Food and Agriculture Organization of the United Nations

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Protecting the world's plant resources from pests

ISPM 27
ANNEX 13

ENG

DIAGNOSTIC PROTOCOLS INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DP 13:
Erwinia amylovora

Produced by the Secretariat of the
International Plant Protection Convention (IPPC)

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ISPM 27
Diagnostic protocols for regulated pests

DP 13: *Erwinia amylovora*

Adopted 2016; published 2016

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1. Pest Information

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 40 countries. It has not been recorded in South America and most African and Asian countries (with the exception of countries surrounding the Mediterranean Sea), and it has been eradicated in Australia after one report there (van der Zwet, 2004). It represents a threat to the pome fruit industry of all these countries (Bonn and van der Zwet, 2000). Details on geographic distribution can be found in the European and Mediterranean Plant Protection Organization (EPPO) Plant Quarantine Data Retrieval System (EPPO, n.d.).

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.*, 2011b).

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 a number of 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, continues into summer with shoot and fruit infection, and ends in winter with the development of cankers throughout the dormant period of the host (van der Zwet and Beer, 1995; Thomson, 2000).

2. Taxonomic Information

Name:	*Erwinia amylovora* (Burrill, 1883) Winslow *et al.*, 1920
Synonyms:	*Micrococcus amylovorus* Burrill, 1883, *Bacillus amylovorus* (Burrill, 1883) Trevisan, 1889, "*Bacterium amylovorus*" [sic] (Burrill, 1883) Chester, 1897, *Erwinia amylovora* f.sp. *rubi* (Starr *et al.*, 1951)
Taxonomic position:	Proteobacteria, Y subdivision, Enterobacteriales, Enterobacteriaceae
Common name:	Fire blight (EPPO, 2013)

3. Detection

Diagnosis of fire blight can be achieved using isolation and serological and molecular tests. The assays indicated below are recommended after having been evaluated in one or more of the following ring tests: in 2003 in a Diagnostic Protocols for Organisms Harmful to Plants (DIAGPRO) project involving ten laboratories (López *et al.*, 2006); in 2009 in a European Phytosanitary Research Coordination (EUPHRESCO) project involving five laboratories (Dreo *et al*., 2009); and in 2010 by fourteen laboratories worldwide (López *et al*., 2010). The tests indicated in Figures 1 and 2 are the minimum requirements for the diagnosis, but further tests may be required by the national plant protection organization (NPPO), especially for the first report in a country. For example, serological tests may facilitate a presumptive diagnosis of symptomatic plant material based on the detection of a specific protein; however, an additional test based on a different biological principle should be used for detection. In all tests, positive and negative controls must be included.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Detection in plants with symptoms

The recommended screening tests are indicated in the flow diagram in Figure 1.

3.1.1 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 similar and easily recognized. The name of the disease is descriptive of its major characteristic: the brownish, 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 exudate, and the characteristic "shepherd's crook" of terminal shoots. Depending on the affected plant part, the disease produces blossom blight, shoot or twig blight, leaf blight, fruit blight, limb or trunk blight, or collar or rootstock blight (van der Zwet and Keil, 1979; van der Zwet and 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, and become dark green and finally brown or black, sometimes oozing droplets of sticky bacterial exudate. 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. Upon infection young fruitlets turn brown but also 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 reddish-brown streaks are often found in the subcortical tissues when the bark is peeled from infected limbs or twigs (van der Zwet and Keil, 1979; Thomson, 2000). 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).

Confusion may occur between fire blight and blight- or blast-like symptoms – especially in blossoms and buds – caused by other pathogenic bacteria and fungi, insect damage or 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*, isolated from necrotic pear blossoms in Spain (López *et al.*, 2011); *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 *et al*., 2001a, 2001b; Palacio-Bielsa *et al*., 2012); and *Pseudomonas syringae* pv. *syringae*, the causal agent of blossom blast. A definitive diagnosis of fire blight should always be obtained through laboratory analysis.

3.1.2 Sampling and sample preparation

Plant material should be analysed as soon as possible after collection, but may be stored at 4–8 ºC for up to one week until processing. Precautions to avoid cross-contamination should be taken when collecting samples, during transport and processing, and especially while isolating the bacterium or extracting DNA.

The samples should be processed with a general procedure valid for isolation, serological tests and polymerase chain reaction (PCR) analysis. The use of freshly prepared antioxidant maceration buffer (polyvinylpyrrolidone (PVP)-10, 20 g; mannitol, 10 g; ascorbic acid, 1.76 g; reduced glutathione, 3 g; phosphate-buffered saline (PBS), 10 mM, 1 litre; pH 7.2; sterilized by filtration) is required for

successful enrichment, as indicated by Gorris *et al*. (1996). The samples can be processed also in sterile distilled water or in PBS, pH 7.2 (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre) but for direct isolation, immunofluorescence or PCR.

Plant parts (flowers, shoots, twigs, leaves or fruit) showing the most typical symptoms, and with bacterial exudate if possible, are carefully selected. Material for processing is selected from the leading edge of disease lesions. The plant tissue is cut into pieces of approximately 0.1–1.0 g, crushed lightly in antioxidant maceration buffer, PBS or sterile distilled water (as described in the previous paragraph) at 1:50 (w/v), left to stand for at least 5 min, and placed on ice for a few minutes. Triplicate samples (1 ml each) of each macerate are transferred to sterile microcentrifuge tubes, with one tube stored at –20 ºC for subsequent analysis by PCR and another tube's contents adjusted to 30% glycerol and stored at –80 ºC for confirmation testing, if necessary. The third tube is kept on ice for performing enrichment before enzyme-linked immunosorbent assay (ELISA) or PCR, and isolation on selective media (Figure 1). If immunofluorescence is to be performed (i.e. immunofluorescence analysis is optional), the slides are prepared and fixed on the same day that the samples are macerated. The PCR analysis should be performed as soon as is convenient, using the macerated sample stored at –20 ºC.

3.1.3 Isolation

3.1.3.1 Isolation from symptomatic samples

In general, plating on three media is advised for maximum likelihood of recovery of *E. amylovora*, especially when samples are not in good condition. Depending on the amount and composition of the microbiota of the sample, each medium can be more or less efficient. Three media (CCT, King's B and levan) have been validated in two ring tests, with levan having the highest plating efficiency.

When symptoms are very advanced or 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 and that can be overcrowded 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 described 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.The recipes for the recommended media are described below:

- CCT medium is prepared in two parts. Part 1 consists of: sucrose, 100 g; sorbitol, 10 g; Niaproof,4 1.2 ml; crystal violet, 2 ml (solvent 0.1% ethanol); nutrient agar, 23 g; distilled water, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 115 ºC for 10 min. The autoclaved medium is cooled to approximately 45 ºC. Part 2 consists of: thallium nitrate, 2 ml (1% w/v aqueous solution); cycloheximide, 0.05 g; sterilized by filtration. Part 2 is added to 1 litre sterile Part 1 (Ishimaru and Klos, 1984).
- King's B medium consists of: 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, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 120 ºC for 20 min (King *et al*., 1954).
- Levan medium consists of: yeast extract, 2 g; bactopeptone, 5 g; NaCl, 5 g; sucrose, 50 g; agar, 20 g; distilled water, 1 litre; pH 7.0–7.2; sterilized by autoclaving at 120 ºC for 20 min.

Cycloheximide is added at 0.05 g/litre to King's B and levan media when fungi are expected in the isolation. Dilutions of 1:10 and 1:100 of each macerate are prepared in PBS (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre).

Preferably 100 µl of the macerates and their dilutions is spread, by triple streaking, in 130 mm plates, or 50 µl is spread in standard 90 mm Petri dishes. Plates are incubated at 25 ºC for up to four days. The final reading is usually taken at 72 h. Colonies of *E. amylovora* on CCT medium are pale violet, circular, high convex to domed, smooth and mucoid, and they grow more slowly than on King's B or levan media. Colonies on King's B medium are creamy white, circular and non-fluorescent under

ultraviolet (UV) light at 366 nm. Colonies on levan medium are white, circular, domed, smooth and mucoid. Levan-negative colonies of *E. amylovora* have been reported (Bereswill *et al*., 1997).

Pure cultures are obtained from individual suspect colonies of each sample by dilution and streaking onto King's B medium. Presumptive colonies of *E. amylovora* are identified preferably by double antibody sandwich indirect (DASI)-ELISA, PCR or by other appropriate tests (e.g. biochemical, immunofluorescence, fatty acid profile), or by inoculating susceptible organs of any available *E. amylovora* host to test pathogenicity, as indicated in section 4.

When analysing symptomatic samples, good correlation is expected between isolation, immunofluorescence, enrichment-DASI-ELISA (section 3.1.4.1) and PCR.

In the 2003 and 2010 ring tests, the accuracy of isolation was 0.88 and 0.81 for King's B, 0.92 and 0.89 for levan, and 0.92 and 0.95 for CCT media, respectively (López *et al.*, 2006; M.M. Lopez, personal communication, 2012). In the 2009 ring test, accuracy of isolation was 0.96 for CCT (Dreo *et al*., 2009).

3.1.3.2 Enrichment-isolation

Enrichment is used to multiply the initial population of culturable *E. amylovora* in a sample and to perform enrichment-DASI-ELISA or enrichment-PCR. It should be carried out before isolation (even for symptomatic samples) when a low number of culturable *E. amylovora* cells is expected to be present (e.g. for copper-treated samples, samples with old symptoms, samples collected during unfavourable weather conditions for fire blight such as in winter). The enrichment step greatly increases the sensitivity of DASI-ELISA. The use of two validated liquid media for enrichment – one non-selective (King's B) and one semi-selective (CCT) – is advised because the composition and population size of the microbiota are unknown.

The tissue sample is macerated as described in section 3.1.2 and 0.9 ml is immediately dispensed into each of two sterile 10–15 ml tubes (to ensure sufficient aeration) containing 0.9 ml of each liquid enrichment medium (King's B without agar, and CCT made with nutrient broth instead of nutrient agar). The tubes are incubated at 25 ºC for 48–72 h without shaking. A longer incubation is recommended when processing plant samples collected in winter. Both enrichment broths and dilutions (1:10 and 1:100) prepared in PBS are spread onto CCT plates, by triple streaking, to obtain isolated colonies. Plates are incubated at 25 ºC for 72–96 h. Final reading of the CCT plates is at 72 h and must be followed by purification of colonies and identification.

The use of semi-selective medium for plating and dilution is advised because the enrichment step will permit growth of the pathogen but will also allow abundant multiplication of other bacteria. The accuracy of the enrichment isolation on King's B and CCT was 0.97 in the 2010 ring test.

3.1.4 Serological detection

3.1.4.1 Enrichment-DASI-ELISA

A kit for enrichment-DASI-ELISA has been validated in two ring tests and is available commercially from Plant Print Diagnòstics SL[1]. It is based on the mixture of two specific monoclonal antibodies described in Gorris *et al*. (1996) and requires prior enrichment of the samples, as previously described. The following protocol must be followed strictly for maximum accuracy. Before ELISA, the required amount of the enriched extracts and controls is treated by incubation in a water bath at 100 ºC for 10 min. This treatment is necessary for optimum specificity. The boiled samples are processed (at

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room temperature) by ELISA on the same day (or stored at –20 ºC for subsequent analysis) following the instructions provided by the manufacturer of the commercial kit.

The ELISA is negative if the average optical density (OD) reading from duplicate sample wells is <2× the OD in the negative sample extract control wells (providing the OD for the positive control wells are above 1.0 after 90 min incubation and are greater than twice the OD obtained for the negative sample extracts). The ELISA is positive if the average OD reading from duplicate sample wells is >2× the OD in the negative sample extract control wells (providing all negative control wells are lower than 2× the average OD reading of the positive control wells).

Negative ELISA readings in positive control wells indicate that the test has not been performed correctly and/or the reagents were not well prepared. Positive ELISA readings in negative control wells indicate cross-contamination or non-specific antibody binding. In both cases, the test should be repeated or a second test based on a different biological principle, such as PCR, should be performed.

In the 2003 and 2010 ring tests the accuracy of the DASI-ELISA was 0.79 and 0.82, respectively, for enrichment in King's B medium (King's B-DASI-ELISA), and 0.83 and 0.77, respectively, for enrichment in CCT medium (CCT-DASI-ELISA) (López *et al.*, 2006, 2010).

3.1.4.2 Direct tissue print-ELISA

To make tissue prints, freshly cut plant sections are pressed carefully against a nitrocellulose membrane. Prints are prepared for positive and negative controls. Printed membranes can be kept for several months in a dry place at room temperature. A validated source of antibodies to *E. amylovora* such as the Plant Print Diagnòstics SL kit[1] should be used. To develop prints, the manufacturer's instructions should be followed. The prints are observed under low power magnification (×10 or ×20). The test is positive when purple–violet precipitates appear in the sections of plant tissue that are printed on the membrane and not in the plant tissue print of the negative control. If exudates or colonies are printed they should appear violet when positive. The test is negative when no purple–violet precipitates appear, as in the negative control.

3.1.4.3 Immunofluorescence

Immunofluorescence is a recommended alternative serological method, and it is easy to follow the standard protocol (Anonymous, 1998). A validated source of antibodies to *E. amylovora* should be used. Two commercial antibodies have been validated in one ring test: one monoclonal antibody is available through Plant Print Diagnòstics SL[1] and one polyclonal antibody is available from Loewe Biochemicals[1].

Immunofluorescence should be performed on fresh sample extracts fixed onto slides. Undiluted macerates and dilutions of 1:10 and 1:100 in PBS are used to spot windows of the immunofluorescence slides. The monoclonal or polyclonal antibody is used at the appropriate dilution in PBS. The appropriate fluorescein isothiocyanate (FITC) conjugate is diluted in PBS: goat anti-mouse for monoclonal antibody (GAM-FITC), and goat anti-rabbit (GAR-FITC) or anti-goat for polyclonal antibody.

The test on a sample is negative if green fluorescing cells with morphology typical of *E. amylovora* are observed in the positive controls, but not in the sample windows. The test on a sample is positive if green fluorescing cells with typical morphology are observed in the positive controls and in the sample windows, but not in the negative controls. As a population of 10^3 cells/ml is considered the limit for reliable detection by immunofluorescence, for samples with $>10^3$ cells/ml, the immunofluorescence test is considered positive. For samples with $<10^3$ cells/ml, or weakly fluorescing cells, the result of the immunofluorescence may be considered uncertain.

The accuracy of immunofluorescence in the 2003 ring test was 0.70 for the Plant Print Diagnòstics SL[1] monoclonal antibody, and 0.72 for the Loewe Biochemicals[1] polyclonal antibodies confirming that the sensitivity of the technique is approximately 10^3 colony-forming units (c.f.u.)/ml.

3.1.4.4 Lateral flow immunoassay

Two lateral flow devices are available commercially for rapid analysis of plant material: Ea AgriStrip (Bioreba[1]) and Pocket Diagnostics (Forsite Diagnostics[1]). Following the manufacturers' instructions their accuracy in the 2009 and 2010 ring tests was 0.66 and 0.55, respectively, for Ea AgriStrip[1] and 0.64 and 0.56, respectively, for Pocket Diagnostics[1]. These results were obtained for the detection of *E. amylovora* in samples from 1 to10^6 c.f.u./g, but the accuracy was approximately 1.0 when analysing samples with 10^5 to 10^6 c.f.u./g, the minimum number expected in symptomatic samples (López *et al.*, 2010). The kits are recommended for use only with symptomatic samples.

3.1.5 Molecular detection

Several PCR methods and one loop-mediated isothermal amplification (LAMP) protocol[2], available for the detection of *E. amylovora*, were evaluated extensively in ring testing by several laboratories (Lopez *et al.*, 2010; M.M. Lopez, personal communication, 2012). The specificity of some of these methods has been evaluated by Powney *et al*. (2011a). Conventional PCR methods may be more expensive and time consuming and usually require more training than serological methods, and for these reasons, as well as the risk of contamination, they are not always appropriate for large-scale testing. However, real-time PCR and some conventional PCR and nested PCR in one tube protocols have provided highly accurate results and they are therefore recommended molecular methods. All PCR assays should be performed using DNA extracted from the samples because of the high amount of inhibitors of *E. amylovora* hosts, or from enriched samples, which have increased reliability of detection.

3.1.5.1 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control

This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) nucleic acid, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used.

Internal control

For conventional and real-time PCR, plant internal controls (e.g. a housekeeping gene (HKG) such as COX (Weller *et al.*, 2000) or 16S ribosomal (r)DNA (Weisberg *et al.*, 1991)) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control)

This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

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

Positive extraction control

This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality and that the target is detected. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequence obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control

This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control compromises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positive samples are expected.

3.1.5.2 DNA extraction

Three DNA extraction methods – Llop *et al.* (1999), Taylor *et al.* (2001) and the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]) – were evaluated in the 2009 ring test (Dreo *et al*., 2009) with four PCR protocols with accuracies ranging from 0.67 to 0.76. The methods showed comparable results in the 2010 ring test (Lopez *et al.*, 2010), as indicated below in the accuracies given for the different PCR methods. Their efficiencies did not improve after diluting the extracts 1:10, suggesting that few or no inhibitors were present. Based on these findings, the Llop *et al.* (1999) extraction method is recommended as it has been extensively tested in a number of countries and is cheap and easy to set up in the laboratory.

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

One millilitre of a sample macerate prepared according to section 3.1.2 and/or 1 ml enriched macerate is centrifuged at 10 000 *g* for 5 min at room temperature. The supernatant is discarded, and the pellet is resuspended in 500 µl extraction buffer (Tris-HCl pH 7.5, 24.2 g; NaCl, 14.6 g; ethylenediaminetetraacetic acid (EDTA), 9.3 g; sodium dodecyl sulphate (SDS), 5 g; PVP-10, 20 g; distilled water, 1 litre; sterilized by filtration) and incubated for 1 h at room temperature before centrifugation at 4 000 *g* for 5 min. Approximately 450 µl supernatant is mixed with an equal volume of isopropanol, inverted, and left at room temperature for 30 min to 1 h. The precipitated nucleic acid is centrifuged at 10 000 *g* for 5 min, the supernatant is discarded and the pellet is air-dried. If there is still a coloured precipitate (brown or green) at the bottom of the tube, this is carefully removed while discarding the supernatant, thus obtaining a cleaner DNA pellet. The pellet is resuspended in 200 µl water. It should be used for PCR immediately or stored at –20 ºC.

3.1.5.3 DNA amplification by PCR

There are many PCR primers and protocols described for *E. amylovora* detection and some have shown specificity problems (Roselló *et al.*, 2006; Powney *et al.*, 2011a). The primers and protocols validated in ring tests were those of Bereswill *et al.* (1992) and Llop *et al.* (2000), with or without previous enrichment, in 2003; and those of Taylor *et al.* (2001), Stöger *et al.* (2006) and Obradovic *et al.* (2007) in 2009 and 2010. The discovery of fully virulent *E. amylovora* strains without the pEA29 plasmid (Llop *et al.*, 2006) and experiences from different countries (Powney *et al.*, 2011a) indicate that two PCR protocols should be used: one with primers based on pEA29 sequences, and another with primers targeting unique chromosomal sequences. If the PCR is negative with the protocol based on the pEA29 primers and positive with the protocol based on the chromosomal

primers, the PCR test can be considered as positive for *E. amylovora*. PCR can be carried out using the primers and conditions validated in the ring tests, although amplification conditions should be optimized for different thermocyclers.

PCR according to Bereswill* et al. *(1992)

The primers are:

A (forward): 5′-CGG TTT TTA ACG CTG GG-3′

B (reverse): 5′-GGG CAA ATA CTC GGA TT-3′

The targeted sequences are in the plasmid pEA29. The PCR mixture is composed of: ultrapure water, 17.4 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 1.5 µl; dNTPs 10 mM, 0.5 µl; primer A 10 pmol/µl, 0.25 µl; primer B 10 pmol/µl, 0.25 µl; and Taq DNA polymerase 5 U/µl, 0.1 µl. The extracted DNA sample volume is 2.5 µl, and should be added to 22.5 µl of the PCR mix. The cycling parameters are a denaturation step of 93 ºC for 5 min followed by 40 cycles of 93 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 1 min 15 s, with a final elongation step at 72 ºC for 10 min. The amplicon size is 900 base pairs (bp) according to Bereswill *et al*. (1992), although variations in size can occur between 900 and 1 100 bp depending on the number of 8 bp repeats within the amplified fragment (Jones and Geider, 2001).

The accuracy was 0.51 in the 2003 ring test but increased to 0.74 and 0.78 after enrichment of the samples in King's B and CCT media, respectively (López *et al.*, 2006).

PCR according to Taylor* et al. *(2001)

The primers are:

G1-F: 5′-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG-3′

G2-R: 5′-GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC-3′

The targeted sequences are chromosomal. The PCR mixture is composed of: ultrapure water, 14.3 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 0.75 µl; dNTPs 10 mM, 0.25 µl; G1-F 10pmol/µl, 1 µl; G2-R 10pmol/µl, 1 µl; and Taq DNA polymerase 5 U/µl, 0.2 µl. An extracted DNA sample of 5 µl is added to 45 µl PCR mix. The cycling parameters are 95 ºC for 3 min followed by 40 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 1 min, with a final extension step at 72 ºC for 5 min, and cooling at 15 ºC. The expected amplicon size is 187 bp.

The accuracy was 0.77 in the 2010 ring test using the Llop *et al.* (1999) DNA extraction procedure.

PCR according to Stöger* et al. *(2006)

The primers (from Llop *et al*., 2000) are:

PEANT1-F: 5′-TAT CCC TAA AAA CCT CAG TGC-3′

PEANT2-R: 5′-GCA ACC TTG TGC CCT TTA-3′

The targeted sequences are in the plasmid pEA29. Stöger *et al.* (2006) recommended this method be used with DNA extracted using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]). The PCR mixture is composed of: ultrapure water, 5 µl; REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich[1]), 10 µl; PEANT1-F 10 pmol/µl, 0.5 µl; PEANT2-R 10 pmol/µl, 0.5 µl; and extracted DNA, 4 µl. The cycling parameters are 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 5 min, and cooling at 15 °C. The expected amplicon size is 391 bp.

The accuracy was 0.76 in the 2009 ring test and 0.72 in the 2010 ring test with the recommended DNA extraction kit.

PCR according to Gottsberger (2010) (adapted from Obradovic* et al. *(2007))

The primers are:

FER1-F: 5′-AGC AGC AAT TAA TGG CAA GTA TAG TCA-3′

rgER2-R: 5′-AAA AGA GAC ATC TGG ATT CAG ACA AT-3′

The targeted sequences are chromosomal. The PCR mixture is composed of: ultrapure water, 14.3 µl; buffer 10×, 2.5 µl; $MgCl_2$ 50 mM, 0.75 µl; dNTPs 10 mM, 0.25 µl; FER1-F 10 pmol/µl, 1 µl; rgER2-R 10 pmol/µl, 1 µl; Taq DNA polymerase 5 U/µl, 0.2 µl; and extracted DNA, 5 µl. The cycling parameters are 94 ºC for 3 min followed by 41 cycles of 94 ºC for 10 s, 60 ºC for 10 s and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min, and cooling at 15 ºC. The expected amplicon size is 458 bp.

The accuracy was 0.76 in the 2009 ring test and 0.68 in the 2010 ring test using the DNA extraction method described by Llop *et al.* (1999).

Nested PCR according to Llop* et al. *(2000)

The nested PCR of Llop *et al*. (2000) uses two sets of primers, which are combined in a single reaction tube. Because of the different annealing temperatures of the primers the two PCRs are run consecutively. The external primers are those designed by McManus and Jones (1995) and are based on sequences of the pEA29 plasmid. The internal primers are those described by Llop *et al*. (2000).

The external primers are:

AJ75-F: 5′-CGT ATT CAC GGC TTC GCA GAT-3′

AJ76-R: 5′-ACC CGC CAG GAT AGT CGC ATA-3′

The internal primers are:

PEANT1-F: 5′-TAT CCC TAA AAA CCT CAG TGC-3′

PEANT2-R: 5′-GCA ACC TTG TGC CCT TTA-3′

The PCR mixture is composed of: ultrapure water, 36.25 µl; buffer 10×, 5 µl; $MgCl_2$ 50 mM, 3 µl; dNTPs 10 mM, 0.5 µl; AJ75-F 0.1 pmol/µl, 0.32 µl; AJ76-R 0.1 pmol/µl, 0.32 µl; PEANT1-F 10 pmol/µl, 1 µl; PEANT2-R 10 pmol/µl, 1 µl; and Taq DNA polymerase 5 U/µl, 0.6 µl. A DNA sample volume of 2 µl should be added to 48 µl PCR mix. The cycling parameters are a denaturation step of 94 ºC for 4 min followed by 25 cycles of 94 ºC for 60 s and 72 ºC for 90 s. This first round PCR is followed in the same thermocycler by a second denaturation step of 94 ºC for 4 min and 40 cycles of 94 ºC for 60 s, 56 ºC for 60 s and 72 ºC for 60 s, with a final elongation step at 72 ºC for 10 min. The expected amplicon size is 391 bp, although variations in size can occur.

The accuracy was 0.69 and 0.72 in the 2003 and 2010 ring tests, respectively, but increased after enrichment to 0.84 (King's B medium) and 0.86 (CCT medium) in the 2003 ring test, and to 0.79 (King's B) and 0.88 (CCT) in 2010.

3.1.5.4 General considerations for PCR

The PCR protocols may need to be modified (optimized) when using different reagents or thermocyclers.

After PCR amplification the presence of *E. amylovora* can be confirmed by sequencing the PCR products or by restriction fragment length polymorphism (RFLP) analysis. The restriction pattern observed in the amplicons obtained with the primers of Bereswill *et al.* (1992) or with the nested PCR of Llop *et al.* (2000) can be used to confirm the specificity of the PCR analysis when compared with the restriction pattern of a known control strain. Restriction digestion should be performed with the endonucleases DraI and SmaI.

The test on a sample is negative if the E. amylovora-specific amplicon of the expected size (and the restriction enzyme pattern or amplicon sequence, when applicable) is not detected in the sample but is detected in all positive controls. The test on a sample is positive if the E. amylovora-specific amplicon of the expected size is detected, providing there is no amplification from any of the negative controls and the restriction enzyme pattern or amplicon sequence (when applicable) is indicative of E. amylovora.

3.1.5.5 Real-time PCR

Based on an evaluation of real-time PCR protocols in the ring tests in 2009 and 2010 (Dreo *et al.*, 2009; Lopez *et al.*, 2010) the protocol described by Pirc *et al.* (2009), which targets chromosomal sequences, was recommended. A duplex real-time PCR based on chromosomal sequences is also available but has not been ring tested (Lehman *et al.*, 2008).

Real-time PCR according to Pirc et al. (2009)

The following oligonucleotides are used:

Ams116F primer: 5′-TCC CAC ATA CTG TGA ATC ATC CA-3′

Ams189R primer: 5′-GGG TAT TTG CGC TAA TTT TAT TCG-3′

Ams141T probe: FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA

The reaction is carried out in a final volume of 25 µl. The PCR mixture is composed of: ultrapure water, 2.5 µl; 2× TaqMan Fast Universal PCR Master Mix (Applied Biosystems[1]), 12.5 µl; Ams116F 10 pmol/µl, 2.25 µl; Ams189R 10 pmol/µl, 2.25 µl; FAM-labelled Ams141T 10 pmol/µl, 0.5 µl; and 5 µl DNA extract (added to the 20 µl PCR mix). The cycling parameters are: 2 min at 50 ºC; 10 min at 95 ºC; and 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. The standard mode for temperature ramping rates on analysers 7900HT and 7900HT Fast (Applied Biosystems[1]) are: 1.6 ºC/s up and 1.6 ºC/s down. It is possible to run reactions at slower ramp rates, but with faster ramp rates (up and down at approximately 3.5 ºC/s) the results were not acceptable. The expected amplicon size is 74 bp.

For analysis of the real-time PCR results, there are usually different options available, automatic or manual, for setting the signal and noise limits. The instructions for the appropriate software should be followed. The baseline should be set automatically, and the threshold should be set manually crossing the exponential phase of the control amplification curves.

The accuracy in the 2010 ring test was 0.80, 0.85 and 0.76 with the DNA extraction method of Llop *et al.* (1999), the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich[1]) and Taylor *et al.* (2001), respectively.

Real-time PCR according to Gottsberger (2010)

The following oligonucleotides that target the *E. amylovora* chromosome are used:

hpEaF primer: 5′-CCG TGG AGA CCG ATC TTT TA-3′

hpEaR primer: 5′-AAG TTT CTC CGC CCT ACG AT-3′

hpEaP probe: FAM-TCG TCG AAT GCT GCC TCT CT-MGB

The reaction is carried out in a final volume of 20 µl. The PCR mixture is composed of: ultrapure water, 6 µl; 2× TaqMan Universal PCR Master Mix (Applied Biosystems[1]), 10 µl; hpEaF 10 pmol/µl, 1 µl; hpEaR 10 pmol/µl, 1 µl; hpEaP 1 pmol/µl, 1 µl; and 1 µl DNA extract (added to the 19 µl PCR mix). The cycling parameters are: 2 min at 50 °C; 10 min at 95 °C; and 50 cycles of 15 s at 95 °C and 1 min at 60°C. The expected amplicon size is 138 bp.

For analysis of the real-time PCR results, there are usually different options available, automatic or manual, for setting the signal and noise limits. The instructions for the appropriate software should be followed. The baseline should be set automatically, and the threshold should be set manually crossing the exponential phase of the control amplification curves.

The accuracy of this real-time PCR could not be tested in the 2010 ring test; however, it was tested in parallel with the real-time PCR of Pirc *et al.* (2009) by one laboratory and gave the same qualitative results with the DNA extraction from Llop *et al.* (1999).

3.1.5.6 Interpretation of results from PCR

Conventional PCR

The pathogen-specific PCR will be considered valid only if:

(1) the positive control produces the correct size amplicon for the bacterium

(2) no amplicons of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

If the 16S rDNA internal control primers are also used, the negative (healthy plant tissue) control (if used), positive control and each of the test samples must produce a 1.6 kilobase (kb) amplicon (16S rDNA). Note that synthetic or plasmid-positive controls will not produce a 1.6 kb amplicon. Failure of the samples to amplify with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size.

Real-time PCR

The real-time-PCR will be considered valid only if:

(1) the positive control produces an amplification curve with the pathogen-specific primers

(2) no amplification curve is produced (i.e. cycle threshold (Ct) value is 40) in the negative extraction control and the negative amplification control.

If the COX internal control primers are also used, the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The test on a sample will be considered positive if it produces a typical amplification curve in an exponential manner. The Ct value needs to be verified in each laboratory when implementing the test for the first time.

3.1.5.7 Loop-mediated isothermal amplification

The LAMP protocol was developed and described by Temple *et al.* (2008) and Temple and Johnson (2011). It was evaluated in the 2010 ring test because it was considered appropriate for laboratories not equipped for PCR and it is easy to perform. In the ring test, the LAMP protocol using primers to detect the chromosomal gene *amsL* of *E. amylovora* was found to lack appropriate sensitivity for analysis of samples with low bacterial populations. Consequently, the LAMP protocol described below to detect chromosomal *amsL* is recommended only for the analysis of symptomatic samples with more than 10^5–10^6 c.f.u./ml. The protocol from Temple and Johnson (2011) using primers to detect pEA29 was not evaluated in the ring test.

The LAMP primers to detect *amsL* Bare:

ALB Fip: 5′-CTG CCT GAG TAC GCA GCT GAT TGC ACG TTT TAC AGC TCG CT-3′

ALB Bip: 5′-TCG TCG GTA AAG TGA TGG GTG CCC AGC TTA AGG GGC TGA AG-3′

ALB F: 5′-GCC CAC ATT CGA ATT TGA CC-3′

ALB B: 5′-CGG TTA ATC ACC GGT GTC A-3′

Primers Fip and Bip were used at 2.4 µM and primers F and B at 0.2 µM final concentrations. Melting temperatures for primers were between 58 and 60 °C. The LAMP reaction mixture is composed of: 10× ThermoPol buffer (New England Biolabs[1]), 5 µl; dNTPs 10 mM, 5 µl; $MgSO_4$ 100 mM, 2 µl; bovine serum albumin (BSA) 10 mg/ml, 2 µl; ALB Fip 100 µM, 1.2 µl; ALB Bip 100 µM, 1.2 µl; ALB F 10 µM, 1 µl; ALB B 10 µM, 1 µl; *Bst* DNA polymerase 8 U/µl, 2 µl; template DNA, 5 µl; and ultrapure water, 24.6 µl. Note that the *Bst* DNA polymerase, template DNA and ultrapure water are not added to the master mix, but are added separately after aliquoting the master mix. Before starting the LAMP reaction, a water bath or a thermocycler is set at 65 °C. The mix is prepared and 18.4 µl is pipetted into each individual 0.2 ml PCR tube. The *Bst* DNA polymerase, template DNA and ultrapure water are then pipetted separately into each tube with master mix. The tubes are spun down in a plate spinner (1 000 r.p.m. for 30 s) and are placed in the water bath (65 °C) in a holder so the reaction end is submerged, or in the thermocycler (65 °C) for 55 min. The tubes are removed and allowed to cool for 10 s.

The test on a sample is positive if the presence of precipitate as cloudiness in the tube or the presence of a solid white magnesium pyrophosphate precipitate at the bottom of the tube is observed, as for the positive control. A clear solution indicates a negative test result, as should be observed for the negative control.

The accuracy in the 2010 ring test was 0.64, but for samples with 10^5–10^6 c.f.u./ml the accuracy was 0.80. For this reason LAMP is recommended only for the analysis of symptomatic samples.

3.2 Detection in asymptomatic plants

The recommended screening tests are indicated in the flow diagram in Figure 2.

3.2.1 Sampling and sample preparation

Asymptomatic samples can be processed individually (preferred) or in groups of up to 100 (EPPO, 2013). 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 protocols:

- 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 ºC (van der Zwet and 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 ten 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 plant material is weighed and macerated in antioxidant buffer following the protocol described in section 3.1.2.
- A sampling procedure reported but not validated for the analysis of twigs of asymptomatic woody material from nurseries is as follows. A sample comprises 100 twigs, each about 10 cm in length, from 100 plants. 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). From each sample 30 twigs are randomly taken and 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. The filtrate is used directly for analysis or centrifuged at 10 000 *g* for 20 min. The pellet is suspended in 4.5 ml sterile PBS. The detection techniques indicated below are performed. A similar protocol can be applied for leaves, shoots, flowers and buds.

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, PCR and isolation using the protocols described for each technique for symptomatic samples in López *et al.* (2006). Immunofluorescence is optional; if done, it must be done directly on the extracts, before enrichment.

3.2.2 Screening tests

Direct analysis of asymptomatic samples is normally negative for *E. amylovora* because of the low bacterial population. Consequently, when analysing asymptomatic material, it is an absolute requirement to perform enrichment from samples prepared in the antioxidant buffer (section 3.2.1) (Gorris *et al.*, 1996) for 72 h at approximately 25 ºC. It is advisable to perform at least two of these screening tests based on different biological principles:

- Enrichment-isolation. Follow the procedure for symptomatic samples (section 3.1.3.2).
- Enrichment-DASI-ELISA. Follow the procedure for symptomatic samples (section 3.1.4.1).
- Enrichment-PCR or enrichment-real-time PCR. Use 500–1000 µl of the samples enriched in King's B and/or CCT media for DNA extraction, then follow the procedure for amplification according to Taylor *et al.* (2001) or Llop *et al.* (2000) (section 3.1.5.3) or the real-time PCR protocols (section 3.1.5.5).

If any of the screening tests are positive but isolation is negative, isolation of the pathogen from the extract stored at –80 ºC with glycerol or from the enriched samples should be attempted. When three tests or more are positive and the isolation is negative, it is reasonable to strongly suspect the presence of *E. amylovora* in the sample, but identification and confirmation require isolation of the pathogen from new samples and subsequent identification of the bacterium.

4. Identification

Identification should be based on results obtained from several techniques because other species of *Erwinia* such as *E. piriflorinigrans* (López *et al.*, 2011), *E. pyrifoliae* (Kim *et al.*, 1999; Rhim *et al.*, 1999), *E. uzenensis* (Matsuura et al., 2012) and other *Erwinia* spp. (Kim *et al.*, 2001a, 2001b; Palacio-Bielsa *et al.*, 2012) share similar morphological, serological and molecular characteristics to that of *E. amylovora*. Differentiation of *E. amylovora* from these closely related *Erwinia* species (that can be found in similarly symptomatic tissues in some hosts) can be achieved with a combination of three techniques based on different biological principles:

- PCR based on chromosomal DNA (sections 3.1.5.2 and 4.3.1)
- DASI-ELISA using specific monoclonal antibodies as described for detection (section 3.1.4.1, excluding the enrichment step)
- Inoculation into fire blight hosts to fulfil the requirements of Koch's postulates, including re-isolation of the inoculated pathogen (section 4.4).

For identification of colonies, at least two of these three techniques are recommended to be used. Other tests can also be used depending on the experience of the laboratory; these are described below. When required, the final confirmation of a culture's identification should include a pathogenicity test.

The *E. amylovora* isolates recommended for use as positive controls are NCPPB 683 and CFBP 1430. The following collections, among others, can provide different *E. amylovora* reference strains: National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, York, United Kingdom; Collection Française de Bactéries Phytopathogènes (CFBP), French National Institute for Agricultural Research (INRA), Station Phytobactériologie, Angers, France; Belgian Co-ordinated Collection of Micro-organisms BCCM/LMG Bacteria Collection, Ghent, Belgium; International Collection of Microorganisms from Plants (ICMP), Manaaki Whenua Landcare Research, Auckland, New Zealand; and American Type Culture Collection (ATTC), Manassas, VA, United States. The authenticity of the strains can be guaranteed only if directly obtained from the culture collections.

4.1 Nutritional and enzymatic identification

Key phenotypic tests are useful and are still used for identification, but it is advised to combine them with pathogenicity assays and a serological or molecular test. Members of the genus *Erwinia* are defined as Gram-negative, facultative anaerobes, motile by peritrichous flagella, rod-shaped, and able to produce acid from glucose, fructose, galactose and sucrose. Key phenotypic properties (Paulin, 2000) that are common to most strains in *E. amylovora*, according to the methods of Jones and Geider (2001), are: oxidase test (–), oxidative/fermentative (O/F) test (+/+), fluorescent pigment in King's B medium under UV light (–), levan production (+), nitrate reduction (–), citrate utilization (+), gelatine liquefaction (+), urease and indol (–) and colony morphology on CCT medium.

The following tests differentiate *E. amylovora* from *E. pyrifoliae* and *E. piriflorinigrans*, although some physiological and biochemical characteristics may vary for some strains (Table 1).

Table 1. Differences among *Erwinia amylovora*, *Erwinia pyrifoliae* and *Erwinia piriflorinigrans*

Microbiological test	*Erwinia amylovora*	*Erwinia pyrifoliae*	*Erwinia piriflorinigrans*
Gelatin hydrolysis	+	–	–
Inositol†	–	ND	+
Sorbitol†	+	+	–
Aesculin†	V	–	+
Melibiose†	–	–	+
D-Raffinose†	–	–	+
β-Gentiobiose†	+	–	+
Amplification with‡ EP16A/EPI62C CPS1/CPS2C	–	+	ND

† From Roselló *et al.* (2006) and López *et al.* (2011). Oxidation of substrates in API 50 CH strips (bioMérieux) using the method described by López *et al.* (2011). More than 90% of strains give the results indicated.

‡ According to Kim *et al.* (2001b).

ND, not determined; V, variable.

4.1.1 Biochemical characterization

4.1.1.1 Nutritional and enzymatic profiling

Identification of *E. amylovora* can be obtained biochemically by profiling on the API system 20 E and 50 CH strips (bioMérieux[1]).

API 20 E[1]. The manufacturer's instructions should be followed for preparing the suspension and inoculating the strip. The strip is incubated at 25–26 ºC. The reading after 48 h for a typical *E. amylovora* culture should be as follows: the tests lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (SH_2), urease (URE), tryptophan deaminase (TDA), indole production (IND) and rhamnose oxidation (RHA) should be negative, while sucrose oxidation (SAC) should be positive. Other tests may vary by strain, according to Donat *et al.* (2007).

API 50 CH[1]. A suspension of OD 1.0 (at 600 nm wavelength) is prepared in PBS. One millilitre of the suspension is added to 20 ml Ayers medium ($NH_4H_2PO_4$, 1 g; KCl, 0.2 g; $MgSO_4$, 0.2 g; bromothymol blue 0.2%, 75 ml; distilled water, 1 litre; pH 7; sterilized at 120 ºC for 20 min) (Ayers *et al.*, 1919). The manufacturer's instructions should be followed for inoculating the strip. The strip is incubated at 25–26 ºC under aerobic conditions. Utilization of the different carbohydrates is observed by the

development of a yellow colour in the well. The reading after 72 h for a typical *E. amylovora* culture should be positive for L-arabinose, ribose, D-glucose, D-fructose, mannitol, sorbitol, N-acetylglucosamine, sucrose, trehalose and β-gentiobiose. The remaining sugars are not utilized by *E. amylovora* in these conditions, but some strains can utilize glycerol and D-fucose, according to Donat *et al.* (2007).

4.1.1.2 Automated identification

An automated identification system based on differential results of 94 phenotypic tests in a microtiter plate and accompanying analysis software are commercially available (OmniLog[1], Biolog[1]). The manufacturers' instructions should be followed for presumptive identification of suspected *E. amylovora* isolates.

4.1.1.3 Fatty acid profiling

In fatty acid profiling (FAP), levan-positive, non-fluorescent colonies are grown on commercially available trypticase soy agar at 28 ºC for 48 h (Sasser, 1990). An appropriate fatty acid extraction procedure is applied and the extract is analysed using the commercially available Sherlock Microbial Identification System (MIS) (MIDI[1]) or other appropriate software for presumptive identification of *E. amylovora*, according to Wells *et al.* (1994).

4.2 Serological identification

4.2.1 Agglutination

Suspected *E. amylovora* colonies can be presumptively identified by slide agglutination. A dense suspension of cells is mixed with a drop of PBS and a drop of *E. amylovora* specific antiserum (undiluted, or at 1:5 to 1:10 dilution only) on a slide. Monoclonal antibodies can be used providing they agglutinate the reference strains. The specificity of the antibodies must be established in advance.

4.2.2 Immunofluorescence

A suspension of approximately 10^6 cells/ml is prepared in PBS from levan-positive, non-fluorescent colonies and the immunofluorescence procedure described in section 3.1.4.3 is followed. The specificity of the antibodies must be established in advance.

4.2.3 ELISA

Direct tissue print-ELISA (section 3.1.4.2), DASI-ELISA (section 3.1.4.1) and indirect ELISA (see below) for isolate identification can be performed using specific monoclonal antibodies as described for detection. A mixture of monoclonal antibodies has been validated in two ring tests for DASI-ELISA. A suspension of approximately 10^8 cells/ml is prepared in PBS from suspected colonies. The DASI-ELISA procedure in section 3.1.4.1 can be used, but without the enrichment step.

Indirect ELISA

Pure cultures of the suspected isolates are treated at 100 ºC for 10 min in a water bath or on a heating block to reduce non-specific reactions with commercial monoclonal antibodies. Aliquots of 200 µl culture are mixed with an equal volume of carbonate buffer (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre; pH 9.6) and this solution is applied to at least two wells of a microtiter plate. The plate is incubated at 37 ºC for 1 h or at 4 ºC overnight. Extracts are flicked out from the wells and the plate is washed three times with washing buffer (see the DASI-ELISA protocol). The specific commercial anti-*E. amylovora* antibodies from Plant Print Diagnòstics SL[1] are prepared at the recommended dilutions. To each well is added 200 µl of the diluted anti-*E. amylovora* antibody solution and the plate is incubated at 37 ºC for 1 h. The antibody solution is flicked out from the wells and the wells are washed as before. The appropriate dilution of secondary antibody-alkaline phosphatase conjugate (GAM-AP) is prepared in PBS containing 0.5% BSA. To each well is added 200 µl of the diluted conjugate antibody and the plate is incubated at 37 ºC for 1 h. The conjugated

antibody is flicked out from the wells and the wells are washed as before. A 1 mg/ml alkaline phosphatase substrate (p-nitrophenylphosphate) is prepared in substrate buffer (diethanol amine, 97 ml; 800 ml distilled water; adjusted to pH 9.8 with concentrated HCl; then the volume is adjusted to 1 000 ml with distilled water). To each well is added 200 µl alkaline phosphatase substrate solution. The plate is incubated in the dark at room temperature and read at 405 nm at regular intervals within 90 min. A positive test is indicated by substrate conversion to a yellow colour.

4.2.4 Lateral flow immunoassay

A suspension of 10^7 c.f.u./ml of the pure culture is prepared for presumptive identification. Buffers and procedures provided by the manufacturers of the kits are used, as described in section 3.1.4.4.

4.3 Molecular identification

4.3.1 PCR

A suspension of approximately 10^6 cells/ml is prepared in molecular grade sterile water from purified levan-positive, non-fluorescent colonies and is treated at 100 ºC for 10 min. The appropriate PCR procedures or the LAMP protocol are applied as described in sections 3.1.5.2 to 3.1.5.4 (directly, without DNA extraction). When using PCR to identify isolated colonies, 1 U of Taq DNA polymerase should be used (instead of 2 U as for plant material).

4.3.2 Macro-restriction and pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) analysis of genomic DNA after *Xba*I digestion according to Jock *et al.* (2002) shows six patterns for *E. amylovora* European strains. The method can provide useful information for strain differentiation and has been applied to understanding the spread of fire blight in Europe (Jock *et al.*, 2002; Donat *et al.*, 2007).

4.4 Pathogenicity techniques

Suspected *E. amylovora* colonies should be inoculated back into host plants to fulfil Koch's postulates and verify their pathogenicity. For plant inoculation, susceptible cultivars of pear (e.g. Conference, Doyenne du Comice, Williams, Passa Crassane), apple (e.g. Fuji, Gala, Idared, Jonathan), loquat (e.g. Algerie, Tanaka), *Crataegus* spp., *Cotoneaster* spp. or *Pyracantha* spp. are used. Young shoots are inoculated by cutting across a young leaf through the central vein with scissors dipped in a 10^9 c.f.u./ml suspension of each isolate prepared in PBS. The plants are maintained at 20–25 ºC at approximately 80% relative humidity for one to two weeks. Detached young shoots that have been surface-sterilized (treated with 70% ethanol for 30 s then washed three times with sterile distilled water) from greenhouse-grown plants can also be inoculated in the same way and kept in tubes with sterile 1% agar. The tubes should be kept at 20–25 ºC with 16 h light per day.

Inoculation can also be performed on detached immature fruits of susceptible cultivars of pear, apple and loquat by placing 10 µl of 10^9 c.f.u./ml suspensions of the isolates in PBS into a fresh wound on the surface of disinfected fruits (treated with 70% commercial chlorine for 30 min then washed three times with sterile distilled water). The fruits should be incubated in a humid chamber at 25 ºC for three to five days.

E. amylovora-like colonies are re-isolated and characterized from inoculated organs showing typical fire blight symptoms. A positive test is evident by the oozing of bacteria and browning around the inoculation site after two to seven days, as seen in the positive *E. amylovora* control, providing no lesions are or only a small necrotic lesion is observed at the wound site in the negative control.

Other inoculation techniques are possible. Hypersensitive reactions in tobacco leaves may indicate expression of the *hrp* genes of *E. amylovora*, but this test may be positive for many other plant pathogenic bacteria. Tobacco plants of cultivars Xanthi or Samsun with more than five to six leaves should be used. Bacterial suspensions of 10^9 c.f.u./ml (OD at 600 nm, 1.0) are prepared and a needle and syringe used to inject the suspensions into the intracellular space of mature leaves. Complete

collapse of the infiltrated tissue after 24–48 h at room temperature is recorded as positive, as observed in the positive *E. amylovora* control.

5. Records

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

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*) and where the pest is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: the original sample, culture(s) of the pest, preserved or slide-mounted specimens or test materials (e.g. photographs of gels, ELISA plate results printouts and PCR amplicons).

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Centro de Protección Vegetal, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López; e-mail: mlopez@ivia.es; tel.: +34 963424000; fax +34 963424001).

Plant Health and Environment Laboratory, Investigation and Diagnostic Centres, Ministry for Primary Industries, 231 Morrin Road, St Johns, Auckland 1140, New Zealand (Robert Taylor; e-mail: Robert.Taylor@mpi.govt.nz; tel.: +64 99093548; fax: +64 99095739).

A request for a revision to a diagnostic protocol may be submitted by NPPOs, regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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Most techniques described were ring tested in a DIAGPRO project financed by the European Union in 2003, in an EUPHRESCO project in 2009 and in a Spanish project in 2010.

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

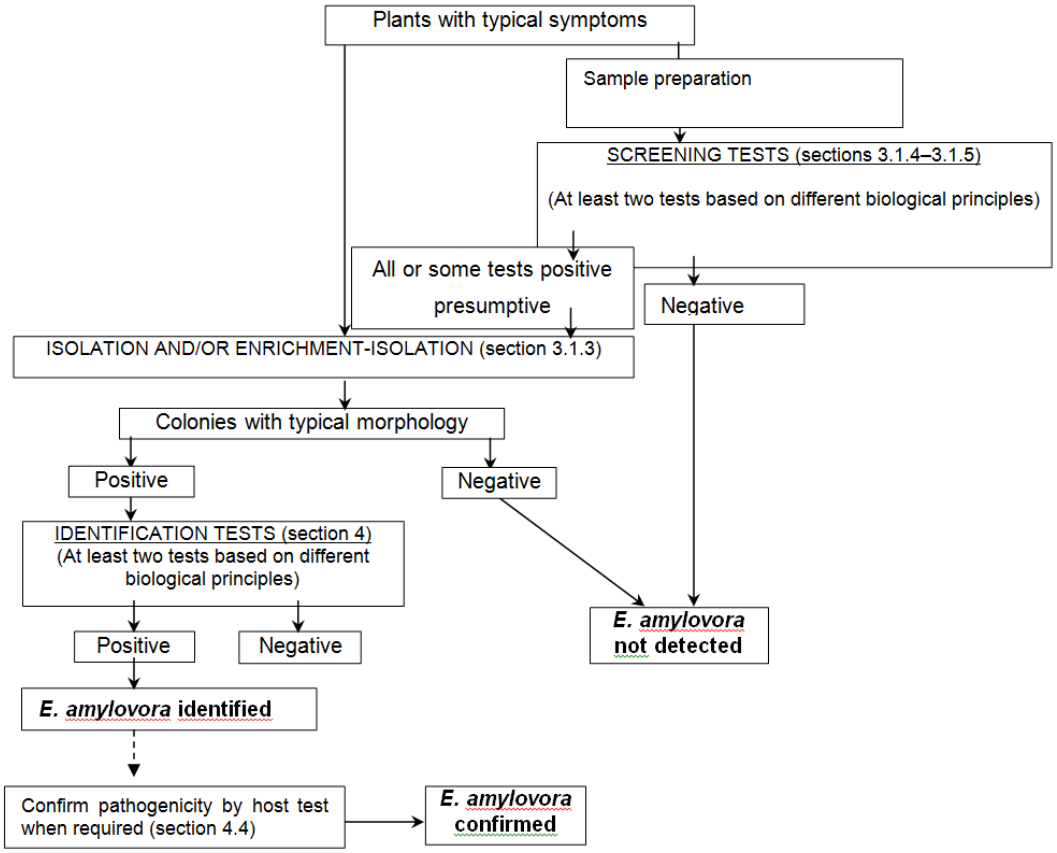

Figure 1. Flow chart for the identification of *Erwinia amylovora* in samples showing symptoms of fire blight.

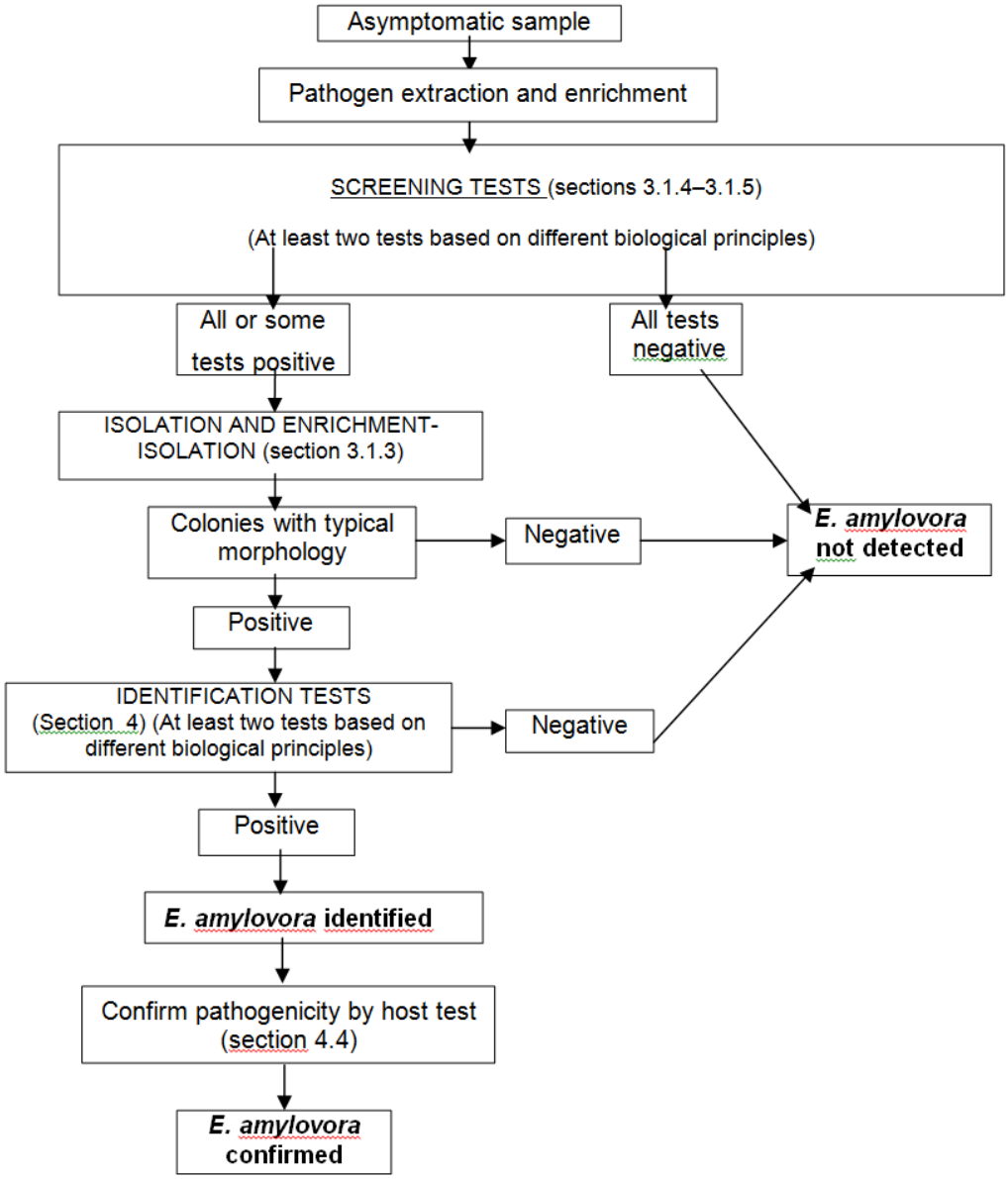

Figure 2. Flow chart for the identification of *Erwinia amylovora* in asymptomatic samples.
* It is reasonable to strongly suspect the presence of *E. amylovora* in the sample, but identification requires isolation of the pathogen from new samples and subsequent identification of the bacterium.

Publication history

This is not an official part of the standard.

2004-11 SC introduced original subject: *Erwinia amylovora* (2004-009).

2006-04 CPM-1 added subject under work programme topic: Bacteria.

2012-11 First draft presented to TPDP (meeting).

2013-06 Draft presented to the TPDP (meeting).

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2014-07 Member consultation.

2015-12 DP drafting group reviewing draft DP and responses to member comments.

2016-03 TPDP e-decision for approval for adoption (2016_eTPDP_Mar_01).

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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