* isolates are recommended for use as positive controls: NCPPB683 (type strain) and CFBP 1430. The following collections can provide different *E. amylovora* reference strains:

(i) National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, Sand Hutton, York (GB); https://www.fera.co.uk/ncppb
(ii) International Center for Microbial Ressources – French Collection for Plant-associated Bacteria (CIRM-CFBP), IRHS – INRAE Beaucouzé (FR); https://www6.inrae.fr/cirm/CFBP-Bacteries-associees-aux-Plantes
(iii) Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig (DE); www.dsmz.de/dsmz
(iv) Laboratorium voor Microbiologie Bacterial Collection (LMG), Universiteit Gent (BE); http://bccm.belspo.be/

Authenticity of the strains can be guaranteed only if obtained directly from the culture collections.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

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9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this diagnostic protocol or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

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APPENDIX 1 - EXTRACTION PROCEDURES

1. Samples from symptomatic material

The samples may be processed in different buffers according to the tests to be performed. The use of freshly prepared antioxidant maceration buffer (Gorris et al., 1996a) has been evaluated in a test performance study and can be used with all subsequent methods. Sterile phosphate-buffered saline, pH 7.2 10 mM (PBS) (Appendix 2) or sterile water can also be used for direct isolation, immunofluorescence or PCR.

Carefully select the plant parts showing the freshest symptoms, with exudates if possible. The leading edge of lesions on each organ should be selected for analysis. The exudates can be processed separately, in 1–4.5 mL sterile water or buffer. For shoots, take pieces of symptomatic shoots, including leaves, at the margin between the necrotic and healthy tissue. Take one or several flowers, with peduncles. Take one or several leaves and petioles, preferably select leaves with vein necrosis, but not fully necrosed. Take one or several fruits. For stems or trunk, peel off the external bark of stems showing symptoms using a sterile scalpel and take pieces underneath with typical subcortical discolouration symptoms.

The protocol evaluated in a test performance study was as follows: cut 0.1 g of shoots, flowers, leaves, stems, trunks or fruits into pieces and place in plastic bags. Add to each bag 4.5 mL of the antioxidant maceration buffer described by Gorris et al. (1996a) (Appendix 2). Allow the samples to macerate for at least 5 min. Crush the plant material slightly in the plastic bag with a rubber hammer, or with a Bioreba homogenizer or similar equipment, avoiding droplets splashing out of the bag. Hold the samples on ice for a few minutes and decant approximately 2, 1 and 1 mL of each macerate into three sterile Eppendorf tubes. Use the tube containing 2 mL for the analysis. Store one tube with 1 mL of each sample at approximately −20°C for subsequent analysis or confirmation; add 30% glycerol (Difco) to the other tube and store it at approximately −80°C.

The isolation should be done on the same day as the maceration of the samples, as well as the enrichment and the fixation of the slides for immunofluorescence. PCR analysis can be performed at the earliest convenience, using the 1 mL stored at approximately −20°C.

2. Samples from asymptomatic material

Asymptomatic samples can be processed individually (preferred) or in groups of up to 30. Precautions to avoid cross-contamination should be taken when collecting the samples and during the extraction process. Sampling and sample preparation can be performed following one of the following procedures:

- Blossoms, shoots, fruitlets or stem segments are collected in sterile bags or containers in summer or early autumn, after favourable conditions for the multiplication of E. amylovora have occurred and when average temperatures rise above about 15°C (van der Zwet & Beer, 1995). Young shoots approximately 20 cm in length, or blossoms when available, are cut from the suspect plant. If analyses need to be performed in winter, five to 10 buds are collected per plant. In the laboratory, blossoms when available, the peduncle and base of the limb of several leaves from the base of the shoots, or the stem segments are cut from the selected plants. About 0.1–1.0 g of plant material is weighed and macerated in 4.5 mL of antioxidant buffer (not in PBS or water) (Appendix 2) before enrichment (Appendix 4). It is not recommended to analyse larger amounts of plant material in one sample.
- A sampling procedure reported for the analysis of twigs of asymptomatic woody material from nurseries is as follows. Twigs (each about 10 cm in length) are collected according to PM 3/76. If there are several plant genera in the lot, these should be represented equally in the sample (with a maximum of three genera per sample). The laboratory sample consists of 30 twigs randomly selected. Each twig is cut into four pieces (producing 120 stem pieces). The samples are covered with sterile PBS containing 0.1% Tween 20 in Erlenmeyer flasks, and the flasks are stirred vigorously on a rotary shaker for 1.5 h at room temperature. The extract is filtered through filter paper held in a sintered glass filter using a vacuum pump, and the filtrate is collected. Alternatively, the extract is centrifugated for 10 min at 1500 g and transferred to a new tube (Pirc et al., 2009). The filtrate/supernatant is centrifuged at 7000–10 000 *g* for 20 min. The pellet is suspended in 4.5 mL sterile PBS. A similar protocol can be applied for leaves, shoots, flowers and buds. This procedure was validated in combination with enrichment isolation and enrichment real-time PCR (Pirc et al., 2009, see Appendix 9).

Whichever procedure is followed, prepare three Eppendorf tubes for each sample with about 2, 1 and 1 mL of macerate. Use the tube containing 2 mL for enrichment (see Appendix 4). Store one tube with 1 mL of each sample at −20°C for subsequent analysis or confirmation; add 30% glycerol (Difco) to the other tube and store it at approximately −80°C.

Depending on the timing of the sampling, the expected recovery of *E. amylovora* will vary, with maximum recovery in summer (providing weather conditions are favourable to *E. amylovora*) and reduced recovery in winter. Samples should be processed immediately by performing enrichment followed by DASI-ELISA and/or PCR and/or isolation (Appendices 3–10).

APPENDIX 2 - PREPARATION OF MEDIA AND BUFFERS

1. Buffers

Phosphate buffered saline 10 mM, pH 7.2 (PBS)

NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L

Sterilize by filtration.

Antioxidant maceration buffer (Gorris et al., 1996a)

Polyvinylpyrrolidone (PVP 10)	20.0 g
Mannitol	10.0 g
Ascorbic acid	1.76 g
Reduced glutathione	3.0 g
PBS 10 mM, pH 7.2	1 L

Adjust pH to 7.0. Sterilize by filtration. This buffer should be prepared immediately before use.

Extraction buffer (Llop et al., 1999)

Tris HCl	31.52 g
NaCl	14.6 g
EDTA	9.3 g
SDS	5.0 g
Polyvinylpyrrolidone (PVP 10)	20.0 g
Distilled water to	1 L

Adjust pH to 7.5. Sterilize by filtration.

2. Media

Media are sterilized by autoclaving at 120°C for 15 min unless stated otherwise.

Ayers' medium (Ayers et al., 1919)

$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4$	0.2 g
Bromothymol blue (solution 0.2%)	75 mL
Distilled water to	1 L

Adjust pH to 7.0.

CCT medium (Ishimaru & Klos, 1984)

Sucrose	100 g
Sorbitol	10.0 g
Niaproof	1.2 mL
Crystal violet (sol. 0.1% in absolute ethanol)	2 mL
Nutrient agar	23.0 g
Distilled water to	1 L

Adjust pH to 7.0–7.2; sterilize by autoclaving at 115°C for 10 min. Then prepare thallium nitrate 2 mL (1% w/v aqueous solution), 0.05 g cycloheximide. Sterilize by filtration (0.22 µm). Add to 1 L of sterile medium (at about 45°C).

King's B medium (King et al., 1954)

Proteose peptone No. 3	20 g
Glycerol	10 mL
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g
Distilled water to	1 L

Adjust pH to 7.0–7.2.

Enrichment media: Use CCT medium /or King's B medium prepared in liquid form, without agar, for enrichment as described in Appendix 4.

NSA medium

Yeast extract	2 g
Bactopeptone	5 g
NaCl	5 g
Sucrose	50 g
Agar	20 g
Distilled water to	1 L

Adjust pH to 7.0–7.2.

Nutrient agar (NA): Commercially available.

Trypticase soy broth agar (TSBA): Commercially available.

APPENDIX 3 - RAPID SEROLOGICAL SCREENING TESTS

1. Immunofluorescence

Follow the standard instructions described in PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009).

Antibodies to *E. amylovora* currently used in detection and identification tests:

- *E. amylovora*, polyclonal antibodies, for detection using IF test (validated in test performance studies), Loewe Biochemica GmbH.
- IVIA EPS 1430, polyclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics, S.L.
- IVIA Mab 7 A, monoclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics, S.L.

Use undiluted macerates and 1:10 and 1:100 dilutions in PBS (Appendix 2) to spot windows of IF slides. Prepare one slide for each sample and its dilutions. Use the monoclonal or polyclonal antibodies at the appropriate dilutions in PBS (Appendix 2). Determination of the contamination level is usually not required. Immunofluorescence is not recommended after enrichment of samples.

Performance characteristics available

- Analytical sensitivity data

  10^3–10^4 cfu/mL plant extract

- Analytical specificity data

  Not tested for polyclonal antibodies.

  For monoclonal antibody IVIA Mab 7 A

  Target organisms tested: 50 *E. amylovora* strains. All positive in the test conditions.

  Non-target organisms tested: 123 unidentified strains from *E. amylovora* hosts, 121 negative and two *Erwinia*-related bacteria positive (*Erwinia persicina* and *Dickeya* sp.).

- Data on repeatability

  Validation data from IVIA (ES): 100%

- Data on reproducibility

  Validation data from IVIA (ES): 60%

2. Enrichment DASI-ELISA

After the enrichment step (see Appendix 4), the use of validated specific monoclonal antibodies is recommended to avoid cross-reactions. A complete kit based on polyclonal and monoclonal antibodies (3B + 5H IVIA), including extraction buffer, semi-selective media, ELISA plates and reagents, is available from Plant Print Diagnostics S.L. This commercial kit for Enrichment DASI-ELISA (Gorris et al., 1996b) has been validated in two test performance studies. It is based on the monoclonal antibodies and technique described in Gorris et al. (1996a,b). As positive controls, use aliquots of a sample extract that previously gave a negative result on testing, mixed with 10^8 cells of *E. amylovora* per mL. As negative controls include a sample extract that has previously given a negative result for *E. amylovora* and a suspension of a non-*E. amylovora* strain in PBS (Appendix 2).

Before ELISA, treat the necessary amount of enriched extracts and controls in a water bath (or in a thermoblock) at 100°C for 10 min, ensuring the tubes are not opened. Keep the remaining enriched samples for isolation and/or PCR. Process the boiled samples (once at room temperature) by ELISA on the same day or store them at −20°C for subsequent analysis. This heat treatment is necessary for optimum sensitivity and specificity using the monoclonal antibodies obtained by Gorris et al. (1996a). Then follow the instructions for DASI-ELISA given in PM 7/101 (1) *ELISA tests for plant pathogenic bacteria* (EPPO, 2010) and those of the manufacturers of the commercial kit.

Positive ELISA readings in negative control wells indicate cross-contaminations or non-specific antibody binding. In either case, the test should be repeated, or a second test based on a different biological principle should be performed.

Performance characteristics available

- Analytical sensitivity data

  10 cfu/mL plant extract in King's B and in CCT (Gorris et al., 1996b).

  10–10^2 cfu/mL plant extract in King's B and 10^3–10^4 cfu/mL plant extract in CCT (in the performance study in 2010).

- Analytical specificity data

  For monoclonal antibodies 3B + 5H.

  Target organisms tested: 250 *E. amylovora* strains. All positive in the test conditions (Gorris et al., 1996a, 1996b; and IVIA tests).

  Non-target organisms tested: 258 unidentified strains from *E. amylovora* hosts and 45 strains of other plant pathogenic bacteria. They were all negative (Gorris et al., 1996a, 1996b).

  A strong cross reaction was observed in loquat with *Rosenbergiella epipactidis* (IVIA, ES).

- Data on repeatability

  Validation data from IVIA (ES): 100%

- Data on reproducibility

  Validation data from IVIA (ES): 98%

3. Lateral flow devices

Two lateral flow devices [Ea Agri-strip (Bioreba) and Pocket Diagnostics (Abingdon Health, York, GB)] were evaluated in performance studies in 2009, 2010 and 2019 and showed relatively similar results. They were appropriate for the analysis of symptomatic plants only and are based on *E. amylovora* polyclonal antisera. Follow the manufacturer's instructions when performing the analysis.

Performance characteristics available

3.1. Analytical sensitivity data (in a test performance study performed in 2010)

Ea Agri-strip: 10^5–10^6 cfu/mL plant extract
Pocket Diagnostics: 10^5–10^6 cfu/mL plant extract

3.2. Analytical specificity data

Ea Agri-strip:
Validation data from a test performance study performed in 2010:
Target organisms tested: 39 strains all positive
Non-target organisms tested: 61 strains (all negative except *E. pyrifoliae*, *E. tasmaniensis* and *E. piriflorinigrans*).
False-positive results with *E. pyrifoliae*, *E. tasmaniensis* and *E. piriflorinigrans* are also reported in AGES (AT) (Ea Agri-strip and Pocket Diagnostics) and Braun-Kiewnick et al. (2011) (Ea Agri-strip).

3.3. Diagnostic sensitivity and specificity for Pocket Diagnostics (validation data from VALITEST TPS, 2019)

The panel used for analysis consisted of eight samples composed of six spiked plant extracts of *Malus* (×3), *Pyrus* (×1), *Amelanchier* (×1) and *Pyracantha* (×1) at a concentration above the expected limit of detection and two healthy plant extracts of *Malus* and *Pyrus*:
Diagnostic sensitivity: 98%

3.4. Data on repeatability (validation data from IVIA, ES)

Ea Agri-strip: 94%
Pocket Diagnostics: 94%

3.5. Data on reproducibility (validation data from IVIA, ES)

Ea Agri-strip: 96%
Pocket Diagnostics: 96%

APPENDIX 4 - ENRICHMENT

Enrichment is used to multiply the initial culturable subpopulation and/or to recover the VBNC subpopulation of *E. amylovora* in the sample. It is needed before detection by ELISA because of the low level of analytical sensitivity of this technique when using specific monoclonal antibodies. It should also be used before detection when a low number of culturable *E. amylovora* is expected (copper-treated samples, old symptoms, unfavourable weather conditions for fire blight, winter, asymptomatic samples etc.) or when a high level of non-pathogenic, endophytic or saprophytic organisms are expected.

After preparation of the samples in the freshly prepared antioxidant maceration buffer, use of one or two validated media is recommended [one non-selective (King's B) and/or one semi-selective (CCT) (Appendix 2)] because the composition and number of microbiota is unknown.

As soon as the macerates have been made (Appendix 1), dispense at least 0.9 mL of each sample into two sterile 2- or 5 mL tubes prepared in advance with the same volume of the enrichment medium. As additional negative controls prepare three tubes with 0.9 mL maceration buffer (Appendix 2) and add the same volume of the enrichment medium (Appendix 2). Incubate at 25°C for 48 h without shaking. Incubate for 72 h when very low numbers of *E. amylovora* are expected, as indicated above for asymptomatic samples.

APPENDIX 5 - ISOLATION

1. Direct isolation

Use CCT, King's B and NSA media (Appendix 2). Plating on at least two media is recommended for maximum recovery of *E. amylovora*, particularly when samples are in poor condition. Prepare 1:10 and 1:100 dilutions of each macerate (Appendix 1) in PBS (Appendix 2). Pipette 50 µL of the diluted and undiluted macerates onto separate plates of each medium. Start with the 1:100 dilution and proceed to the undiluted macerate. Carefully spread the pipetted volumes by triple streaking. Plate a 10^3, 10^4 and 10^5 cfu/mL dilution of a pure culture of *E. amylovora* as a quality control of the media. Incubate the plates at approximately 25°C for 48–72 h. Final reading is at 72–96 h.

Performance characteristics available

- Analytical sensitivity data (in a performance study in 2010)

  10^3 cfu/mL in King's B; 10–10^2 cfu/mL in NSA and CCT

- Analytical specificity data

  Not evaluated

- Data on repeatability

  In IVIA (ES): 100%

- Data on reproducibility

  In IVIA (ES): 100%

2. Enrichment isolation

Plate the enrichments (Appendix 4) on CCT plates (Appendix 2). Spread 50 µL of each enriched extract and of the 1:10, 1:100 and 1:1000 dilutions prepared in PBS

(Appendix 2) by triple streaking (as for isolations) to obtain isolated colonies. Incubate at approximately 25°C for 72–96 h. The use of CCT semi-selective medium and dilutions is recommended because of the possible abundant multiplication of different bacteria during the enrichment step.

Performance characteristics available

- Analytical sensitivity data (in a performance study in 2010)

  10 cfu/mL after enrichment in CCT
  $10–10^2$ cfu/mL after enrichment in King's B

- Analytical specificity data

  Not evaluated

- Data on repeatability

  In IVIA (ES): 100%

- Data on reproducibility

  In IVIA (ES): 100%

APPENDIX 6 - DNA EXTRACTION

Two protocols for DNA extraction from plant samples (Llop et al., 1999; Taylor et al., 2001) and one commercial kit [RED-Extract N-Amp T Plant kit (Sigma-Aldrich, USA)] have been validated in the test performance studies in 2009 with four conventional PCR protocols and showed comparable results. Two of them are detailed below.

In the test performance study performed in the framework of VALITEST (Ališ et al., 2020; Trontin et al., 2021), the following DNA extraction kits were used by several participants that reported valid results: QuickPick™ SML Plant DNA Kit (Bio-Nobile), DNeasy Plant Mini Kit (Qiagen) and DNeasy Mericon Food kit (Qiagen). The following were used by one participant and led to production of valid data: Mericon Bacteria Kit (Qiagen), NucleoSpin Plant II (Macherey-Nagel), E.Z.N.A. DNA® Plant Kit, Exgene Plant SV mini Kit (GeneAll Biotechnology), Extract N-Amp T Plant kit, Sigma, Llop et al., 1999 (starting with 100 µL of plant extract) and PREP-GS kit (AgroDiagnostica). The DNeasy Plant Mini Kit (Qiagen) protocol which was used by most participants and QuickPick™ SML Plant DNA Kit protocol which was tested in the preliminary study led by NIB are detailed below. The latter protocol was validated with three real-time PCR protocols (Pirc et al. (2009) (ITS and AmsC amplicons) and Gottsberger (2010) and one LAMP test (Shin et al., 2018). Other commercial kits for extracting DNA are available, but they have not been evaluated.

1. DNA extraction according to Llop et al. (1999)

Use 1 mL of each macerate and/or 1 mL of the enriched macerate prepared according to Appendices 1 and 4. Centrifuge the macerates at 10 000 *g* for 5 min at room temperature. Discard the supernatant, resuspend the pellet in 500 µL of extraction buffer (Llop et al., 1999 Appendix 2) and shake for 1 h at room temperature. Centrifuge at 4000 *g* for 5 min. Take 450 µL of the supernatant and add the same volume of isopropanol, invert and leave for 30 min to 1 h at room temperature. Centrifuge at 10 000 *g* for 5 min, discard the supernatant and dry. Resuspend the pellet in 200 µL of water. Use for PCR reaction or store at approximately −20°C.

2. DNA extraction based on the procedure described by Taylor et al. (2001) but with minor modifications (elimination of Gene Releaser which was considered unnecessary).

Add 200 µL of each macerate and/or 200 µL of the enriched macerate in 500 µL of buffer [140 mM NaCl, 50 mM KCl, 0.05% Tween 20, 2% polyvinylpyrrolidone (average molecular weight 10 000 g/mol) (PVP 10), 0.4% BSA, distilled water] for 15 min at room temperature. The resulting suspension can be used for PCR reaction or stored at approximately −20°C.

3. QuickPick™ SML Plant DNA Kit (Bio-Nobile)

DNA from the pure bacterial cultures and plant extracts was extracted and purified using magnetic-bead-based QuickPick™ SML Plant DNA kits (Bio-Nobile, Turku, Finland). This was automated on a KingFisherR mL system (Thermo Labsystem), as described previously for *E. amylovora* (Pirc et al., 2009), and with a minor modification (440 µL of lysate used in the purification).

4. DNeasy Plant Mini Kit (Qiagen)

The DNeasy Plant Mini Kit was used according to the manufacturer's protocol for purification of total DNA from plant tissue with final DNA elution into 2 × 50 µL AE buffer.

Performance characteristics

Performance characteristics are provided together with the different conventional PCR, real-time PCR and LAMP tests (Appendices 7 to 11).

APPENDIX 7 - CONVENTIONAL PCR ACCORDING TO TAYLOR ET AL. (2001)

The test below is described as it was carried out at IVIA and in a test performance study in 2010 to generate the validation data provided in section 4. Other equipment, kits

or reagents may be used provided that a verification is carried out (see PM 7/98).

1. General information

1.1. This test is suitable for the detection of *Erwinia amylovora* in plant material and for identification of *E. amylovora* in pure bacterial colonies.
1.2. This test is universal for all known *E. amylovora* strains to date. The protocol was validated in a test performance study in 2010.
1.3. The targeted sequences are chromosomal (Taylor et al., 2001).
1.4. Oligonucleotides:

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	G1-F	5′-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG−3′	187 bp
Reverse primer	G2-R	5′-GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC−3′	

1.5. Enzyme: the test performance study in 2010 was performed with a DNA polymerase from Biotools.

2. Methods

2.1. Nucleic acid extraction and purification: for plant material, two DNA extraction methods (the one according to Llop et al. (1999) and the one modified from Taylor et al. (2001)) described in Appendix 6 were evaluated in a test performance study.
2.2. Polymerase chain reaction

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		14.3	
PCR buffer	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	10 mM	0.25	0.1 mM of each dNTP
G1-F primer	10 µM	1.00	0.4 µM
G2-R primer	10 µM	1.00	0.4 µM
DNA polymerase	5 U/µL	0.2	1 U
Subtotal		20.00	
DNA		5.00	
Total reaction volume of a single PCR reaction		25.00	

2.3. PCR cycling conditions: 3 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, a final step of 5 min at 72°C and cooling at 15°C.
2.4. Observations: if the expected target concentration is high, i.e. in enriched samples, 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 present in the sample. Amplification is performed on stock solution and the dilution.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCR tests not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that

amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Amplification of samples spiked with exogenous nucleic acid (control sequence) that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2. Interpretation of results

Verification of controls

- NIC and NAC no band is visualized
- PIC and PAC a band of 187 bp is visualized.
- If IPC are used, a band of the expected size is visualized.

When these conditions are met

- A test will be considered positive if a band of 187 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Performance characteristics are provided for the PCR test without enrichment

4.1. Analytical sensitivity data (according to a test performance study in 2010)

DNA extraction following Llop et al. (1999): 10^3–10^4 cfu/mL in plant extract

DNA extraction modified after Taylor et al. (2001): 10^4–10^5 cfu/mL in plant extract

4.2. Analytical specificity data (according to Taylor et al., 2001)

Target organisms tested: 69 strains all positive. Negative reaction with strains from *Rubus* sp.

Non-target organisms tested: 49 strains all negative.

4.3. Data on repeatability

In IVIA (ES): 100%

4.4. Data on reproducibility

In IVIA (ES): 100%

APPENDIX 8 - PCR ACCORDING TO GOTTSBERGER ADAPTED FROM OBRADOVIC ET AL. (2007)

The test below is described as it was carried out at IVIA and in a test performance study in 2010 to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification is carried out (see PM 7/98).

1. General information

1.1. This test is suitable for the detection of *Erwinia amylovora* in plant material and for identification of *E. amylovora* in pure bacterial colonies.

1.2. The original protocol and primers from Obradovic et al. (2007) were modified by Gottsberger for optimized specificity and maximum sensitivity in plant samples. The protocol was validated in the 2010 test performance study.

1.3. The targeted sequences are chromosomal.

1.4. Oligonucleotides:

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	FER1-F	5′-AGC AGC AAT TAA TGG CAA GTA TAG TCA−3′	458 bp
Reverse primer	rgER2R	5′-AAA AGA GAC ATC TGG ATT CAG ACA AT−3′	

1.5. Enzyme: the test performance study in 2010 was performed with a DNA polymerase from Biotools.

2. Methods

2.1. Nucleic acid extraction and purification: for plant material, two DNA extraction methods [the one according to Llop et al. (1999) and the one modified from Taylor et al. (2001)] described in Appendix 6 were evaluated in a test performance study.

2.2. Polymerase chain reaction

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		14.3	
1× PCR buffer	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM

	Working concentration	Volume per reaction (µL)	Final concentration
dNTPs	10 mM	0.25	0.1 mM of each of the dNTP
FER1-F	10 µM	1.00	0.4 µM
rgER2R	10 µM	1.00	0.4 µM
DNA polymerase	5 U/µL	0.2	1 U
Subtotal		20.00	
DNA		5.00	
Total reaction volume of a single PCR reaction		25.00	

2.3. PCR cycling conditions: 3 min at 94°C, 41 cycles of 10 s at 94°C, 10 s at 60°C and 30 s at 72°C, a final step for 5 min at 72°C and cooling at 15°C.

2.4. Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, to dilute inhibitor compounds. Amplification is performed on the stock solution and the dilution.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Amplification of samples spiked with exogenous nucleic acid (control sequence) that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2. Interpretation of results

Verification of controls

- NIC and NAC no band is visualized.
- PIC and PAC a band of 458 bp is visualized.
- If IPC are used, a band of the expected size is visualized.

When these conditions are met

- A test will be considered positive if a band of 458 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Performance characteristics are provided for the PCR test without enrichment

4.1. Analytical sensitivity data (according to the test performance study in 2010)

DNA extraction following Llop et al. (1999): 10^3–10^4 cfu/mL in plant extract

DNA extraction following Taylor et al. (2001) modified: 10^4–10^5 cfu/mL in plant extract

4.2. Analytical specificity data

According to Obradovic et al. (2007)

Target organisms tested: 44 strains all positive

Non-target organisms tested: 30 strains all negative

4.3. Data on repeatability

In IVIA (ES): 92%

4.4. Data on reproducibility

In IVIA (ES): 90%

APPENDIX 9 - REAL-TIME PCR (PIRC ET AL., 2009)

The tests below are described as they were carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification is carried out (see PM 7/98).

1. General information

1.1. The Ams and ITS tests are two independent tests that are suitable for the detection of *E. amylovora* in plant material and for identification of *E. amylovora* in pure bacterial colonies. They are performed as simplex. Pirc et al. (2009) recommend using the ITS test in screening when detection of lower concentrations is desired and/or to use the Ams test when a more specific test is necessary.

1.2. Real-time PCR tests designed by Pirc et al. (2009) are based on chromosomal sequences.

1.3. The targeted genes are *ams*C (Ams test) and 16S-23S rRNA intergenic spacer region (ITS test). Only the primer pair from *ams*C gene was evaluated in the test performance studies in 2009 and 2010. The two primer pairs were evaluated in the test performance study organized within the framework of the VALITEST project (Ališ et al., 2020; Trontin et al., 2021).

1.4. Oligonucleotides:

1.4.1. Ams test

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	Ams116F	5′-TCC CAC ATA CTG TGA ATC ATC CA−3′	74 bp
Reverse primer	Ams189R	5′-GGG TAT TTG CGC TAA TTT TAT TCG−3′	
Probe	Ams141T	5′-FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA−3′	

1.4.2. ITS test

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	ITS15F	5′-TGA GTA ATG AGC GAG CTA AGT GAA G−3′	79 bp
Reverse primer	ITS93R	5′-CGC AAT GCT CAT GGA CTC AA−3′	
Probe	ITS43T	5′-FAM-AGG CGT CAG CGC GCA GCA AC-TAMRA−3′	

1.5. Enzyme: included in the TaqMan Universal master mix (Applied Biosystems, USA).

1.6. Real-time PCR system (ABI PRISM 7900 HT Sequence Detection System or ViiA™ 7 Real-Time PCR System, Applied Biosystems) using the universal cycling conditions for all amplicons.

2. Methods

2.1. Nucleic acid extraction and purification:

Three DNA extraction methods were used as described in Appendix 6: (i) the silica-column based DNeasy Plant Mini Kit (Qiagen); (ii) the magnetic bead based QuickPick™ SML Plant DNA Kit (Bio-Nobile, Turku, Finland) with KingFisherR mL system (Thermo Labsystem); and (iii) the simple extraction method from Llop et al., 1999 [for the latter only 100 µL aliquots of crude sample extract were used (Pirc et al., 2009)].

2.2. Real-time polymerase chain reaction

2.2.1. Master Mix

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		1.00	
TaqMan universal master mix (Applied Biosystems)	2×	5.00	1×
Ams116F (or ITS15F)	10 µM	0.90	0.9 µM
Ams189R (or ITS93R)	10 µM	0.90	0.9 µM
Ams141T (or ITS43T)	10 µM	0.20	0.2 µM
Subtotal		8.00	

	Working concentration	Volume per reaction (µL)	Final concentration
DNA		2.00	
Total		10.00	

2.2.2. PCR 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.

2.2.3. Note: If the expected target concentration is high, i.e. in enriched samples, 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

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic acid (control sequence) that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

Verification of controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- The test should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Performance characteristics are provided for the PCR test without enrichment

4.1 Analytical sensitivity data

4.1.1 Ams test

Validation data available from the test performance study in 2010

DNA extraction following Llop et al. (1999) and Taylor et al. (2001) modified: 10^3–10^4 cfu/mL in plant extract

Validation data available from the National Biology Institute, SL

DNA extraction using DNeasy Plant Mini Kit (Qiagen): 2×10^3 cfu/mL

DNA extraction using QuickPick™ SML Plant DNA Kit (Bio-Nobile): 9×10^2 cfu/mL

DNA extraction following Llop et al. (1999): 1×10^4 cfu/mL

Validation data available from VALITEST preliminary study (National Biology Institute, SL)

5×10^3 cells/mL using DNA isolated from the target bacteria in pure culture

5×10^2–5×10^3 cells/mL using DNA isolated from plant extracts from four host plants (*Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*)

4.1.2 ITS test

Validation data available from VALITEST preliminary study (National Biology Institute, SL)

5×10^2 cells/mL using DNA isolated from the target bacteria in pure culture

5×10^2–5×10^3 cells/mL using DNA isolated from plant extracts from four host plants (*Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*)

4.2 Analytical specificity data

4.2.1 Ams test

Validation data from TPS carried out in 2010

Target organisms tested: 423 strains all positive

Non-target organisms tested: 97 strains all negative

Validation data available from VALITEST preliminary study (National Biology Institute, SL)

Analytical specificity was tested on 54 isolates, of these 30 target isolates of *E. amylovora* (28) and *E. amylovora* f. sp. *rubi* (2), and 24 non-target isolates including isolates of *E. piriflorinigrans* (6), *E. billingiae* (3), *E. tasmaniensis* (1), *E. pyrifoliae* (1), *E. gerundensis* (5), *P. agglomerans* (5) and three isolates at the border of *E. amylovora* species (MB2 from *Rosa rugosa*, Germany, 2000; 223b from *Prunus communis*, Hokkaido, which is closely related to *E. pyrifoliae*; ICMP 10125, *Erwinia* sp. from *P. pyrifoliae*, Australia).

Inclusivity: 100%

Exclusivity: 100%

4.2.2 ITS test

Validation data available from VALITEST preliminary study (National Biology Institute, SL)

Analytical specificity was tested on the same isolates as for Ams test.

Inclusivity: 93% (false-negative results with *E. amylovora* f. sp. *rubi* isolates ICMP 1841 and NCPPB 1859)

Exclusivity: 88% (false-positive results with isolates at the border of the species: MB2, 223b, ICMP 10125)

4.3 Diagnostic sensitivity and specificity

Validation data available from VALITEST TPS (National Biology Institute, SL)

The panel sent to participants consisted of 20 samples composed of 12 naturally infected *Malus* and *Pyrus* samples (two concentrations for each matrix), four spiked plant extracts of *Amelanchier* and *Pyracantha* and four healthy plant extracts of *Malus*:

Diagnostic sensitivity was 91% for the *ITS* test and 84% for the Ams test.

Diagnostic specificity was 94% for the *ITS* test and 97% for the Ams test.

The results support the original proposal of Pirc et al. (2009) to use the ITS test in screening when detection of lower concentrations is desired and/or to use the Ams test when a more specific test is necessary.

4.4 Data on selectivity

Validation data available from VALITEST TPS (National Biology Institute, SL)

The panel of samples included test items prepared from plant material of genera *Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*. Both tests performed well in *Malus*, *Amelanchier* and *Pyracantha*. The Ams test did not perform as well as the ITS test in *Pyrus*.

4.5 Data on repeatability

In IVIA (ES): 98%

4.6 Data on reproducibility

In IVIA (ES): 94%

APPENDIX 10 - REAL-TIME PCR (GOTTSBERGER, 2010)

The test below is described as it was carried out to obtain the validation data generated by Gottsberger et al., *2010 provided in section 4. Other equipment, kits or reagents may be used provided that a verification is carried out (see PM 7/98).*

1. General information

1.1. This test is suitable for the detection of *E. amylovora* in plant material and for identification of *E. amylovora* in pure bacterial colonies.

1.2. Real-time PCR targeting a hypothetical protein-coding gene was designed (Gottsberger, 2010). The accuracy in the 2010 test performance study could not be tested with this real-time PCR; however, it was tested by one laboratory in parallel with the real-time PCR described in Pirc et al. (2009) and gave the same qualitative results with the DNA extraction from Llop et al. (1999) protocol. This test was further evaluated in the test performance studies organized within the framework of the VALITEST project (Ališ et al., 2020; Trontin et al., 2021).

1.3. The target sequences are located in the chromosome.

1.4. Oligonucleotides:

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	hpEaF	5′-CCG TGG AGA CCG ATC TTT TA-3′	138 bp

	Primer	Sequence	Amplicon size (including primer sequences)
Reverse primer	hpEaR	5′-AAG TTT CTC CGC CCT ACG AT-3′	
Probe	hpEaP	5′-FAM-TCG TCG AAT GCT GCC TCT CT-MGB-3′	

1.5. Enzyme: included in the TaqMan Universal master mix (Applied Biosystems).
1.6. Real-time PCR system [Eppendorf Realplex Mastercycler Epgradient S, Eppendorf, Hamburg, Germany or ViiA™ 7 Real-Time PCR System (Applied Biosystems™)].

2. Methods

2.1. Nucleic acid extraction and purification: several DNA extraction methods were tested as described in Appendix 6: (i) the silica-column based DNeasy Plant Mini Kit (Qiagen) (DNA elution into 1 × 100 µL of AE buffer); (ii) the magnetic bead based QuickPick™ SML Plant DNA Kit (Bio-Nobile) and (iii) the simple extraction method from Llop et al., 1999. Further protocols were used are described in Stöger et al. (2006) and Persen et al. (2011).
2.2. Real-time polymerase chain reaction
2.2.1. Master Mix

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		6.00	
TaqMan Universal master mix (Applied Biosystems)	2×	10.00	1×
hpEaF	10 µM	1.00	0.5 µM*
hpEaR	10 µM	1.00	0.5 µM*
hpEaP	1 µM	1.00	0.05 µM*
Subtotal		19.00	
DNA		1.00*	
Total		20.00	

* In the framework of the VALITEST TPS, higher concentrations of primers (0.9 µM) and probe (0.2 µM), and a bigger volume of DNA (4 µL) were used.

2.2.2. PCR cycling conditions: 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C and 1 min at 60°C. In the framework of the VALITEST TPS, the amplification step consisted of 45 cycles.
2.2.3. Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a ten-fold 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

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Amplification of samples spiked with exogenous nucleic acid (control sequence) that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2. Interpretation of results

Verification of controls

- The PIC and PAC (as well as IPC if relevant) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- The test should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical sensitivity data

Validation data available from Gottsberger (2010) *(AGES, AT)*

2×10^3 cfu/mL.

Validation data available from VALITEST project preliminary study, National Biology Institute, SL

5×10^3 cells/mL using DNA isolated from the target bacteria in pure culture

5×10^2 cells/mL using DNA isolated from plant extracts from four host plants (*Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*)

4.2. Analytical specificity data

Validation data available from Gottsberger (2010) *(AGES, AT)*

Target organisms tested: 71 strains all positive

Non-target organisms tested: 41 strains all negative

Validation data from VALITEST project preliminary study, (National Biology Institute, SL)

Analytical specificity was tested on 54 isolates, of these 30 target isolates of *E. amylovora* (28) and *E. amylovora* f. sp. *rubi* (2), and 24 non-target isolates including isolates of *E. piriflorinigrans* (6), *E. billingiae* (3), *E. tasmaniensis* (1), *E. pyrifoliae* (1), *E. gerundensis* (5), *P. agglomerans* (5) and three isolates at the border of *E. amylovora* species (MB2 from *Rosa rugosa*, Germany, 2000; 223b from *Prunus communis*, Hokkaido which is closely related to *E. pyrifoliae*; ICMP 10125, *Erwinia* sp. from *P. pyrifoliae*, Australia).

Inclusivity: 100%

Exclusivity: 96% (false-positive result with ICMP 10125)

4.3. Diagnostic sensitivity and specificity

Validation data available from VALITEST TPS

The panel sent to participants consisted of 20 samples composed of 12 naturally infected *Malus* and *Pyrus* samples (two concentrations for each matrix), four spiked plant extracts of *Amelanchier* and *Pyracantha* and four healthy plant extracts of *Malus*:

Diagnostic sensitivity was 81%.

Diagnostic specificity was 97%.

4.4. Data on selectivity

Validation data available from VALITEST TPS

The panel of samples included test items prepared from plant material of genera *Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*. The test performed well in *Malus*, *Amelanchier* and *Pyracantha* but did not perform as well in *Pyrus*.

4.5. Data on repeatability

In AGES, AT: 100%

4.6. Data on reproducibility

In AGES, AT: 100%

APPENDIX 11 - LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) SHIN ET AL. (2018)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This test is suitable for the detection of *E. amylovora* in symptomatic plant material or for identification in pure bacterial colonies.

1.2. The test was developed by Shin et al. (2018) and was evaluated in the test performance studies organized within the framework of the VALITEST project (Ališ et al., 2020; Trontin et al., 2021).

1.3. The target sequence is located at a histidine-tRNA ligase gene of *E. amylovora*.

1.4. LAMP primers

	Primer	Sequence
Forward outer primer	Ea_Shin2018_F3	5′-ATA ATA AGA GAA TGG CGC TAT G-3′
Reverse outer primer	Ea_Shin2018_B3	5′-TCT ACA TCT CCA CCT TTG G-3′

	Primer	Sequence
Forward inner primer	Ea_Shin2018_FIP*	5′-TAA TGA AGT TGA ATC TCA GGC ATG AGA AAA AAT CCA TTG TAA AAC CTT CG-3′
Reverse inner primer	Ea_Shin2018_BIP*	5′-GAT GGA TTG CTT AGT GAG CTC AGC CAA TCT CTC CAC AAC CG-3′
Forward primer	Ea_Shin2018_LoopF	5′-AAA GTT GTT TTC ATC CCA CGG A-3′

1.5. LAMP reactions were run on QuantStudio™ Real-Time PCR System (Applied Biosystems).

2. Methods

2.1. Nucleic acid extraction and purification

The magnetic bead based QuickPick™ SML Plant DNA Kit (Bio-Nobile) was used as described in Appendix 6.

2.2. LAMP

2.2.1. Master mix:

To prevent pre-amplification it is important to prepare LAMP reactions on ice and keep them on ice until analysis.

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		Up to 20	
Isothermal Master Mix ISO 001 (Optigene Ltd., Horsham, UK)	10x	2.50	1×
ROX Reference Dye (supplied with Takara, Premix Ex Taq™)	50x	0.05*	0.1×
Ea_Shin2018_F3	10 µM	0.50	0.20 µM
Ea_Shin2018_B3	10 µM	0.50	0.20 µM
Ea_Shin2018_FIP	20 µM	2.00	1.60 µM
Ea_Shin2018_BIP	20 µM	2.00	1.60 µM
Ea_Shin2018_LoopF	20 µM	1.00	0.80 µM
Subtotal		20.00	
DNA		5.00	
Total		25.00	

* As different equipment requires different concentrations of ROX the value here is indicative. Please modify the concentration and the volume needed as suitable for your equipment.

2.2.2. LAMP cycling conditions: amplification for 30 min at 65°C followed by a melting curve (60–95°C, 0.05°C/s).

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue (when working with plant material) or clean extraction buffer (when working with pure culture); one per DNA extraction series.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism); one per DNA extraction series.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix; one per LAMP run.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product); one per LAMP run. For tests not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

3.2. Interpretation of results

Verification of controls

- NIC and NAC should produce no fluorescence.
- PIC and PAC: for real-time measurement, a positive reaction is defined by time of positivity of 30 min and melting temperature (TM) (84.65°C ± 0.171 on the Viia Real-time PCR System).

When these conditions are met

- A test will be considered positive if it produces a positive reaction as defined for PIC and PAC (see above).
- A test will be considered negative if it produces no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical sensitivity data (from VALITEST preliminary study, National Biology Institute, SL):

10^5 cells/mL using DNA isolated from the target bacteria in pure culture.

10^4–10^5 cells/mL using DNA isolated from plant extracts from four host plants (*Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*).

4.2. Analytical specificity data (from VALITEST preliminary study, National Biology Institute, SL):

Analytical specificity was tested on 54 isolates, of these 30 target isolates of *E. amylovora* (28) and *E. amylovora* f. sp. *rubi* (2), and 24 non-target isolates including isolates of *E. piriflorinigrans* (6), *E. billingiae* (3), *E. tasmaniensis* (1), *E. pyrifoliae* (1), *E. gerundensis* (5), *P. agglomerans* (5) and three isolates at the border of *E. amylovora* species (MB2 from *Rosa rugosa*, Germany, 2000; 223b from *Prunus communis*, Hokkaido, which is closely related to *E. pyrifoliae*; ICMP 10125, *Erwinia* sp. from *P. pyrifoliae*, Australia).

Inclusivity: 100%

Exclusivity: 100%

4.3. Diagnostic sensitivity and specificity (from VALITEST Test performance Study)

The panel sent to participants consisted of 20 samples composed of 12 naturally infected *Malus* and *Pyrus* samples (two concentrations for each matrix), four spiked plant extracts of *Amelanchier* and *Pyracantha* and four healthy plant extracts of *Malus*:

Diagnostic sensitivity was 65%.

Diagnostic specificity was 98%.

4.4. The relatively low diagnostic sensitivity observed in the VALITEST TPS is mostly explained by the presence in the panel of samples of materials with low concentration of *E. amylovora*. Diagnostic sensitivity in plant materials with active fire blight disease and characteristic symptom development was 95%. Data on selectivity (from VALITEST Test performance Study).

The panel included test items prepared from plant material of genera *Malus*, *Pyrus*, *Amelanchier* and *Pyracantha*. The test performed well in *Malus* and relatively well in *Pyrus* but the test did not performed well in *Amelanchier* and *Pyracantha*.

APPENDIX 12 - PATHOGENICITY TESTS

1. Detached organ assay

Biological tests made by inoculation of fruitlets (preferentially of susceptible cultivars of pear or, if not available and depending on the season, apple or loquat) can be performed on whole disinfected immature fruits or on slices of them, using 10 µL of 10^7 cfu/mL suspensions of colonies in PBS (Appendix 2). Alternatively, a needle laden with the freshly grown bacteria can be used to prick immature pears. Incubate in a humid chamber at 25°C for 3–7 days. A positive test on fruit is shown by browning around the wounding site and oozing of bacteria in 3–7 days (provided the negative control gives no lesion or only a necrotic lesion).

Detached young shoots from glasshouse-grown plants can also be inoculated in the same way, after disinfection for 30 s with 70% ethanol and washing three times with sterile distilled water, and kept in tubes with sterile 1% agar. Maintain the plants or tubes at 20–25°C at 80–100% relative humidity with 16 h light. Read results after 3, 7 and 15 days. Typical *E. amylovora* symptoms include wilting, discoloration, necrotic tissue and ooze.

For pathogenicity tests, using whole-plant inoculation, use susceptible cultivars of pear, apple or loquat, or susceptible species of *Crataegus*, *Cotoneaster* or *Pyracantha*. To inoculate a potted plant, cut a young leaf from a young shoot to the main vein with scissors dipped into a 10^9 cfu/mL suspension of each test colony prepared in PBS (Appendix 2).

For all the tests, positive and negative controls should be performed in parallel.

E. amylovora-like colonies should be re-isolated from inoculated fruitlets, plants or shoots showing typical symptoms and their identity confirmed.

2. Hypersensitive response in tobacco

Tobacco plants of cv. Xanthi, Samsun or White Burley with more than 5–6 leaves are used. Bacterial suspensions of 10^8–10^9 cfu/mL (OD at 620 nm = 1.0) are infiltrated by using a syringe without needle and by applying gentle and steady pressure while holding the open end of the syringe against the leaf until at least a 2- to 4-mm diameter area of mesophyll tissue is water-soaked (see Wick, 2010). Complete collapse of the infiltrated tissue after 24 h at room temperature is recorded as positive.