DOI: 10.1111/epp.12916

EPPO STANDARD ON DIAGNOSTICS

PM 7/65 (2) *Xanthomonas fragariae*

Specific scope: This Standard describes a diagnostic protocol for *Xanthomonas fragariae*.[1]
This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.
Specific approval and amendment: First approved in 2005–09. Revision approved in 2023–01. Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Xanthomonas fragariae Kennedy & King, 1962 is the causal agent of bacterial angular leaf spot disease of *Fragaria* × *ananassa* (strawberry). Other *Fragaria* species have been shown to be susceptible through artificial inoculation (CABI, 2021) but *Fragaria moschata* and some other variable ploidy species, have exhibited at least partial resistance (Bestfleisch et al., 2015; Kennedy, 1965; Kennedy & King, 1962; Maas, 1998). Bacterial angular leaf spot disease is an insidious and potentially serious disease, which was first reported in the USA (Kennedy & King, 1962). It was later described in New Zealand and Australia from where it was subsequently eradicated, and has been reported in some other countries around the world but also in most European countries where strawberry is cultivated. More information on the geographical distribution of *X. fragariae* is available from EPPO Global Database.

Xanthomonas fragariae is readily transmitted via asymptomatic plants with latent infections and international movement of latently infected plants is blamed for the introduction of *X. fragariae* in many countries (López et al., 1985). Symptoms appear under favourable conditions (see section 3.1) as well as after cold storage (Rat, 1993). *X. fragariae* is widespread in nurseries in many countries and has been responsible for important production losses in Europe (Bosshard & Schwind, 1997; López et al., 1985; Mazzucchi et al., 1973). Analyses of *X. fragariae* strains isolated at different times in diverse locations around the world indicate some genetic and phenotypic diversity among these strains (Opgenorth et al., 1996; Pooler et al., 1996; Roberts et al., 1996). In addition, some differential pathogenicity has been noted among *X. fragariae* strains (Maas et al., 2000). However, there is no correlation between these variations and the geographic origin of the isolates.

This Standard describes different methodologies for the diagnosis of *X. fragariae* in symptomatic and asymptomatic plants of *Fragaria* spp. A flow diagram describing the diagnostic procedure for *X. fragariae* is presented in Figure 1.

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

2 | IDENTITY

Name: *Xanthomonas fragariae* Kennedy & King, 1962.
Synonyms: None.
Taxonomic position: Bacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae.
EPPO Code: XANTFR.
Phytosanitary categorization: EPPO A2 list n°135, EU RNQP (Annex IV).

3 | DETECTION

3.1 | Symptoms

Small (1–4 mm diameter) angular water-soaked spots (lesions) bounded by the smallest leaf veins appear initially on the lower leaf surface. In the early stages of infection, these spots are barely visible in the field and appear translucent yellow when viewed under transmitted light (Figures 2 and 5a).

The lesions enlarge and coalesce, eventually appearing on the lower leaf surface as angular water-soaked spots that become reddish brown (Figure 3). Viscous bacterial exudates that are white, milky, cream or yellow in colour develop from lesions under wet conditions or when the relative humidity is high. The exudates become dry scale-like masses that are opaque and whitish or silvery at first, then turn brown (Figure 4) (Janse, 2005).

As the disease progresses, coalesced reddish-brown lesions become necrotic. Necrotic lesion tissue may tear or break off the leaf, and diseased leaves may appear blighted or ragged. Leaf infections often develop and form long lesions along major veins. In advanced stages of disease development, the foliar tissue around old coalesced reddish-brown lesions is generally chlorotic

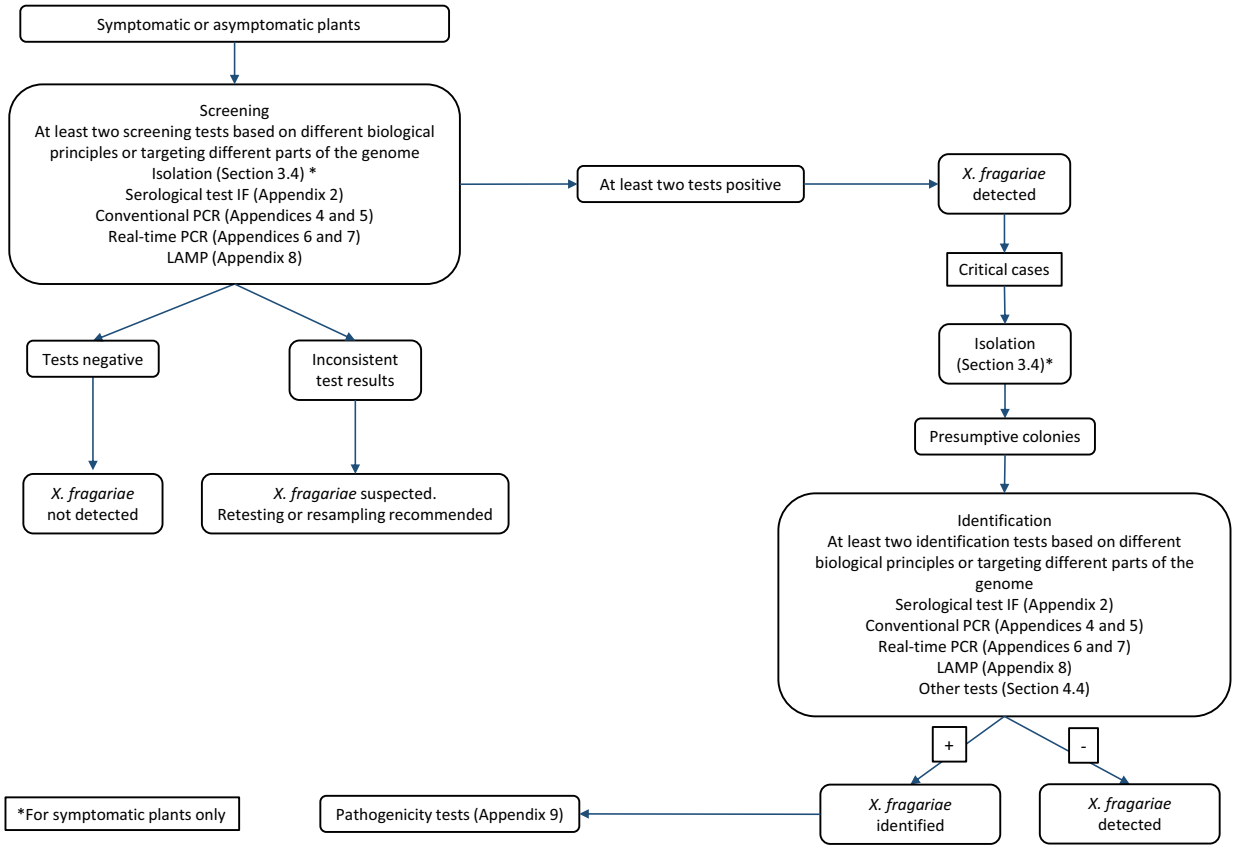

FIGURE 1 Flow diagram describing the detection and identification of *Xanthomonas fragariae* in samples of symptomatic or asymptomatic *Fragaria* spp.

FIGURE 2 Early-stage symptoms of angular leaf spot of strawberry. Courtesy Elphinstone J (Fera Science Ltd, GB) © Crown Copyright.

FIGURE 3 Angular water-soaked spots on the lower leaf surface. Courtesy Aspin A (Fera Science Ltd) © Crown Copyright.

(Figures 5 and 6) (EPPO, 1997; Kennedy & King, 1962; Maas, 1998; Rat, 1993).

Severe infections of *X. fragariae* may spread from the leaves to the crown, where discrete water-soaked areas develop (Hildebrand et al., 1967). Severe crown infection can result in plants with decreased vigour that may collapse and eventually die. Leaves that develop from infected crowns are often systemically infected, with lesions that appear along the veins at the base of the leaves (Figure 7). Bacterial exudate may ooze from vascular bundles when the crown is cut transversely.

In severe cases of disease, *X. fragariae* may attack flowers and cause blossom blight, but it does not directly infect fruits (Gubler et al., 1999). Water-soaked lesions on infected calyx tissue are similar in appearance to foliar lesions.

Xanthomonas fragariae can move systemically into the roots, crowns and runners without exhibiting

FIGURE 4 Angular spots with dried slime. Courtesy Cruz L (INIAV, PT).

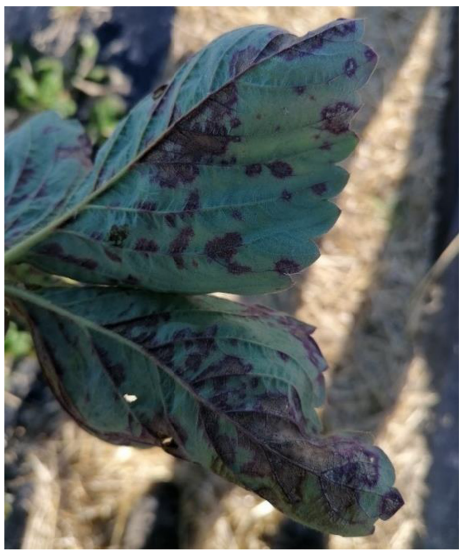

FIGURE 6 Lesions on the lower surface of a leaf. Courtesy Olivier V (ANSES, FR).

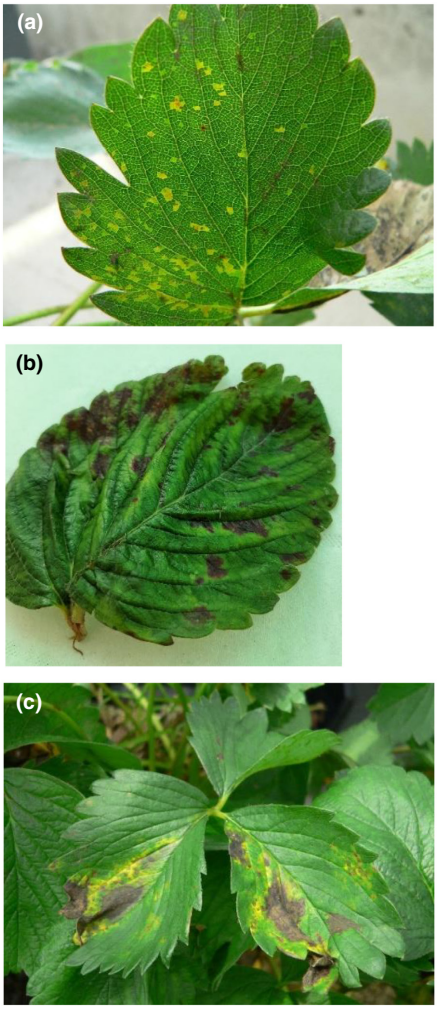

FIGURE 5 Early (a) and late (b, c) symptoms on the upper surface of a leaf. (a, c) Courtesy van Vaerenbergh J (ILVO, BE), (b) Courtesy Olivier V (ANSES, FR).

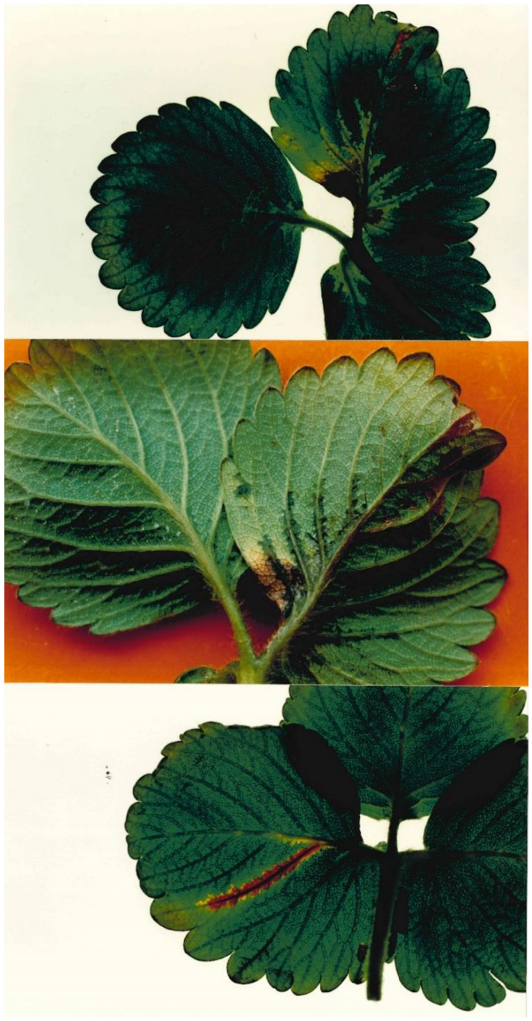

FIGURE 7 Vascular lesions caused by *Xanthomonas fragariae*. Courtesy Cruz L (INIAV, PT).

FIGURE 8 Crown infection pocket symptoms. Courtesy Feng J (Northwest A&F University, CN).

obvious symptoms (Bestfleisch et al., 2015; Mahuku & Goodwin, 1997; Milholland et al., 1996; Stefani et al., 1989). Although only rarely seen, this infection may result in the appearance of water-soaked areas at the base of newly emerged leaves rapidly followed by sudden plant collapse and death. One strain was also shown recently to form crown infection pockets in strawberry in China (Figure 8) (Li et al., 2021).

3.1.1 | Possible confusion

In contrast to angular leaf spot disease of strawberry, bacterial leaf blight of strawberry caused by *Xanthomonas arboricola* pv. *fragariae* is characterized by small reddish-brown lesions on the lower leaf surface that are neither water-soaked nor translucent; reddish spots on the upper leaf surface (Figure 9); lesions coalescing into large, dry brown spots surrounded by a chlorotic halo; and large brown V-shaped lesions along the leaf margin, midrib and major veins (Janse et al., 2001). In addition, no bacterial exudation is associated with bacterial leaf blight lesions (Janse et al., 2001). In advanced stages, bacterial angular leaf spot is difficult to distinguish from fungal leaf-spotting diseases such as common leaf spot (*Ramularia grevilleana*) and leaf scorch (*Diplocarpon fragariae*) (Janse et al., 2001).

FIGURE 9 Symptoms on the upper surface of strawberry leaves caused by *Xanthomonas arboricola* pv. *fragariae*. Courtesy Olivier V (ANSES, FR).

3.2 | Test sample requirements

3.2.1 | Sample collection

For plants with symptoms, leaves with initial water-soaked spots should be sampled as this facilitates successful isolation of *X. fragariae*. Alternatively, leaves with dry spots and with or without exudates can be used. Crown tissue should also be examined.

For symptomless plants, statistically representative samples of leaves, petioles and crowns of several plants should be taken. In the Netherlands, five samples per ha are collected. Each sample consists of 30 complete leaves including the petiole and the leaf base. In Portugal, the field sample is composed of 20 strawberry runners. In the United Kingdom up to 300 petioles are taken.

After collection, samples are wrapped in paper, placed in polythene bags, kept cool and stored at 4°C upon arrival at their destination and processed as soon as possible.

3.2.2 | Sample preparation in the laboratory

For symptomatic plants, the surfaces of leaf and stem plant tissue can be surface sterilized by wiping with 70% ethanol. If the plants show vascular symptoms, it is recommended that the roots and the leaves are removed, keeping the crown and petioles. The sample is rinsed in tap water to remove excess soil and surface sterilized by immersing for 1 min in 70% ethanol followed by rinsing three times in sterile distilled water.

Approximately 0.1 g of leaf or crown and petiole tissue per sample are added to approximately 9 mL phosphate-buffered saline (PBS) (Appendix 1) or water. The plant tissue is homogenized and incubated at room temperature for 15 min.

Alternatively, leaf pieces with up to 25 typical (water-soaked angular) leaf spots, suspect for *Xanthomonas fragariae*, are cut out. The dissected leaf pieces are placed in a beaker and covered with 1 cm of tap water and five drops (e.g. 5 × 10 μL) Tween-20. The leaf pieces are washed thoroughly in the liquid, rubbing off any air bubbles and dirt when present. The sample is incubated at room temperature for up to 10 minutes and then rubbed again. The washing fluid is discarded. The leaf pieces are covered in excess clean tap water, rubbed as before and left for 1 minute. The step is repeated twice more with clean tap water.

The leaf pieces are blotted dry on tissue paper then placed in a beaker and covered with ethanol (70%). The leaf pieces are stirred for up to 5 seconds before being removed and immediately blotted dry and the ethanol is allowed to evaporate completely.

Leaf fragments are cut into very small pieces (1–4 mm^2) and placed in a tube with 5 mL 0.01 M PBS. They are mixed and incubated for 30 min at room temperature to extract the bacterium from the plant tissue. A 1:100 dilution of the original extract in 0.01 M PB is prepared. Both extracts are used for dilution plating on nutrient media, as for Method 1.

For asymptomatic plants, the protocol used in the Netherlands consists of soaking the sample overnight at 4°C in 500 mL PBS supplemented with 0.02% Tween-20 (Appendix 1). The next day the sample is homogenized for 2 min at maximum setting in a bag-mixer (e.g., Interscience JumboMix). Ten mL of plant extract are transferred into a 15 mL centrifuge tube and centrifuged at 3 400*g* for 20 min. The supernatant is discarded, and the pellet is resuspended in Lysismix buffer (Appendix 1) with two steel ball bearings (2.778 mm Ø). The pellet is resuspended by putting the tubes in a Geno/Grinder for 3 min at 1 500 rpm and is then incubated for 1 hour at 55°C (homogenized by shaking a couple of times). The sample is cooled on ice and centrifuged for 20 min at 3 400*g*. Four hundred μL of supernatant are used for DNA extraction via the Kingfisher platform using the Sbeadex maxi plant kit (LGC Genomics).

In Portugal, a slice of the basal part of each crown of the field sample is taken above the roots to prepare the laboratory sample. Crown areas are excised and surface cleaned with paper soaked with 70% ethanol. The epidermis tissues are removed with a sterile scalpel and the inner crown tissues collected are macerated overnight at 4°C in SCPAP buffer (Appendix 1). The maceration extract is centrifuged at 13 500*g* and 4°C for 10 min and the pellet is resuspended in 1 mL SCPAP buffer (Appendix 1). Dilutions are prepared for further testing.

In the United Kingdom, the sample of up to 300 petioles is divided into 50 petiole or micro-propagated plant batches, covered in PBS (Appendix 1) and agitated on an orbital shaker for 30 min at room temperature, before the supernatant is removed and concentrated by centrifugation at 10 000*g*, for 10 min at 4–10°C, and the pellets from each batch of the sample are combined in 1 mL of buffer.

3.3 | Screening tests

Symptomatic and asymptomatic plant samples may be screened using indirect immunofluorescence (IF), and molecular methods. Isolation can be used for screening symptomatic plant samples. At least two screening tests (isolation, immunofluorescence and/or molecular tests) should be positive to confirm detection of *X. fragariae*.

3.3.1 | Serological tests

Instructions for performing an IF are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Recommended antisera and validation data are given in Appendix 2.

3.3.2 | Molecular tests

3.3.2.1 | *Conventional PCR*

Several conventional PCR tests, each targeting different loci in the *X. fragariae* genome, have been developed for the detection of *X. fragariae*.
These tests can be used for the detection of *X. fragariae* in symptomatic plant material, and several of them have also been used for the detection of latent *X. fragariae* infection).
The following conventional PCR tests are recommended for the detection of *X. fragariae*:
The semi-nested PCR from Roberts et al. (1996), described in Appendix 4.
The multiplex PCR from Hartung and Pooler (1997), described in Appendix 5.

Other conventional PCR tests that can be used for the detection of *X. fragariae* are the Zimmermann et al. (2004); Moltmann and Zimmermann (2005) and the conventional PCR from Vermunt and van Beuningen (2008). Nested PCR protocols have been reported to increase sensitivity up to 100 times compared with conventional PCR protocols, but they present a higher risk of contamination.

3.3.2.2 | *Real-time PCR*

The following real-time PCR tests are recommended for the detection of *X. fragariae*:
The real-time PCR from Weller et al. (2007), described in Appendix 6.
The real-time PCR from Vandroemme et al. (2008), described in Appendix 7.
These tests are potentially useful for detecting low levels of *X. fragariae* in asymptomatic or latent infections.

Other real-time PCR tests that can be used for the detection of *X. fragariae* are the real-time PCR from Turechek et al. (2008) and the propidium monoazide (PMA) real-time PCR from Wang and Turechek (2020).

3.3.2.3 | *LAMP*

The LAMP from Gétaz et al. (2017) is described in Appendix 8.

The LAMP from Wang and Turechk (2016) can also be used for the detection of *X. fragariae*.

3.4 | Isolation

Direct isolation of *X. fragariae* is difficult, even in the presence of symptoms and exudates, because *X. fragariae* grows very slowly on artificial nutrient media and is rapidly overgrown by saprophytic organisms (Hazel & Civerolo, 1980; López et al., 1985; Schaad et al., 2001; Saddler & Bradbury, 2005). Therefore, isolation is recommended from symptomatic plant material only. Specific procedures for direct isolation of *X. fragariae* are given in López et al. (2005). Three media are recommended for isolation: Sucrose Peptone Agar (SPA), Wilbrink's medium with nitrate (Wilbrink-N) or without nitrate, and Yeast extract Peptone Glycerol Agar with cycloheximide (YPGA+C) (Appendix 1). Isolation on YPGA+C medium is less successful than on the other two media but it is still recommended. Streaking can improve the success of isolation (L. Cruz IVIA, PT and T. Dreo NIB, SI personal communication).

3.4.1 | Isolation method

Isolation of *X. fragariae* from tissue may be performed from aliquots of fresh exudates from lesions directly onto Wilbrink(-N), YPGA+C and/or SPA media (Appendix 1). Alternatively, 10–100 µL aliquots (depending on the plating technique used) of lesion tissue supernatant or homogenate (see 3.2.2), as well as dilutions (1:10, 1:100, 1:1 000 and 1:10 000) are plated out onto the surface of Wilbrink(-N), YPGA+C and/or SPA media (Appendix 1). Similar aliquots of *X. fragariae* cell suspensions (10^4, 10^5 and 10^6 cfu/mL) should also be plated out to verify the quality of the media and to compare the cultural characteristics of any bacterial colonies that develop. The plates are incubated at 24°C for 7 days. Colonies appearing after 2–3 days should be disregarded as these will not be *X. fragariae*. Final reading is performed after seven to 10 days of incubation at 24°C.
Xanthomonas fragariae colonies on Wilbrink(-N) (Appendix 1) medium are initially off-white, becoming pale yellow, circular, slightly convex, smooth and mucoid after 4–6 days (Figure 10). On YPGA+C and SPA media (Appendix 1), the colonies are similar in morphology to those on Wilbrink(-N), but they have a more intense yellow colour.

3.4.2 | Interpretation of isolation results

The isolation is negative if no bacterial colonies with morphology characteristic of *X. fragariae* colonies are observed after seven to 10 days on any of the three media (provided no growth inhibition due to competition or antagonism has occurred) and typical *X. fragariae* colonies are found in the positive controls.
Detection based on isolation is considered positive if presumptive *X. fragariae* colonies are isolated on at least one of the media used.
Considering that isolation of this bacterium frequently fails, if immunofluorescence and PCR tests are positive, the sample should be considered as *X. fragariae* detected but not identified. The best isolation results are expected when using freshly prepared sample extracts from young lesions.

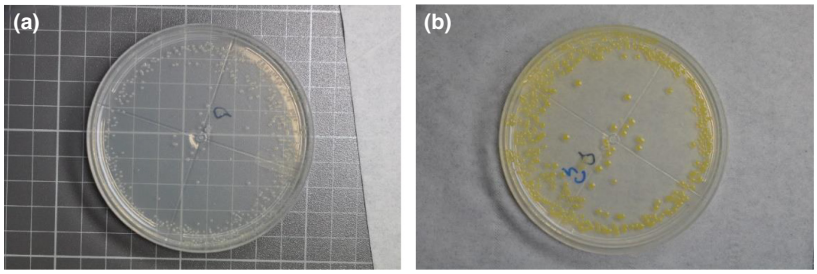

FIGURE 10 Colonies of *Xanthomonas fragariae* on Wilbrink's medium with nitrate after five (a) and ten (b) days incubation at 24°C. Courtesy Olivier V (ANSES, FR).

4 | IDENTIFICATION

Identification is recommended in critical cases only. At least two tests based on different biological principles or targeting different parts of the genome should be performed.

4.1 | Serological tests

4.1.1 | Immunofluorescence

Immunofluorescence (IF) can be used for the identification of suspect *X. fragariae* strains.
Instructions for performing an IF are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Recommended antisera and validation data are given in Appendix 2.

4.2 | Molecular tests

4.2.1 | Conventional PCR

Suspect *X. fragariae* cultures can be identified using the semi-nested PCR of Roberts et al. (1996) described in Appendix 4 and the multiplex PCR from Hartung and Pooler (1997) described in Appendix 5.

Other conventional PCR tests that can be used for the identification of *X. fragariae* are the conventional PCR from Pooler et al. (1996); the conventional PCR from Zimmermann et al. (2004); the nested PCR from Moltmann and Zimmermann (2005) and the conventional PCR from Vermunt and van Beuningen (2008).

4.2.2 | Real-time PCR

The following real-time PCR tests can be used for the identification of *X. fragariae*.
The real-time PCR from Weller et al. (2007), described in Appendix 6.
The real-time PCR from Vandroemme et al. (2008), described in Appendix 7.
Other real-time PCR tests that can be used for the identification of *X. fragariae* are the real-time PCR from Turechek et al. (2008), and the the propidium monoazide (PMA) real-time PCR from Wang and Turechek (2020).

4.2.3 | LAMP

The LAMP from Gétaz et al. (2017) described in Appendix 8 can be used for the identification of *X. fragariae*.

The LAMP from Wang and Turechk (2016) can also be used for the identification of *X. fragariae*.

4.3 | Pathogenicity tests

The identity of bacterial strains suspected of being *X. fragariae* can be confirmed by a pathogenicity test. Several procedures are available: Hazel and Civerolo (1980), Civerolo et al., 1997 and Hildebrand et al. (2005). Pathogenicity tests are described in Appendix 9.

4.4 | Other tests

4.4.1 | Rep-PCR

The Rep-PCR from Opgenorth et al. (1996) can be used for the identification of *X. fragariae*. Guidelines on how to perform Rep-PCR are provided in the EPPO Standard PM 7/100 *Rep-PCR tests for identification of bacteria*.

4.4.2 | DNA barcoding methods

Single locus sequence typing of the partial *gyrB* gene can be used for the identification of *X. fragariae*. Sequence analysis should follow the guidelines described in the EPPO Standard PM 7/129 on *DNA barcoding as an identification tool for plant pests*.

4.4.3 | Biochemical and physiological tests

X. fragariae has the common cultural characteristics of all xanthomonads. Cells are Gram-negative, aerobic rods with a single polar flagellum. Different biochemical characteristics can be used for the identification of *X. fragariae* as described in Schaad et al. (2001), and Janse et al. (2001). The tests differentiate between *X. fragariae* and *X. arboricola* pv. *fragariae*.

4.4.4 | MALDI-TOF mass spectrometry

A MALDI-TOF mass spectrometry method for proteomic analysis has been described by Vandroemme et al., 2013 (see also Catara et al., 2021) which allows rapid, reliable and robust identification of strains.

4.4.5 | Multilocus sequence typing/analysis (MLSA)

A multilocus sequence analysis (MLSA) approach (Almeida et al., 2010; Gétaz et al., 2018; Young et al., 2008) based on the sequencing of 4–8 housekeeping genes can be used to support the identification of *X. fragariae*.

However, it should be noted that this methodology has not yet been validated for the identification of *X. fragariae*.

5 | REFERENCE MATERIAL

Type strain: NCPPB 1469^T = CFBP 2157^T = LMG 708^T = DSM 3587^T = PD 885^T, PD 2905, PD 5202, NCPPB 1822,
CFBP 2510, LMG 25863.
Xanthomonas fragariae strains are commercially available from:
National Collection of Plant Pathogenic Bacteria (NCPPB), Fera Science Ltd, York (GB).
International Center for Microbial Resources-French Collection for Plant-associated Bacteria (CIRM-CFBP), IRHS-INRAE, Beaucouzé (FR).
Belgian Co-ordinated Collections of Microorganisms / Laboratorium voor Microbiologie (BCCM/LMG), Gent (BE).
German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig (DE).
EPPO-Q-bank (https://qbank.eppo.int/) includes sequences for *gyrB* for properly documented species and strains present in collections.

The authenticity of the strains can be guaranteed only if they are directly obtained from one of the indicated culture collections.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *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:
Pothier JF, Zurich University of Applied Sciences ZHAW, Einsiedlerstrasse 31, Wädenswil 8820 (Switzerland). E-mail: joel.pothier@zhaw.ch
Aspin A and Cole J, Fera Science Ltd, Sand Hutton, YO41 1LZ (United Kingdom). E-mails: andrew.aspin@fera.co.uk and jennifer.cole@fera.co.uk
Cottyn B, Flanders research institute for agriculture, fisheries and food, Burg. Van Gansberghelaan 96, Merelbeke (Belgium). E-mail: bart.cottyn@ilvo.vlaanderen.be
van der Wolf JM, Wageningen University, PO Box 16, 6700 AA Wageningen (the Netherlands). E-mail: jan.vanderwolf@wur.nl

9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Standard, 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.

ACKNOWLEDGEMENTS

This protocol was originally drafted by López MM, Domínguez F, Morente C, Salcedo CI and Olmos A (IVIA, ES), and Civerolo E (USDA- ARS, USA). The first revision was prepared by Aspin A and Cole J (Fera Science Ltd., GB), and Pothier JF (ZHAW, CH). It was reviewed by the EPPO Panel on Diagnostics in Bacteriology.

REFERENCES

Almeida NF, Yan S, Cai R, Clarke CR, Morris CE, Schaad NW, Schuenzel EL, Lacy GH, Sun X, Jones JB, Castillo JA, Bull CT, Leman S, Guttman DS, Setubal JC & Vinatzer BA (2010) PAMDB, a multilocus sequence typing and analysis database and website for plant-associated microbes. *Phytopathology*, 100(3), 208–215.
Bestfleisch M, Richter K, Wensing A, Wünsche JN, Hanke MV, Höfer, M, Schulte E and Flachowsky H (2015). Resistance and systemic dispersal of *Xanthomonas fragariae* in strawberry germplasm (Fragaria L.). *Plant Pathology* 64:71–80.
Bosshard E and Schwind M (1997) Detection of different bacterial and fungal pathogens in apparently healthy strawberry plants. *In Diagnosis and Identification of Plant Pathogens* (eds Dehne HW, Adam G, Diekmann M, Frahm J, Mauler-Machnik A & van Halteren P), pp. 37–41. Kluwer, Dordrecht (NL).
CABI 2021 *Crop protection compendium*. CABI, Wallingford (UK). Available at https://www.cabi.org/isc/datasheet/56934 (last accessed 31 October 2022).
Calzolari A & Mazzucchi U (1989) Attempts to detect *Xanthomonas fragariae* in symptomless strawberry plants. *Acta Horticulturae*, 265, 601–604.

Catara V, Cubero J, Pothier JF, Bosis E, Bragard C, Đermić E, Holeva MC, Jacques MA., Petter F, Pruvost O, Robène I, Studholme DJ, Tavares F, Vicente JG, Koebnik R, and Costa J (2021) Trends in molecular diagnosis and diversity studies for phytosanitary regulated *Xanthomonas*. *Microorganisms* 9,862.

Civerolo EL, Feliciano AJ, Melvin JA & Gubler WD (1997) A detached leaf bioassay for *Xanthomonas fragariae*. In *Proceedings of the 9th International Conference of Plant Pathogenic Bacteria* (ed Mahadevin A), pp. 89–94. University of Madras, Madras (IN).

EPPO (European and Mediterranean Plant Protection Organization) (1997) Data sheet on *Xanthomonas fragariae*. *In* EPPO/CABI (eds Smith IM, McNamara DG, Scott PR & Holderness M), ed. *Quarantine pests for Europe*, 2nd edn, pp. 1124–1128. CABI, Wallingford (UK).

EPPO (European and Mediterranean Plant Protection Organization) (2009) Indirect immunofluorescence test for plant pathogenic bacteria. EPPO Standards PM 7/97 (1). *EPPO Bulletin*, 39, 413–416.

Gétaz M, Bühlmann A, Schneeberger PHH, van Malderghem C, Duffy B, Maes, M, Pothier JF and Cottyn B (2017). A diagnostic tool for improved detection of *Xanthomonas fragariae* using a rapid and highly specific LAMP assay designed with comparative genomics. *Plant Pathology* 66:1094–1102.

Gétaz M, Krijger M, Rezzonico F, Smits THM, van der Wolf JM and Pothier JF (2018). Genome-based population structure analysis of the strawberry plant pathogen *Xanthomonas fragariae* reveals two distinct groups that evolved independently before its species description. *Microbial Genomomics* 4:189. doi: 10.1099/mgen.0.000189.

Gubler WD, Feliciano A, Bordas A, Civerolo EL, Melvin J & Welch N (1999) *X. fragariae* and *C. cladosporioides* cause strawberry blossom blight. *California Agriculture*, 53, 26–28.

Hartung JS & Pooler MR (1997) Immunocapture and multiplexed-PCR assay for *Xanthomonas fragariae*, causal agent of angular leafspot disease. *Acta Horticulturae*, 439, 821–828.

Hayward C (1960) A method for characterizing *Pseudomonas solanacearum*. *Nature*, 186, 405–406.

Hazel WJ & Civerolo EL (1980) Procedures for growth and inoculation of *Xanthomonas fragariae*, causal organism of angular leaf spot of strawberry. *Plant Disease*, 64, 178–181.

Hildebrand DC, Schroth MN & Wilhelm S (1967) Systemic invasion of strawberry by *Xanthomonas fragariae* causing vascular collapse. *Phytopathology*, 57, 1260–1261.

Hildebrand PD, Braun PG, Renderos WE, Jamieson AR, McRae KB & Binns MR (2005) A quantitative method for inoculating strawberry leaves with *Xanthomonas fragariae*, factors affecting infection, and cultivar reactions. *Canadian Journal of Plant Pathology*, 27, 16–24.

IPPC (2017). International Standard for Phytosanitary Measures ISPM 27 Annex 14: *Xanthomonas fragariae*.

Janse JD (2005) Examples of bacterial diseases of cultivated and wild plants – *Xanthomonas fragariae*. In *Phytobacteriology: principles and practice*. pp. 224–225. CABI, Wallingford (UK).

Janse JD, Ross M.P, Gorkink RFJ, Derks JHJ, Swings J, Janssens D & Scortichini M (2001) Bacterial leaf blight of strawberry (*Fragaria* (×) *ananassa*) caused by a pathovar of *Xanthomonas arboricola*, not similar to *Xanthomonas fragariae* Kennedy & King. Description of the causal organism as *Xanthomonas arboricola* pv. *fragariae* (pv. nov., comb. nov.). *Plant Pathology*, 50, 653–665.

Kennedy BW (1965) Infection of Potentilla by *Xanthomonas fragariae*. *Plant Disease Reporter*, 49, 491–492.

Kennedy BW & King TH (1962) Angular leaf spot of strawberry caused by *Xanthomonas fragariae* sp. nov. *Phytopathology*, 52, 873–875.

Koike H (1965) The aluminum-cap method for testing sugarcane varieties against leaf scald disease. *Phytopathology*, 55, 317–319.

Lelliott RA & Stead DE (1987) Methods for diagnosis of bacterial diseases of plants. *In Methods in Plant Pathology* (ed. Preece TF), Vol. 2. Blackwell Scientific Publications, Oxford (GB).

Li YL, Wang DJ, Ma YY, Cai XL, Xiao S, Wen YQ & Feng JY (2021). First report of *Xanthomonas fragariae* strain YL19 causing crown infection pockets in strawberry in Liaoning province, China. *Disease Note* 105(8). DOI 10.1094/PDIS-12-20-2560-PDN

López MM, Aramburu JM, Cambra M & Borrás V (1985) Detection and identification of *Xanthomonas fragariae* in Spain. *Anales del Instituto Nacional de Investigaciones Agrarias, Serie Agricola*, 28, 245–259 (in Spanish).

López MM, Dominguez F, Morente C, Salcedo CI, Olmos A & Civerolo E. (2005) *Diagnostic protocols for organisms harmful to plants*: *Diagnosis* Xanthomonas fragariae. SMT-4-CT98-2252.

Maas JL, ed. (1998) *Compendium of strawberry diseases*, 2nd edn. APS Press, St Paul (MN).

Maas JL, Gouin-Behe C, Hartung JS & Hokanson SC (2000) Sources of resistance for two differentially pathogenic strains of *Xanthomonas fragariae* in *Fragaria* genotypes. *Horticultural Science*, 35, 128–131.

Mahuku GS & Goodwin PH (1997) Presence of *Xanthomonas fragariae* in symptomless strawberry crowns in Ontario detected using a nested polymerase chain reaction (PCR). *Canadian Journal of Plant Pathology*, 19, 366–370.

Mazzucchi U, Alberghina A & Dally A (1973) Occurrence of *Xanthomonas fragariae*. Italy. *Phytopathologische Zeitschrift*, 76, 367–370.

Milholland RD, Ritchie DF, Dayking ME & Gutierrez WA (1996) Multiplication and translocation of *Xanthomonas fragariae* in strawberry. *Advances in Strawberry Research*, 15, 13–17.

Minsavage GV, Thompson CM, Hopkins DL, Leite RMVBC, and Stall RE (1994) Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology* 84: 456–461.

Moltmann E & Zimmermann C (2005) Detection of *Xanthomonas fragariae* in symptomless strawberry plants by nested PCR. *EPPO Bulletin*, 35, 53–54.

Opgenorth DC, Smart CD, Louws FJ, de Bruijn FJ & Kirkpatrick BC (1996) Identification of *Xanthomonas fragariae* field isolates by rep-PCR genomic fingerprinting. *Plant Disease*, 80, 868–873.

Pooler MR, Ritchie DF & Hartung JS (1996) Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology*, 62, 3121–3127.

Rat B (1993) *Xanthomonas fragariae*: Causal agent of angular leaf spot of strawberry. *In* J.G. Swings & E.L. Civerolo, eds. *Xanthomonas*, pp. 69–70, Chapman and Hall, London (UK).

Roberts PD, Jones JB, Chandler CK, Stall RE & Berger RD (1996) Survival of *Xanthomonas fragariae* on strawberry in summer nurseries in Florida detected by specific primers and nested PCR. *Plant Disease*, 80, 1283–1288.

Saddler GS & Bradbury JF (2005) Xanthomonas. In *Bergey's manual of systematic bacteriology* (eds Garrity GM, Brenner DJ, Krieg NR & Stanley JT) 2nd edn, Vol. 2, pp. 63–90. Springer, New York, (US).

Schaad NW, Jones JB & Lacy GH (2001) Xanthomonas. In *Laboratory guide for identification of plant pathogenic bacteria* 3rd edn (eds Schaad NW, Jones JB & W. Chun), pp. 175–200. APS Press, St Paul, MN.

Stefani E, Mazzucchi U & Calzolari A (1989) Evidence of endophytic movement of *Xanthomonas fragariae* Kenn. and King in strawberry. *Phytopathologia Mediterranea*, 28, 147–149.

Stöger A & Ruppitsch W (2004) A rapid and sensitive method for the detection of *Xanthomonas fragariae*, causal agent of angular leafspot disease in strawberry plants. *Journal of Microbiological Methods*, 58, 281–284.
Turechek WW, Hartung, JS & McCallister J (2008) Development and optimization of a real-time detection assay for *Xanthomonas fragariae* in strawberry crown tissue with receiver operating characteristic curve analysis. *Phytopathology*, 98(3), 359–368.
Vandroemme J, Baeyen S, Van Vaerenbergh J, De Vos P. & Maes M (2008) Sensitive real-time PCR detection of *Xanthomonas fragariae* in strawberry plants. *Plant Pathology*, 57(3), 438–444.
Vandroemme J, Cottyn B, Pothier JF, Pflüger V, Duffy B & Maes M (2013) *Xanthomonas arboricola* pv. *fragariae*: what's in a name? *Plant Pathol.* 62, 1123–1131.
Vermunt A & van Beuningen A (2008) Monitoring van *Xanthomonas fragariae* in de aardbeiteelt en de ontwikkeling van een hygiëneprotocol. *Productschap Tuinbouw en Plantum (NL)*.
Wang H and Turechek WW (2020) Detection of viable *Xanthomonas fragariae* cells in strawberry using propidium monoazide and long-amplicon quantitative PCR. *Plant Disease* 104(4): 1105–1112.
Wang H, Turechk WW (2016) A loop-mediated isothermal amplification assay and sample preparation procedure for sensitive detection of *Xanthomonas fragariae* in strawberry. *PLoS ONE* 11(1): e0147122. https://doi.org/10.1371/journal.pone.0147122.
Weller SA, Beresford-Jones NJ, Hall J, Thwaites R, Parkinson N & Elphinstone JG (2007) Detection of *Xanthomonas fragariae* and presumptive detection of *Xanthomonas arboricola* pv. *fragariae*, from strawberry leaves, by real-time PCR. *Journal of Microbiological Methods*, 70, 379–383.
Young JM, Park DC, Shearman HM & Fargier E (2008) A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology*, 31, 366–377. doi: 10.1016/j.syapm.2008.06.004.
Zimmermann C, Hinrichs-Gerger J, Moltmann E & Buchenauer H (2004) Nested PCR for detection of *Xanthomonas fragariae* in symptomless strawberry plants. *Journal of Plant Diseases and Protection*, 111, 39–51.

How to cite this article: EPPO (2023) PM 7/65 (2) *Xanthomonas fragariae*. *EPPO Bulletin*, 53, 184–204. Available from: https://doi.org/10.1111/epp.12916

APPENDIX 1 - BUFFERS AND MEDIA

The use of bacteriological grade agar (eg., Oxoid, BD Difco) is recommended for all media as impurities in other commercial agars can inhibit the growth of *X. fragariae*.

Because *X. fragariae* grows very slowly on artificial nutrient media and is rapidly overgrown by saprophytic organisms, cycloheximide can be added to all media used for isolation to reduce the growth of saprophytic fungi (e.g., 250 mg/L by using 5 mL/L of medium of a stock solution with 5 g cycloheximide[2] per 100 mL absolute ethanol).

PBS (phosphate saline buffer 10 mM, 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 to 1 L; adjust pH to 7.2 before autoclaving.

Lysismix (per sample):

Lysis buffer PVP (LGC genomics)	400 µL
Protease solution (LGC Genomics)	44 µL
DTT 5 M	16 µL

Succinate citrate phosphate supplemented with ascorbic acid and polyvinylpyrrolidone (SCPAP) (Minsavage et al., 1994)

Disodium succinate	1.0 g
Trisodium citrate	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Sodium ascorbate	0.02 M
Acid-washed insoluble polyvinylpyrrolidone	5%

Distilled water to 1 L; adjust pH to 7 before autoclaving.

Cetyl trimethylammonium bromide (CTAB)

1 M Tris–HCl	50 mL
5 M ethylenediaminetetraacetic acid (EDTA)	50 mL
NaCl	40.9 g
Polyvinylpyrrolidone (PVP)-40	5 g
CTAB	12.5 g

Distilled water to 500 mL.

Wilbrink with nitrate (Wilbrink-N) (Koike, 1965).

Sucrose	10 g
Proteose peptone (L85; Oxoid)	5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$NaNO_3$ (can be omitted for Wilbrink's medium without nitrate)	0.25 g
Bacteriological grade agar	15 g

Distilled water to 1 L; adjust pH to 7.0–7.2 before autoclaving.

Yeast extract Peptone Glycerol Agar (YPGA) (Lelliott & Stead, 1987)

Yeast extract	5 g
Bacto-Peptone	5 g
Glucose	10 g
Bacteriological grade agar	15 g

Distilled water to 1 L; adjust pH to 7.0–7.2 before autoclaving.

[2]Nystatin may be used as an alternative to cycloheximide. In the United Kingdom, 35 mg/L are used (A. Aspin, personal communication)

Sucrose Peptone Agar (SPA) (Hayward, 1960)

Sucrose	20 g
Bacto-Peptone	5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Bacteriological grade agar	15 g

Distilled water to 1 L; adjust pH to 7.2–7.4.

APPENDIX 2 - SEROLOGICAL TESTS

The following polyclonal antibodies to *X. fragariae* are recommended for use in serological tests:

Polyclonal antibodies from Plant Research International (Wageningen UR, NL) catalogue reference:

SKU Xf_I (https://shop.wur.nl/primediagnostics/xf-i.html) were validated for use in immunofluorescence.

Analytical sensitivity is 1 000 cfu/mL. Inclusivity is 100%, calculated on 30 isolates of *X. fragariae*. Exclusivity is 100%, calculated on 20 isolates (5 isolates per species) of *Ralstonia solancearum*, *Rhodococcus fascians*, *Xanthomonas axonopodis* pv. *vesicatoria* and *Xanthomonas campestris* pv. *campestris*.

IPO9534 BCD1, SWAR-FITC antiserum/conjugate, validated on sixty-three isolates of *X. fragariae* and a number of related bacteria among which *X. arboricola* pv. *fragariae*.

One commercially available polyclonal anti-*X. fragariae* serum has been validated using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (López et al., 2005). Immunofluorescence with these antibodies allows the detection of 10^3–10^4 cfu/mL *X. fragariae* in strawberry tissue (Calzolari and Mazzucchi, 1989). Instructions for performing immunofluorescence are provided in EPPO Standard PM 7/097 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009).

It can be noted that Loewe (DE), Catalogue No. 07332/01 (https://www.loewe-info.com/categories/immuno-fluorescence/antisera-and-controls.html) has not been validated.

APPENDIX 3 - DNA EXTRACTION

Protocols for DNA extraction from plant samples and PCR described in Pooler et al. (1996) and Hartung and Pooler (1997) have been validated (López et al., 2005). A modified protocol using the REDExtract-N-Amp Plant PCR Kit (Sigma) has also been reported to be suitable for DNA extraction before amplification for testing large numbers of samples of asymptomatic leaves (Stöger & Ruppitsch, 2004). Other commercial kits for extracting DNA and for nested PCR and PCR using other primers (Roberts et al., 1996) are available; however, these have not yet been validated (López et al., 2005).

Plant material

The DNeasy Plant Mini Kit (Qiagen), as modified for mycoplasma like organism (MLO) DNA extraction, provided the best results during the European Union DIAGPRO project (SMT-4-CT98-2252).

Two-hundred and fifty µL of tissue macerate prepared as described in section 3.2.2 is used for DNA extraction, by adding 250 µL CTAB extraction buffer and 4 µL RNase A (100 mg/mL). The sample is mixed by inverting gently five times and incubated at 65°C for 10 min with occasional mixing by inversion. The manufacturer's instructions are followed until the DNA elution step.

To elute the DNA, 100 µL of 10 mM Tris–HCl, pH 9 (preheated to 65°C) are added to the column and centrifuged at ≥6 000 *g* for 1 min. An additional 100 µL Tris–HCl is added and the centrifugation step is repeated. The DNA solution is adjusted to a total volume of 300 µL with Tris-EDTA (TE) buffer and 200 µL of 5 M ammonium acetate and 1 mL absolute ethanol are added. The sample is mixed well and incubated at −20°C for 1 h to overnight. After incubation, it is centrifuged at 17 000 *g* for 10 min. The supernatant is discarded and the DNA pellet washed in 1 mL absolute ethanol and centrifuged at 16 000 *g* for 5 min. Washing is repeated by substituting the absolute ethanol with 500 µL of 80% ethanol. The supernatant is discarded. After the pellet has dried, it is resuspended in 50 µL sterile distilled water.

Cell cultures

For crude DNA extraction from presumptive *X. fragariae* cultures and from cultures of reference strains, suspend approximately 1 µL of cell material (e.g., using a 1 µL disposable inoculating loop) or one colony in 100 µL of sterile distilled water. Heat in closed microvials at approximately 95–100°C for 5–10 min. A freezing step after the heating may be performed.

Alternatively, a cell suspension in 0.05 mM NaOH can be prepared. One hundred µL of the cell suspension in closed tubes is heated at approximately 95°C for 5 min.

The lysate can be stored at approximately −20°C.

APPENDIX 4 - SEMI-NESTED PCR (ROBERTS ET AL., 1996)

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 and identification of *X. fragariae* in symptomatic and asymptomatic plant material and cell cultures.

1.2. The test is based on Roberts et al., 1996.
1.3. The test targets the *hrp* gene of *X. fragariae*.
1.4. Oligonucleotides:

Primer	Sequence	Amplicon size (bp)
Forward primer XF9	5′-TGG GCC ATG CCG GTG GAA CTG TGT GG-3′	537 or 458, depending on the reverse primer used
Reverse primer XF11	5′-TAC CCA GCC GTC GCA GAC GAC CGG-3′	537
Reverse primer XF12	5′-TCC CAG CAA CCC AGA TCC G-3′	458

1.5. Cycler or real-time PCR system DNA Thermal Controller PT-100 (MJ Research Watertown, MA)

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: plant material and cell cultures
2.1.2. See Appendix 3 for extraction procedures from plant material and cell cultures
2.1.3. DNA should preferably be stored at approximately −20°C
2.2. Conventional PCR
2.2.1. Master Mix round 1

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	39.25	N.A.
PCR buffer with $MgCl_2$ (Promega)	10 ×	5.0	1 ×
dNTPs (Promega)	10 mM	0.5	0.1 mM
Forward primer XF9	10 µM	1.0	0.2 µM
Reverse primer XF11	10 µM	1.0	0.2 µM
Taq DNA polymerase[a]	5 U/µL	0.25	0.025 U/µL
Subtotal		47	
Nucleic acid extract		3	
Total		50	

[a] The manufacturer of the *Taq* polymerase was not given in the original paper.

2.3. Conventional PCR
2.3.1. Master Mix round 2

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	39.25	N.A.
PCR buffer with $MgCl_2$ (Promega)	10 ×	5.0	1 ×
dNTPs (Promega)	10 mM	0.5	0.1mM
Forward primer XF9	10 µM	1.0	0.2 µM
Reverse primer XF12	10 µM	1.0	0.2 µM
Taq DNA polymerase[a]	5 U/µL	0.25	0.025 U/ µL
Subtotal		47	
First round PCR product		3	
Total		50	

[a] The manufacturer of the *Taq* polymerase was not given in the original paper.

2.3.2. PCR conditions first round: 95°C for 2 min followed by 30 cycles of (95°C for 30 s, 65°C for 30 s and 72°C for 45 s) followed by a final extension step of 5 min at 72°C
2.3.3. PCR conditions second round: 95°C for 2 min followed by 30 cycles of (95°C for 30 s, 58°C for 30 s and 72°C for 45 s) followed by a final extension step of 5 min at 72°C

3. Essential Procedural Information

Controls:

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available 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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the 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 isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be (transcripts of) genes present in the matrix nucleic acids or added to the nucleic acid extracts.

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 18 S 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).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC (and if relevant IC) a band of the expected size (458 and 537 bp) is visualized.

When these conditions are met:

- A test will be considered positive if a band of the expected size is visualized. A product of 458 bp is expected in the second round of amplification.
- 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

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

Validation data from Roberts et al. (1996).

4.1. Analytical sensitivity data

Approximately 18 cells in plant tissue.

4.2. Analytical specificity data

Inclusivity
100% validated on 49 isolates of *X. fragariae*.
Exclusivity
100% validated on 17 isolates of *X. campestris* (representing 16 pathovars) and 9 isolates of non-pathogenic Xanthomonads isolated from strawberry.

APPENDIX 5 - MULTIPLEX PCR (HARTUNG & POOLER, 1997)

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 and identification of *X. fragariae* in symptomatic and asymptomatic plant material and cell cultures.
1.2. The test is based on Hartung & Pooler, 1997.
1.3. The test targets the Random Amplified Polymorphic DNA region.
1.4. Oligonucleotides:

Primer	Sequence	Amplicon size (bp)
Forward primer 241A	5′-GCC CGA CGC GAG TTG AAT C-3′	550
Reverse primer 241B	5′-GCC CGA CGC GCT ACA GAC TC-3′	
Forward primer 245A	5′-CGC GTG CCA GTG GAG ATC C-3′	300
Reverse primer 245B	5′-CGC GTG CCA GAA CTA GCA G-3′	
Forward primer 295A	5′-CGT TCC TGG CCG ATT AAT AG-3′	615
Reverse primer 295B	5′-CGC GTT CCT GCG TTT TTT CG-3′	

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. Matrices: symptomatic and asymptomatic plant material and cell cultures
2.1.2. See Appendix 3 for extraction procedures from plant material and cell cultures

2.1.3. DNA should preferably be stored at approximately −20°C

2.2. Conventional PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR buffer (*Perkin Elmer*)[a]	10 ×	2.5	1 ×
dNTPs	1 mM	5	0.2 mM
Forward primer 241A	0.4 µM	2	0.032 µM
Reverse primer 241B	0.4 µM	2	0.032 µM
Forward primer 245A	0.4 µM	2	0.032 µM
Reverse primer 245B	0.4 µM	2	0.032 µM
Forward primer 295A	0.4 µM	2	0.032 µM
Reverse primer 295B	0.4 µM	2	0.032 µM
Taq DNA Polymerase[b]	2 U/µL	0.5	0.04 U/µL
Subtotal		20	
Nucleic acid extract		5	
Total		25	

[a]Provider as indicated in the publication, but this company does not exist anymore. Further optimization is recommended for use with other PCR buffers.

[b]The manufacturer of the *Taq* polymerase was not given in the original paper.

2.2.2. PCR conditions

Initial denaturation at 95°C for 15 min, followed by 35 cycles of (95°C for 1 min, 57°C for 1 min, 72°C for 1 min), and 72°C for 7 min.

3. Essential Procedural Information

Controls:

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available 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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the 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 isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be (transcripts of) genes present in the matrix nucleic acids or added to the nucleic acid extracts.

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)
- 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).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.

PIC, PAC (and if relevant IC) a band of the expected sizes (550, 300 and/or 615 bp) are visualized.

When these conditions are met:

- A test will be considered positive if a band of the expected size (300, 550 and/or 615 bp) is visualized. The 300 bp band is usually present when the extracts are

from plants infected with *X. fragariae* but the other bands (550 and 615 bp) may appear occasionally.
- 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

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

Validation data from Hartung and Pooler (1997).

4.1. Analytical sensitivity data

10^3 cfu/mL in plant tissue.

4.2. Analytical specificity data

Inclusivity: 100% validated on 30 isolates of *X. fragariae*.

Exclusivity: 100% validated on 36 isolates of *X. campestris* (representing 19 pathovars) and 56 isolates of epiphytic bacteria commonly isolated from strawberry.

APPENDIX 6 - REAL-TIME PCR (WELLER ET AL., 2007)

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 and identification of X. fragariae in symptomatic and asymptomatic plant material and cell cultures.
1.2. The test is based on Weller et al., 2007.
1.3. The test targets the gyrB gene.
1.4. Oligonucleotides:

Primer	Sequence
Forward primer XF *gyrB*-F	5'-CCG CAG CGA CGC TGA TC -3'
Reverse primer XF *gyrB*-R	5'-ACG CCC ATT GGC AAC ACT TGA-3'
Probe XF *gyrB*-P	5'-JOE-TCC GCA GGC ACA TGG GCG AAG AAT TC-TAMRA-3'

1.5. ABI Prism 7700 Sequence Detection system; PE Biosystems, Foster City, California.

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. Matrices: symptomatic and asymptomatic plant material and cell cultures
2.1.2. See Appendix 3 for extraction procedures from plant material and cell cultures
2.1.3. DNA should preferably be stored at approximately -20 °C
2.2. Real-time Polymerase Chain Reaction – real-time PCR
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	8.875	N.A.
TaqMan Buffer A (*Applied Biosystems*)	10 ×	2.5	1 ×
$MgCl_2$	25 mM	5.5	5.5 mM
dNTPs Promega	25 mM	2.0	2 mM
Forward Primer XF *gyrB*-F	10 µM	0.75	300 nM
Reverse Primer XF *gyrB*-R	10 µM	0.75	300 nM
Probe 1 XF *gyrB*-P	5 µM	0.5	100 nM
AmpliTaq Gold DNA polymerase (*Applied Biosystems*)	5 U/µL	0.125	0.025 U/µL
Subtotal		21	
Nucleic acid extract		4	
Total		25	

2.2.2. PCR conditions: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of (95°C for 10 s and 60°C for 1 min)

3. Essential Procedural Information

Controls:

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available 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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the 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 isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be (transcripts of) genes present in the matrix nucleic acids or added to the nucleic acid extracts.

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 18 S 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).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) 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.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with EPPO Standard PM 7/98.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

Validation data from NVWA (NL).

4.1. Analytical sensitivity data

8.0×10^4 cfu/mL for detection in *Fragaria* spp. extract and 7.8×10^4 cfu/mL for pure cultures

4.2. Analytical specificity data

Inclusivity: 100% validated on 19 isolates of *X. fragariae*.

Exclusivity: 100% validated on 6 non-target organisms. The test allows to differentiate between *X. fragariae* and *X. arboricola* pv. *fragariae* (IPPC, 2017).

4.3. Data on repeatability

100%.

4.4. Data on reproducibility

100%.

4.5. Diagnostic sensitivity

100%.

4.6. Diagnostic specificity

100%.

APPENDIX 7 - REAL-TIME PCR (VANDROEMME ET AL., 2008)

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 and identification of *X. fragariae* in symptomatic and asymptomatic plant material and cell cultures.

1.2. The test is based on Vandroemme et al., 2008.

1.3. The test targets a 1 014 bp gene coding for a hypothetical protein that shows high homology with PilW family proteins (type IV pilus).

1.4. Oligonucleotides:

Primer	Sequence
Forward primer Xfr-QPCR-241-f	3′- CTT GTT CCG CGC GCA T – 5'
Reverse primer Xfr-QPCR-241-r	3′- TCG GTG ATT GCG AAT CTG C -5'
Probe Xfr-QPCR-241-p	3′- FAM-AGT CCC AAT GAA CCA ACG AGC AGC A-BHQ1–5'

1.5. The real-time PCR systems used to generate the validation data below is 7900HT Sequence Detection System (Applied Biosystems)

1.6. Software and settings (automatic or manual) for data analysis.

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: symptomatic and asymptomatic plant material, and cell cultures

2.1.2. See Appendix 3 for extraction procedures from plant material and cell cultures

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix[a]

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	8.34	N.A.
Taqman PCR Core Reagent Kit (*Thermofisher Scientific*)	10 x	2.5	1 ×
$MgCl_2$	25 mM	4	4 mM
dNTPs	25 mM	0.16	0.16 mM
T4 protein 32 GP 32 (*New England Biolabs*)	10 µg/µl	0.125	50 ng/µl
Forward Primer (Xfr-QPCR-241-f)	10 µM	0.75	300 nM
Reverse Primer (Xfr-QPCR-241-r)	10 µM	0.75	300 nM
Probe (Xfr-QPCR-241-p)	10 µM	0.25	100 nM
AmpliTaq Gold® DNA polymerase (*Thermofisher Scientific*)	5 U/µL	0.125	0.025 U/µL
Subtotal		20	
Nucleic acid extract		5	
Total		25	

[a]The Taqman PCR Core Reagent Kit (*Applied Biosystems*) was used for the published work.

2.2.2. PCR conditions: 95°C for 10 min, followed by 40 cycles of (95°C for 15 s and 60°C for 1 min)

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 extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available 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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the 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 isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be (transcripts of) genes present in the matrix nucleic acids or added to the nucleic acid extracts.

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 18 S 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).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) 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.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

Validation data from Vandroemme et al. (2008).

4.1. Analytical sensitivity data

300 cfu in 100 mg strawberry leaf sample; 25 fg (i.e. 5 cells) in water or in non-target DNA from strawberry tissue or *X. campestris* pv. *campestris* culture.

4.2. Analytical specificity data

Inclusivity: 100%, validated on 27 bacterial reference strains and isolates of *X. fragariae* from different geographical origins.

Exclusivity: 100%, validated on 40 bacterial strains: *Xanthomonas albilineans* (1), *Xanthomonas arboricola* pv. *juglandis* (1), pv. *fragariae* (5), *Xanthomonas arboricola* pv. *populi* (1), *Xanthomonas axonopodis* pv. *axonopodis* (1), *Xanthomonas axonopodis* pv. *begonia* (1), *Xanthomonas bromi* (1), *Xanthomonas campestris* pv. *campestris* (1), *Xanthomonas cassavae* (1), *Xanthomonas citri* (1), *Xanthomonas codiaei* (1), *Xanthomonas cucurbitae* (1), *Xanthomonas hortorum* pv. *hederae* (1), pv. *pelargonii* (1), *Xanthomonas hyacinthi* (1), *Xanthomonas melonis* (1), *Xanthomonas oryzae* pv. *oryzae* (1), *Xanthomonas pisi* (1), *Xanthomonas populi* (1), *Xanthomonas sacchari* (1), *Xanthomonas theicola* (1), *Xanthomonas translucens* (1), *Xanthomonas vasicola* (1), *Xanthomonas vesicatoria* (1), and 12 non identified bacterial isolates obtained from healthy strawberry tissue in Belgium in 2006.

APPENDIX 8 - LAMP (GÉTAZ ET AL., 2017)

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 and identification of *X. fragariae* in symptomatic and asymptomatic plant material and cell cultures.
1.2. The test is based on Gétaz et al., 2017.
1.3. The test targets the 5'end of a gene annotated as encoding a putative type IV secretion system protein VirD4 (GenBank ENZ93874) located on contig 93 of the draft genome of *X. fragariae* LMG 25863 (GenBank WGS AJRZ01000093).
1.4. Oligonucleotides:

Primer	Sequence
Outer Primer F3	5'- CGT CTC AGG TCA TGC CTT -3'
Outer Primer B3	5'- CGA TCC TGA TCT TCA TCG C -3'
Inner Primer FIP	5'- CCT ACG TGT TGG AGT GTG GCT ACC ATG AAC CGA GGC AA -3'
Inner Primer BIP	5'- TTA GGA ACC GCA CTG GCT TTG CAA GGT GAT GTA ACC G -3'
Loop Primer loopF	5'- CTC AAT CCA CCC AGG CAA -3'
Loop Primer loopB	5'- TGC TTC TAC TCG CCG CAT -3'

1.5. The LAMP system used to generate the validation data below is ABI 7900 HT (Applied Biosystems) or Genie II (OptiGene Ltd)

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. Matrices: symptomatic and asymptomatic plant material and cell cultures

2.1.2. See Appendix 3 for extraction procedures from plant material and cell cultures
2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. LAMP
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water	N.A.	To adjust final volume to 20 μL	N.A.
Isothermal mastermix (OptiGene Ltd)	2 ×	15	1.2 ×
Outer Primer F3	100 μM[a]	0.01[a]	0.16 μM
Outer Primer B3	100 μM[a]	0.01[a]	0.16 μM
Inner Primer FIP	100 μM[a]	0.1[a]	1.6 μM
Inner Primer BIP	100 μM[a]	0.1[a]	1.6 μM
Loop Primer loopF	100 μM[a]	0.05[a]	0.8 μM
Loop Primer loopR	100 μM[a]	0.05[a]	0.8 μM
Subtotal		20	
Nucleic acid extract		5	
Total		25	

[a] Although primer stocks are all at 100 μM, a primer master mix combining all six primers is prepared to reach the final concentrations mentioned.

2.2.2. LAMP conditions: 65°C for 30 min
2.2.3. Melting curve analysis during cooling from 92°C to 82°C with a temperature decrease of 0.05°C s^{-1}. Specific melting temperature is observed at 88.2±0.2°C.

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 extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available 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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the 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 isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be (transcripts of) genes present in the matrix nucleic acids or added to the nucleic acid extracts.

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 18 S 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).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no fluorescence
- PIC, PAC (and if relevant IC) should produce: Fluorescence. For end point measurement a positive reaction is defined by FU and/or T_m (°C±known variation). For real-time measurement a positive reaction is defined by time of positivity (minutes) and/or T_m (°C±known variation).

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

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

Validation data from Gétaz et al. (2017).

4.1. Analytical sensitivity data

Tested using strawberry crude leaf tissue extracts spiked with serial dilutions of *X. fragariae* LMG 25863 using boiled cells ranging from 10^6 to 10^1 cfu/mL after dilution in lysis and dilutions buffers: about 5–50 cfu per reaction (10^2 cfu/mL).

4.2. Analytical specificity data

Inclusivity: 100% validated on 37 strains from a culture collection of *X. fragariae*.

Exclusivity: 100% validated on 81 strains of other *Xanthomonas* species and pathovars *Xanthomonas arboricola* pv. *fragariae* (15), *Xanthomonas arboricola* (4), *Xanthomonas arboricola* pv. *corylina* (28), *Xanthomonas arboricola* pv. *juglandis* (1), *Xanthomonas albilineans* (6), *Xanthomonas alfalfae* subsp. *alfalfae* (1), *Xanthomonas alfalfae* subsp. *citrumelonis* (1), *Xanthomonas axonopodis* (1), *Xanthomonas axonopodis* pv. *aurantifolii* (1), *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (1), *Xanthomonas bromi* (1), *Xanthomonas campestris* pv. *campestris* (1), *Xanthomonas campestris* pv. *vesicatoria* (1), *Xanthomonas cassavae* (1), *Xanthomonas citri* pv. *citri* (1), *Xanthomonas citri* pv. *malvacearum* (1), *Xanthomonas codiaei* (1), *Xanthomonas cucurbitae* (2), *Xanthomonas hortorum* pv. *cynarae* (1), *Xanthomonas hortorum* pv. *gardneri* (1), *Xanthomonas hortorum* pv. *hederae* (2), *Xanthomonas hyacinthi* (1), *Xanthomonas melonis* (1), *Xanthomonas oryzae* pv. *oryzae* (1), *Xanthomonas perforans* (1), *Xanthomonas pisi* (1), *Xanthomonas sacchari* (1), *Xanthomonas theicola* (1), *Xanthomonas translucens* pv. *translucens* (1), *Xanthomonas vasicola* pv. *holcicola* (1) and 11 strains of other bacterial genera isolated from strawberry leaves: *Erwinia pyrifoliae* (1), *Kocuria kristinae* (1), *Microbacterium barkeri* (1), *Pantoea agglomerans* (1), *Pseudomonas fluorescens* (1), *Pseudomonas syringae* pv. *syringae* (1), *Rhizobium rubi* (1), *Rhodococcus fascians* (1), *Salmonella typhimurium* (1), *Staphylococcus cohnii* subsp. *cohnii* (1), *Variovorax paradoxus* (1).

APPENDIX 9 - PATHOGENICITY TEST

Pathogenicity test on detached leaf

Tissue sample preparations can be used for inoculating detached strawberry leaves as soon as they are prepared in extraction buffer or distilled water (Civerolo et al., 1997). Young (7–14 days old) leaves of a cultivar susceptible to *X. fragariae* (e.g. cvs. 'Camarosa', 'Pajaro', 'Seascape', 'Selva', 'Korona', 'Elsanta') from greenhouse-grown, *X. fragariae*-free plants should be used.

Three leaves (each one with three leaflets) from the greenhouse-grown plants are removed aseptically, the basal portion of the petioles is cut off and the petioles immediately placed in glass tubes containing sterile water.

Four sites on the abaxial surface of each leaflet (two on each side of the main vein) are infiltrated using a needleless syringe (3 mL plastic disposal BD, 2 mm orifice).

The excess inoculum is rinsed off with sterile water 1 h after inoculation. The leaves with their petioles are placed in the tubes in a humid chamber (relative humidity 95–100%) and incubated at 18–20°C with a 12 h photoperiod for up to 21 days. The inoculated leaves should not have visible injuries, and water-soaking caused by the inoculum infiltration should disappear within 24 h.

Specific symptoms (i.e. angular dark water-soaked lesions) similar to those observed on naturally infected leaves begin to appear a few days after inoculation. Symptoms are recorded every 2 days for up to 21 days.

The detached leaf assay is negative if no typical *X. fragariae* angular leaf spots (i.e. dark and water-soaked when viewed with reflected light; translucent yellow when viewed with transmitted light) and/or chlorotic halos appear at any of the inoculated sites after 21 days. No water-soaked spots that appear translucent yellow when viewed with transmitted light should appear within inoculation sites infiltrated with negative controls (Civerolo et al., 1997).

The detached leaf assay is positive if typical *X. fragariae* angular leaf spots (i.e. dark and water-soaked when viewed with reflected light; translucent yellow when viewed with transmitted light) develop at the infiltration inoculation sites within 10 to 21 days. These should be similar in appearance to those that develop at inoculation sites infiltrated with the positive control suspensions.

Pathogenicity test on plants

For pathogenicity test on plants, *X. fragariae*-free strawberry plants of a susceptible cultivar (e.g. cvs. 'Camarosa', 'Seascape', 'Selva', 'Korona', 'Pajaro', 'Elsanta') should be used. Plants should be kept overnight in an environmental chamber at 20–25°C with

high (>90%) relative humidity and exposed to light for 4 h before inoculation to induce stomatal opening.

Bacterial cell suspensions (10^6 cfu/mL) in sterile distilled water or 10 mM PBS are prepared. Each strain is inoculated into the abaxial surfaces of three trifoliate leaves on each of two or three plants with a low-pressure spray gun, airbrush or similar device (e.g., from DeVilbiss) so as not to induce water soaking. Infection may be facilitated by wounding leaves (e.g., puncturing the abaxial surface with a needle) before applying inoculum, although it is not necessary to do this. After inoculation, plants are incubated in a chamber maintained at 20–25°C with high humidity (>90%) and a 12–14 h photoperiod. Lesion development is evaluated weekly for 21 days post-inoculation. The pathogen is re-isolated from such lesions, and identified by immunofluorescence or molecular tests.

If the bacterial cell suspension contains *X. fragariae*, initial symptoms will be dark, water-soaked (when viewed with reflected light) lesions on the lower leaf surfaces. These lesions appear translucent yellow when viewed with transmitted light. Later these lesions develop into necrotic spots surrounded by a yellow halo or marginal necrosis.