Diagnostics[1]
Diagnostic

Xanthomonas fragariae

Specific scope

This standard describes a diagnostic protocol for *Xanthomonas fragariae*.

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2005-09.

Introduction

Xanthomonas fragariae is the causal agent of bacterial angular leaf spot of strawberry. It is an insidious and potentially serious disease which was first reported in USA (Kennedy & King, 1962a). It was later described in New Zealand, Australia, a few Asian and African countries and in most European countries where strawberry is cultivated (EPPO/CABI, 1998). The disease has been reported to cause the loss of 75% of fruit in Wisconsin (US) (Epstein, 1966). It is widespread in nurseries in many countries and has been responsible for important production losses in Europe (Mazzucchi *et al*., 1973; López *et al*., 1985; Bosshard & Schwind, 1997). *X. fragariae* is easily transmitted via asymptomatic plants with latent infections and international movement of latently infected plants is blamed for the introduction of *X. fragariae* from the USA to many other countries (López *et al*., 1985). The pathogen spreads from plants harvested in infested nurseries and symptoms appear under favourable conditions as well as after cold storage (Rat, 1993).

Identity

Name: *Xanthomonas fragariae* Kennedy & King.
Taxonomic position: *Bacteria, Gracilicutes*.
EPPO code: XANTFR.
Phytosanitary categorization: EPPO A2 list no. 135, EU Annex II/A2.

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

Detection

The natural hosts of *X. fragariae* are *Fragaria x ananassa*, its parents *Fragaria chiloensis* and *Fragaria virginiana*, and various wild strawberries such as *Fragaria vesca*.

Symptoms

Small (1–4 mm) angular water-soaked spots appear initially only on the lower leaf surface surrounded by the veins. In the early stage, the spots are only visible on the lower surface and appear translucent when viewed with transmitted light. The bacteria are disseminated from the spots by irrigation, rain or dew to initiate new infections, frequently along the main veins of the leaf (Kennedy & King, 1962b). With high relative humidity, white, milky, cream-coloured or yellow exudates can appear, accumulating as yellow glistening opaque or brown masses. The leaves are most sensitive when they are two weeks to two months old. Older and younger leaves are resistant to infection. The size of the lesions increases progressively and, subsequently, the spots may coalesce and become apparent in the upper surface of the leaf (Rat, 1993). Dead tissues appear as reddish-brown irregular spots, and tear and break off. Systemic infections of the crown of the plants have been described in the USA by Hildebrand *et al*. (1967) and the endophytic movement of the bacterium confirmed by Stefani *et al*. (1989).

Symptoms of angular leaf spot caused by *X. fragariae* may be confused with those caused by fungi like *Mycosphaerella fragariae* and with the symptoms caused by a new pathovar of *X. arboricola*, pv. *fragariae* (Janse *et al*., 2001). Preliminary studies show that *X. a. fragariae* may be more widespread

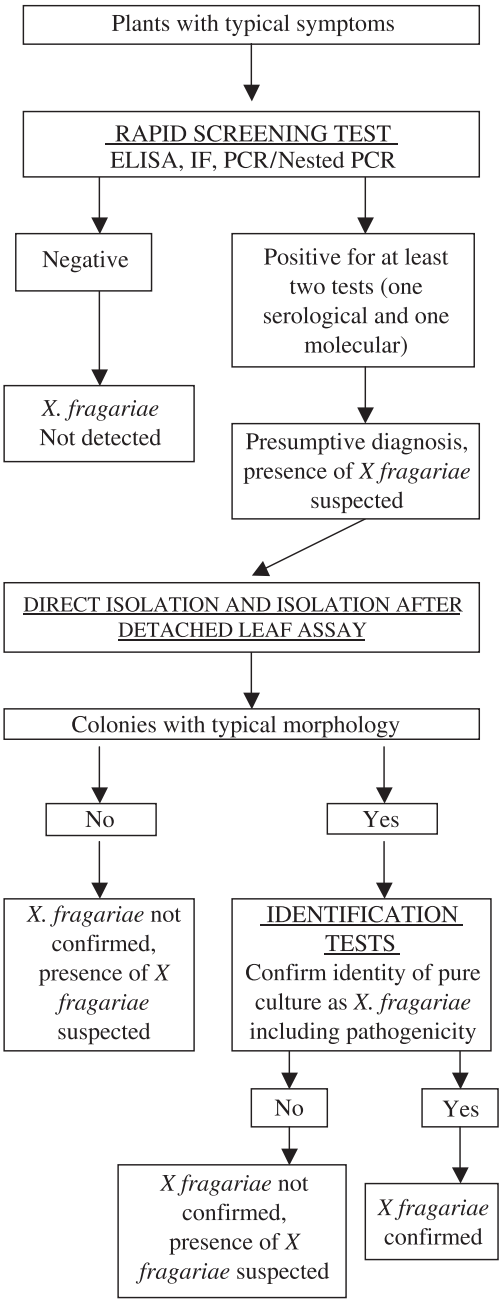

Fig. 1 Flow diagram for the diagnosis of bacterial angular leaf spot (*Xanthomonas fragariae*) on host plants with symptoms.

in the EPPO region than originally estimated. Definitive diagnosis should always be obtained through laboratory analysis.

Identification on plants with symptoms

The procedures to be followed are summarized in the flow diagram of Fig. 1.

Sampling and sample preparation

The sample for analysis of bacterial angular leaf spot on plants with symptoms should if possible be leaves with young water-soaked spots, or otherwise dry spots with or without exudates. In the case of suspected systemic infections, tissue from the crown of the plants should be tested. The general sample preparation procedure (Appendix 1 – symptomatic plants) is valid for isolation, bioassay, serological detection and PCR amplification (before or after biological enrichment) of *X. fragariae*.

Rapid screening tests

Rapid screening tests (Appendix 2) facilitate presumptive diagnosis. Positive results should be obtained in two tests – a serological test (ELISA or Immunofluorescence (IF) and PCR/Nested PCR) to confirm presumptive diagnosis of *X. fragariae*, as the bacterium is very difficult to isolate.

Isolation

Direct isolation of *X. fragariae* is difficult, even in the presence of symptoms and exudates, because the bacterium is very slow-growing on artificial nutrient media. Leaves with young lesions are selected from plants with symptoms and disinfected individually with a cotton-wool stick soaked in 70% ethanol. Isolations should be made from young, water-soaked lesions, or from the margins of older lesions by cutting out a small piece of tissue with a sharp sterile scalpel. The tissue is crushed in a few mL of sterile distilled water or PBS (Appendix 7) and held for 10–15 min, and processed as in Appendix 3 (method 1). Isolation is usually more successful on Wilbrink's medium with nitrate (Wilbrink-N) (Appendix 7) than on YPGA or other common media. Use of purified agar (Difco) is recommended in all media because impurities from other commercial agars can inhibit the growth of *X. fragariae*.

An alternative method for isolation is based on surface sterilization of the infected plant material, followed by diffusion of bacteria into an extraction buffer, plating and growth of the bacteria on nutrient media (Appendix 3, method 2).

Interpretation of isolation results

The isolation is negative if no bacterial colonies with morphology similar to *X. fragariae* are observed after 7 days on either of two media (provided that no inhibition is suspected due to competition or antagonism) and typical *X. fragariae* colonies are found in the plates of the positive control. A negative result does not guarantee the absence of the pathogen. The isolation is positive if presumptive *X. fragariae* colonies are isolated in at least one of the media used.

When analysing symptomatic samples, good correlation is not always observed between isolation and the rapid screening tests, because isolation frequently fails. Best isolation results can be expected if freshly prepared samples extracted from young lesions are used. Even if isolation fails, the sample should

be considered as presumptively affected by *X. fragariae* if a serological test, and PCR, are positive.

Detached leaf assay
Samples prepared in the maceration buffer can be used directly to inoculate detached strawberry leaves, following Civerolo *et al*. (1997) (Appendix 4). The detached leaf assay is negative if no typical angular leaf spots or chlorotic haloes appears in some of the inoculated sites after 28 days. The negative controls should be negative or at least show totally different type of lesions. The detached leaf assay is positive if typical angular leaf spots or chlorotic haloes appear in the inoculated sites after 28 days. The lesions should differ from those which may sometimes be observed in the negative controls.

PCR or dilution plating after detached leaf assay
The inoculated detached leaves can be used for enrichment prior to PCR or dilution plating. One leaf per sample is taken from those inoculated in the detached leaf assay, 48 h after inoculation, and 10–12 small discs 0.5 cm in diameter are taken from each inoculated site and crushed in 4.5 mL PBS (Appendix 7).

For PCR, the samples can be stored at –20°C at this point. The method of the *Rapid screening tests* (above) is used (Appendix 2). Each of the soaked enriched samples is streaked on Wilbrink N medium plates and the bacterial growth is washed off after 4 days for PCR analysis. This is a modification of the bio-enrichment PCR described by Schaad *et al*. (1995).

For dilution plating, the samples are used directly, without freezing. The enrichments are plated on Wilbrink N medium, after preparation of 1 : 10, 1 : 100 and 1 : 1000 dilutions in PBS (Appendix 7). The dilutions are plated by triple streaking (Appendix 3) to obtain isolated colonies. After incubation at 25°C for 5–7 days, *X. fragariae*-like colonies are recorded as for *Isolation* (Appendix 3).

Identification in asymptomatic samples

Whereas a flow diagram is provided for the identification of the bacterium in plants with symptoms (Fig. 1), there is not enough information to do the same for identification in asymptomatic samples.

Sampling and sample preparation

Asymptomatic samples can be processed individually or in groups of up to 50 plants. Where surveys are performed, they should be based on statistically representative samples. Samples taken from stored material can be considered random whereas those from orchards or nurseries may not be.

Sampling and sample preparation for analysis of asymptomatic runners can be performed according to a modification of the procedure of OEPP/EPPO (1994) (Appendix 1 – asymptomatic plants, method 1) or by testing the crown and petioles of the plants (Randhawa California Seed and Plant Laboratory) (Appendix 1 – asymptomatic plants, method 2).

Isolation

The washing liquid, pellet or macerate of crowns can be used for direct isolation as in the method for symptomatic samples (Appendix 3). Unfortunately, isolation of *X. fragariae* from asymptomatic samples is normally negative due to low bacterial populations.

Screening tests

The procedures for ELISA, IF and PCR are as for symptomatic samples (Appendix 2). If two of the screening tests are positive but the isolation is negative, an attempt can be made to isolate the pathogen from new fresh samples.

Confirmation

Biochemical and physiological identification

Conventional tests
X. fragariae has the common characteristics of all xanthomonads: Gram-negative, aerobic, rods, with single polar flagellum, nitrates not reduced, catalase positive, asparagine not used as a sole source of carbon and nitrogen and producer of xanthomonadin, weak production of acids from carbohydrates. Colonies are mucoid, convex, and shiny on YPGA and Wilbrink-N media (Dye, 1962; van den Mooter & Swings, 1990; Swings *et al*., 1993). *Xanthomonas* spp. are easily differentiated from the other genera of aerobic, Gram-negative rods and other yellow pigmented bacteria by the characters shown in Table 1, following (Schaad & Stall, 1989; Schaad *et al*., 2001).

The most relevant or useful characteristics for distinguishing *X. fragariae* from other *Xanthomonas* (Schaad & Stall 1989; Goszczynska *et al*., 2000; Janse *et al*., 2001) are shown in Table 2. Differences from *X. campestris* justify the status of *X. fragariae* as a separate species. Growth is poor on nutrient agar but much better when supplemented with 5% glucose. Purified agar is required for growth of most of the strains. For long-term preservation, cultures should be stored at –80°C, as a suspension of more than 10^{10} cfu mL^{-1} in sterile 30% glycerol.

Biochemical characterization by commercial systems
X. fragariae can be identified biochemically by its specific profiles in API 20 NE and API 50 CH strips (BioMérieux, France). For API 20 NE, the manufacturer's instructions are followed to prepare a suspension from a 48-h culture on Wilbrink-N medium (Appendix 7) and inoculate a strip, which is incubated at 25–26°C and read after 48 h for enzymatic activities and 96 h for substrate utilization (Table 3). For API 50 CH, a suspension of OD = 1.0 is prepared in PBS (Appendix 7), of which 1 mL is added to 20 mL of medium C of Dye (1962) (Appendix 7). The manufacturer's instructions are followed for inoculation of the strip, which is incubated at 25°C aerobically and read after 2, 3 and 6 days. Utilization of the different carbohydrates is shown by a yellow colour in the well after the incubation period (Table 4).

Table 1 Phenotypic characteristics useful for differentiating *Xanthomonas* from *Pseudomonas* and other yellow-pigmented bacteria such as *Flavobacterium*

Test	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagella	1, polar	> 1, polar	none	peritrichous
Xanthomonadin	+	–	–	–
Fluorescence	–	V	–	–
Levan from sucrose	+	V	–	–
H_2S from cysteine	+	–	–	–
Oxidase	–	V	+	–
Fermentation	–	–	–	+
Growth on 0.1% TTC	–	+	+	+

V: variable reaction.

Table 2 Diagnostic tests for differentiation of *Xanthomonas* spp

Tests	*X. campestris*	*X. arboricola* pv. *fragariae*	*X. fragariae*
Growth at 35°C	+	ND	–
Growth on 2% NaCl	+	+	–
Esculin hydrolysis	+	+	–
Gelatin liquefaction	V	+	+
Protein digestion	+	ND	–
Starch hydrolysis	V	+	+
Urease production	–	–	–
Acid from:			
Arabinose	+	ND	–
Galactose	+	+	–
Trehalose	+	ND	–
Cellobiose	+	+	–

V: variable reaction.
ND: Not determined.

Table 3 Reaction of *X. fragariae* in API 20 NE tests

Test	Reaction (48 or 96 h)[1]
Glucose fermentation	–
Arginine	–
Urease	–
Esculin	+
Gelatin	weak +
PNPG	+
Assimilation of	
Glucose	+
Arabinose	–
Mannose	+
Mannitol	–
N-acetyl-glucosamine	+
Maltose	–
Gluconate	–
Caprate	–
Adipate	–
Malate	+
Citrate	–
Phenylacetate	–

[1]Common reactions of 90% strains of *X. fragariae* analysed (Domínguez *et al*., pers. comm.). For first 6 tests, read at 48 h. For others, read at 96 h.

Serological tests

For IF-test, a suspension of approximately 10^6 cells per mL is prepared in PBS buffer and the IF procedure for symptomatic samples is applied (Appendix 2). Alternatively, indirect ELISA or DAS-ELISA can be used (Appendix 2). Recommended antibodies are also given in Appendix 2. If only two identification tests are performed, only one should be serological.

PCR test

A suspension of approximately 10^6 cells per mL in molecular grade sterile water is prepared from a 48 h growing culture on Wilbrink-N medium. The PCR procedure given for symptomatic samples can be used (Appendix 2) or alternatively the protocol of Roberts *et al*. (1996), without any DNA extraction (Appendix 5).

Fatty acid profiling (FAP)

Cultures are grown on trypticase soy agar for 48 h at 28°C. Fatty acid analysis is available from MIDI (Newark, US), NCPPB (CSL, York, GB), and PD (Wageningen, NL). A positive test is achieved if the profile of the presumptive culture is identical to that of the *X. fragariae* positive control.

REP-PCR

The following protocols have been used by Opgenorth *et al*. (1996) to identify *X. fragariae* strains specifically. The PCR reaction mixture and amplification conditions are essentially the same as those described by Louws *et al*. (1994). The sequences of the primers are as follows: REP1R-I, 5′-IIIICGICGIC-ATCIGGC-3′; REP2-I, 5′-ICGICTTATCIGGCCTAC-3′; ERIC1R, 5′-ATGTAAGCTCCTGGGGATTCAC-3′; ERIC2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′. Amplification conditions were: 95°C for 6 min followed by 35 cycles at 94°C for 1 min, 44°C (REP primers) or 52°C (ERIC primers) for 1 min, 65°C for 8 min. The amplification cycles were followed by a final extension cycle of 68°C for 16 min.

Inoculation

Suspected *X. fragariae* colonies from the isolation and enrichment plates should be inoculated onto strawberry plants to prove their

Table 4 Readings after 6 days for a typical *X. fragariae* culture in API 50 CH

Test[1]	Reaction
D-arabinose	Variable
Galactose	+
D-glucose	+
D-fructose	+
D-mannose	+
N-acetylglucosamine	+
Esculin	+
Melibiose	Variable
Sucrose	+
Trehalose	+
D-xilose	+
L-fucose	+

[1]The remaining sugars are not utilized by *X. fragariae* (Domínguez, pers. comm.)

pathogenicity, specially those from new findings. The detached leaf assay described for symptomatic samples may be used (Appendix 4) or other procedures (Hazel & Civerolo, 1980) (Appendix 6).

Reference material

See Appendix 8.

Reporting and documentation

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

Further information

Further information on this organism can be obtained from:

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[2]J. Janse (Plant Protection Service, Wageningen, NL); M. Keck (Bundesamt und Forschungszentrum für Landwirtschaft, Vienna, AT); A. Sletten (Plant Protection Centre, Ås, NO); S. Simpkins (Central Science Laboratory, York GB); L. Cruz (Centro Nacional da Producao Tapada de Ajuda, Lisbon, PT); F. Poliakoff (LNPV Unité Bactériologie, Angers, FR); J. Van Vaerenbergh Rijkstation voor Plantenziekten, Merelbeke (BE); A. Baudry NPV, Villenave d'Ornon (FR); M.M. López (IVIA, Moncada-Valencia, ES).

References

Bosshard E & Schwind M (1997) Detection of different bacterial and fungal pathogens in apparently healthy strawberry plants. In: *Diagnosis and Identification of Plant Pathogens* (Ed. H-W Dehne, *et al*.), pp. 37–41. Kluwer, Dordrecht (NL).

Bradbury JF (1986) *Guide to Plant Pathogenic Bacteria*. CAB International, Wallingford (GB).

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

Civerolo EL & Roberts P (1997) Comparative detection of *Xanthomonas fragariae* in strawberry plants by detached leaf inoculation, ELISA and PCR. In: *Proceedings of the 9th International Conference of Plant Pathogenic Bacteria* (Ed. Mahadevan, A), pp. 95–99. University of Madras, Madras (IN).

Dye DW (1962) The inadequacy of the usual determinative test for the identification of *Xanthomonas* spp. *New Zealand Journal of Science* **5**, 393–416.

EPPO/CABI (1998) *Distribution Maps of Quarantine Pests for Europe*. CAB International, Wallingford (GB).

Epstein AH (1966) Angular leaf spot of strawberry. *Plant Disease* **50**, 167.

EU (1998) Interim testing scheme for the diagnosis, detection and identification of *Ralstonia solanacearum* in potatoes. Annex II to Council Directive 98/57/EC of 20 July 1998 on the control of *Ralstonia solanacearum*. *Official Journal of the European Communities* **L235**, 8–39.

Goszczynska T, Serfontein JJ & Serfontein S (2000) *Introduction to Practical Phytobacteriology*. Swiss Agency for Development and Cooperation, Bern (CH).

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.

Janse JD, Rossi MP, Gorkink RFJ, Derks JHJ, Swings J, Janssens D & Scortichini M (2001) Bacterial leaf blight of strawberry (*Fragaria* x *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 & King TH (1962a) Angular leaf spot of strawberry caused by *Xanthomonas fragariae* sp. nov. *Phytopathology* **52**, 873–875.

Kennedy BW & King TH (1962b) Studies on epidemiology of bacterial angular leaf spot of strawberry. *Plant Disease Reporter* **46**, 360–363.

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

López MM, Aramburu JM, Cambra M & Borras V (1985) [Detection and identification of *Xanthomonas fragariae*. Spain.] *Anales INIA* **28**, 245–257 (in Spanish).

Louws FJ, Fulbright DW, Stephens CT & de Bruijn FJ (1994) Specific genomic fingerprints of phytopathogenic *Xanthomonas and Pseudomonas* pathovar and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**, 2286–2295.

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

OEPP/EPPO (1994) EPPO Standards PM 3/58 Phytosanitary procedures *Xanthomonas fragariae*. *Bulletin OEPP/EPPO Bulletin* **24**, 343–346.

Opgenorth DC, Smart CD, Louws FJ, 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: *Xanthomonas* (Ed. Swings, JG & Civerolo, EL), pp. 69–70. Chapman & Hall, London (GB).

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 polymerase chain reaction. *Plant Disease* **80**, 1283–1288.

Schaad NW, Cheong SS, Tamaki S, Hatziloukas E & Panapoulos NJ (1995) A combined biological and enzymatic amplification (Bio-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* **85**, 243–248.

Schaad NW, Jones JB & Lacy GH (2001) *Xanthomonas*. *Guide for Identification of Plant Pathogenic Bacteria*, 2nd edn., pp. 175–200. APS Press, St Paul (US).

Schaad NW & Stall RE (1989) *Xanthomonas*. *Guide for Identification of Plant Pathogenic Bacteria*, pp. 81–94. APS Press, St Paul (US).

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

Stoger 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.

Swings J, Vauterin L & Kersters K (1993) The bacterium *Xanthomonas*. In: *Xanthomonas* (Ed. Swings, J & Civerolo, EL), pp. 138–144. Chapman & Hall, London (GB).

van den Mooter M & Swings J (1990) Numerical analysis of 295 phenotypic features of 266 *Xanthomonas* strains and related strains and an improved taxonomy of the genus. *International Journal of Systematic Bacteriology* **40**, 348–369.

Zimmermann C, Hinrisch-Berger C, Moltmann E & Buchenhauer H (2004) Nested PCR for detection of *Xanthomonas fragariae* in symptomless strawberry plants. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **111**, 39–51.

Appendix 1 Sample preparation

Symptomatic plants

Select plants with young symptoms (water-soaked spots in the lower part of the leaves). Externally disinfect the parts to be tested with 70% ethanol. Aseptically excise 0.1 g from each leaf containing water-soaked spots. If the plants show vascular symptoms, remove the roots and the leaves, carefully rinse the crown and petioles under tap water and then disinfect them by immersion for 1 min in 70% ethanol and rinse each plant separately three times in sterile distilled water. Take 0.1 g per sample from the leaves or crown and petioles and add 9 mL of PBS (Appendix 7) in a plastic bag with a heavy net. Slightly crush the plant material with a hammer or other appropriate apparatus in the plastic bag. Let all the samples macerate for 15 min at room temperature.

Transfer 2 mL, 1 mL and 1 mL from each macerated sample to three sterile Eppendorf tubes. Immediately (on the same day), use the 2 mL for isolation, inoculation of detached leaves and IF. Store the other two tubes (with 1 mL), respectively, at –20°C, and at –80°C with 30% glycerol. The ELISA and PCR analysis are performed as soon as convenient, using aliquots from the Eppendorf tubes stored at –20°C.

Asymptomatic plants

Method 1

Remove leaves and roots from runners, keeping the petioles. Wash the remaining crowns in running water, drain and cut lengthwise into quarters. Collect a 30 g sample at random from the 200 quarters. Place each sample in 150 mL of PBS (Appendix 7). Shake for 30 min and use the washing liquid directly for detection, or after centrifugation at 10 000 *g* per 10 min. Suspend the final pellet in sterile distilled water to obtain a final volume of 5 mL. After leaving to settle for 15 min collect the upper clarified part and prepare dilutions (1 : 10 and 1 : 100) in sterile distilled water. Keep 2 mL for analysis and put 1 mL at –20°C and 1 mL at –80°C with 30% glycerol.

Method 2

Surface-disinfest runners with 0.5% sodium hypochlorite +0.02% Tween 20 for 2 min, followed by two washes with sterile distilled water. Take small discs of tissue about 0.3–0.5 cm in diameter from the crown and petioles of each plant, wash in running water before crushing and macerating them in 10 mM MOPS buffer pH 7.3 for 15–30 min at room temperature.

Appendix 2 Rapid screening tests

ELISA

Use 2 mL of the sample freshly prepared or stored at –20°C. Two ELISA protocols with commercially available polyclonal antibodies (Appendix 8) have been validated in a ring test performed by 10 laboratories. Use a pure culture of *X. fragariae* and a non *X. fragariae* strain as positive and negative controls in each plate. Because of the frequent cross reactions with polyclonal antibodies, healthy plant controls should be used. The titre should preferably be determined for each new batch of antibodies.

Indirect ELISA

Mix 210 µL of each sample with 210 µL coating buffer pH 9.6 (Appendix 7). Add 200 µL of the mix to two wells of one microtiter plate (Nunc-Polysorp or equivalent). Prepare a 10^9 cfu mL^{-1} suspension of the positive control in PBS (Appendix 7). Mix 210 µL of this suspension with 210 µL of coating buffer and fill two wells. Prepare a 10^9 cfu mL^{-1} suspension of the negative control bacterium, mix 1 : 1 with coating buffer and fill in other two wells. Crush 0.01 g of strawberry (*X. fragariae*-free) material (leaf or crown) in 0.9 mL PBS (Appendix 7) and add 0.9 mL of coating buffer (Appendix 7). Use this as plant material negative control and fill two wells. Wrap the plate tightly in cling film or place in a plastic box with some damp paper towels and close the box. Incubate the plate at 4°C overnight.

Wash the plate three times with PBS (Appendix 7) diluted 1/2 +0.05% Tween 20. Fill the wells of the plate with PBS 1/2 +0.05% Tween 20 and invert to remove the buffer. Repeat twice. Put the plate to dry on paper towels. Add 200 µL of blocking buffer (PBS +1% bovine serum albumin (BSA, or non fat milk powder) to each of the test wells. Wrap the plate as described above and incubate at 37°C for 1 h. Wash the plate as described above.

Prepare the *X. fragariae* antibody at appropriate dilution in PBS (Appendix 7) and add 200 µL to each test well. Wrap the plate as described above and incubate at 37°C for 2 h. Wash the plate as described above. Prepare the antibody-enzyme conjugate at appropriate dilution in PBS (Appendix 7) +0.2% BSA and add 200 µL to each test well. Wrap the plate as described above and incubate at 37°C for 1 h. Wash the plate four times. Prepare the substrate just before use. Add p-nitrophenylphosphate at 1 mg/mL to substrate buffer pH 9.8 (Appendix 7). Add 200 µL of prepared substrate to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min. Read the absorbance at 405 nm.

DAS-ELISA

Prepare antibodies at the appropriate dilution in coating buffer pH 9.6 (Appendix 1). Add 200 µL to each well of two plates. Incubate at 37°C for 4 h. Wash the wells three times with PBS (Appendix 7) 1/2 +0.05% Tween 20. Add 200 µL of each sample to two wells of each plate. Add also in two wells 200 µL of positive and negative controls, as described for indirect ELISA, to each plate. Incubate for 16 h at 4°C. Wash the wells three times with PBS 1/2 +0.05% Tween 20. Incubate at 37°C for 1 h, and wash three times as before. Prepare the appropriate dilution of the conjugate in PBS (Appendix 7) containing 0.2% BSA and add 200 µL to each well. Incubate at 37°C for 3 h. Wash the wells four times with PBS 1/2 +0.05% Tween 20. Prepare the substrate just before use. Add p-nitrophenylphosphate at 1 mg/mL to substrate buffer pH 9.8 (Appendix 7). Add 200 µL of prepared substrate to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min. Read the absorbance at 405 nm.

Interpretation of ELISA test results

The ELISA test is negative if the average absorbance reading from duplicate sample wells is < 2x the average absorbance of that in the negative sample extract control wells. This is provided that the absorbances for the positive controls are all above 1.0 after 60 min incubation and are greater than twice the absorbances obtained for negative sample extracts.

The ELISA test is positive if the average absorbance readings from duplicate sample wells is > 2x the average absorbance in the negative sample extract well provided that 2x average absorbance readings in all negative control wells are lower those in the positive control wells.

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

Immunofluorescence

Follow the standard procedure described in EU (1998), preferably using a validated source of antibodies. Two commercially available polyclonal antibodies (Appendix 8) have been validated using FITC-conjugated antispecies immunoglobulins.

Use undiluted preparations of the samples and their 1 : 10, 1 : 100 and 1 : 1000 dilutions in PBS (Appendix 7) to spot windows of the IF slides. Prepare one IF slide for each sample and its dilutions. Prepare positive controls with 10^6 cfu mL^{-1} suspensions in PBS of a pure culture of a reference strain of *X. fragariae* and a negative controls (healthy plant controls and another bacterium). Include negative buffer controls with antibodies and conjugate, and with conjugate only. Air-dry, and fix by flaming or by absolute or 95° ethanol. Store slides at –20°C until required. Use the antibodies at the recommended dilutions in PBS (Appendix 7). Use of two dilutions of the antibodies is advised to detect cross reactions with other bacteria. Spot the appropriate amount per well. Incubate slides in a moist chamber for 30 min at room temperature. Shake droplets off the slide and rinse slides with PBS. Wash 10 min with the same buffer. Carefully remove excess moisture. Dilute the appropriate FITC conjugate in PBS (Appendix 7). Cover the windows of all slides with the corresponding diluted conjugate and incubate in a moist chamber for 30 min at room temperature. Repeat the washing step. Add 0.1 M phosphate-buffered glycerol mountant (with antifading) on each window and apply a cover slip. View slides under oil immersion at 500–1000× magnification by scanning windows across 2 diameters at right angles and around the perimeters. Count the cells that show fluorescence and have a similar size to that of the reference strain of *X. fragariae* as described in EU (1998).

Interpretation of the IF test result

The test is negative if green fluorescing cells with morphology typical of *X. fragariae* are observed in positive controls but not in sample windows.

The test is positive if green fluorescing cells with typical morphology are observed in positive control and sample windows, but not in negative control windows. As a population of 10^3 cells per mL is considered the limit of reliable detection by the IF test, the IF test is considered positive for samples with > 10^3 cells per mL. For samples with < 10^3 cells per mL, the result of the IF test may be considered doubtful in which case further testing or re-sampling should be performed. Samples with large numbers of incomplete or weakly fluorescing cells compared to the positive control need further testing. The test should be based on a different biological principle, or involve a repeat IF test with different dilutions of antibody or pellet or a second source of antibodies.

PCR

Use the validated PCR reagents and protocol. A nested PCR in two tubes has been developed but is not yet validated (Zimmerman *et al*., 2004). Care should be taken with nested

PCR since the increased sensitivity gained may increase the possibility for false positive results. Every precaution should be taken to avoid contamination of samples with target DNA. As positive controls, use aliquots of strawberry samples which previously gave a negative test result by several techniques, to which 10^4 and 10^6 cells mL^{-1} of a reference X. fragariae strain has been added, and a suspension of 10^4 cells mL^{-1} of the bacterium. Positive controls should be prepared separately. As negative controls, use at least a sample extract which previously gave a negative test result for X. fragariae, and a sample of ultra pure water. Perform the DNA extraction from the positive and negative controls as well as from the samples, and also include negative controls after extraction.

One protocol for DNA extraction from plant samples, and the amplification protocol of Pooler, Ritchie & Hartung (1996), have been validated in the ring test. Other commercial kits for extracting DNA, such as the REDExtract-N-Amp Plant PCR-Kit (Sigma) (Stoger & Ruppitsch, 2004) and other PCR primers (Roberts *et al*., 1996) are available but have not been validated.

DNA extraction protocol using the Qiagen DNeasy Plant Kit

DNA extraction should always be performed before amplification. The Qiagen Dneasy Plant kit, modified from the MLO DNA extraction provided by R. Martin, USDA/ARS, Corvallis (US), has given the best results in preliminary testing.

Take 250 µL from the frozen samples. Always include healthy strawberry plant material and PBS as negative controls and pure culture *X. fragariae* cells as a positive control for the extraction and amplification. Add 250 µL CTAB extraction buffer and 4 µL of RNAse A (provided by Qiagen at 100 mg/mL). Invert gently 5 times and incubate at 65°C for 10 min with occasional mixing by inversion. Add 200 µL of buffer AP2 (provided by Qiagen) and invert gently 5 times. Incubate on ice for 5 min. Transfer contents of tube to QIAshredder column (purple) by pouring. Spin at high speed for 5 min. Repeat 5 min spin if needed. Transfer 450 µL of supernatant into a new 1.5-mL tube, being careful to avoid the pellet. To the new tube containing the supernatant, add 675 µL of buffer AP3/E provided by Qiagen. Mix by inverting gently 5 times. Transfer 650 µL to a Qiaamp column (white) placed in a 2-mL collection tube. Spin at 10 000 rev min^{-1} for 1 min. Discard flow-through and repeat spin. If necessary, repeat a third time. Discard the collection tube and transfer column to a new collection tube. Wash column twice with 500 µL of Qiagen buffer AW. Add first 500 µL and spin at 10 000 rev min^{-1} for 1 min. Discard flow-through. Add second 500 µL and spin at 14 000 rev min^{-1} for 1 min. To elute the DNA, transfer column to a new 1.5-mL tube. Add 100 µL of 10 mM Tris-HCI, pH 9 preheated to 65°C. Spin at 10 000 rev min^{-1} for 1 min. Add additional 100 µL of Tris-HCI and repeat spin. Using TE buffer, bring the volume of DNA solution to a total volume of 300 µL. Add 200 µL of 5 M ammonium acetate and 1 mL of absolute ethanol. Mix well. Incubate for 1 h or overnight at –20°C. After incubation, spin at 14 000 rev min^{-1} for 10 min. Pour off supernatant keeping the pellet. Add 1 mL absolute ethanol to pellet and spin at 14 000 rev min^{-1} for 5 min. Pour off supernatant keeping the pellet. Add 500 µL of 80% ethanol and spin for 5 min at 14 000 rev min^{-1}. Pour off supernatant keeping the pellet. Invert tubes and allow to dry at room temp. After pellet has dried completely, resuspend in 50 µL of sterile distilled water.

Amplification by multiplex PCR

This protocol has given the best results in preliminary tests and validated in the ring test. Prepare the following master mix for each sample: 2.5 µL Perkin Elmer buffer (with 15 mM $MgCl_2$); 5.0 µL dNTP (1 mM); 2.0 µL each primer (5 µM) 6 primers in total; 0.5 µL Taq polymerase; 5.0 µL sample DNA. Final volume 25.0 µL. Use the three sets of primers of Pooler, Richie & Hartung (1996):

241 A-5′-GCC CGA CGC GAG TTG AATC-3′
241 B-5′-GCC CGA CGC GCT ACA GAC TC-3′
245 A-5′-CGC GTG CCA GTG GAG ATC C-3′
245 B-5′-CGC GTG CCA GAA CTA GCA G-3′
295 A-5′-CGT TCC TGG CCG ATT AAT AG-3′
295 B-5′-CGC GTT CCT GCG TTT TTT CG-3′

Perform PCR as follows: 95°C for 15 min, 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, finally 72°C for 7 min.

Prepare 1.5% agarose gel in TAE buffer 0.5 X. Place 3-µL droplets of loading buffer on parafilm, mix 20 µL of PCR product by gentle aspiration with the pipette before loading. Load the wells of the gel and include positive and negative controls. Include DNA marker 100 pb in the first well of the gel. Run the gel for 20 min at 120 V (medium gel tray: 15 × 10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15 × 25 cm). Soak the gel in ethidium bromide solution for 20 min. Visualize the amplified DNA fragments by UV trans-illumination. Observe specific amplicons of 300, 550 and 615 bp as described by Pooler, Ritchie & Hartung (1996). The band of 300 bp is usually present when the plants are infected with *X. fragariae* but the other bands (550 and 615 bp) appear only occasionally.

Interpretation of the PCR results

The PCR test is negative if the *X. fragariae* amplicons are not of expected size, or no bands appear for the sample in question, but the amplicon is detected for all positive control samples.

The PCR test is positive if the *X. fragariae*-specific amplicon of expected size is detected, provided that it is not amplified from any of the negative control samples. Reliable confirmation of a positive result can be obtained by repeating the test. Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control sample containing *X. fragariae* in water, but negative results are obtained from positive controls with *X. fragariae* in plant extract. It is then advisable to repeat the amplification with dilutions 1 : 10, 1 : 100 and 1 : 1000 of the extract or to repeat the DNA extraction.

Appendix 3 Isolation

Method 1

Prepare 1 : 10, 1 : 100 and 1 : 1000 dilutions of each crushed sample in PBS (Appendix 7). Plate them as well as the 1 : 10, 1 : 100, 1 : 1000 dilutions and 1 : 10.000 on Wilbrink N and YPGA plates (Appendix 7). Pipette 50 µL of the diluted and undiluted samples onto separate plates of each medium. Use a set of four plates of each medium for each sample. Start with the 1 : 1000 dilution and proceed to the undiluted sample. Using sterile loops, carefully spread the sample aliquots by triple streaking over the media, or alternatively use a flame-sterilized glass spreader. Although not validated in the ring test, the use of SPA medium for fastidious bacteria (Hayward, 1960) can also be useful. Plate suspensions with 10^4, 10^5 and 10^6 cfu mL^{-1} of a *X. fragariae* reference strain as a quality control for the media and to compare the cultural characteristics of the colonies. Incubate the plates at 25°C for 7 days but mark the colonies appearing after 2–3 days, as these will not be *X. fragariae*. Final readings should be performed after incubation for 5–7 days at 25°C. *X. fragariae* colonies on Wilbrink N medium are white at the beginning and then pale yellow, circular, slightly convex, smooth and mucoid and appear after 4–6 days. On YPGA, the colonies are similar in morphology but they have a more intense yellow colour.

Obtain pure cultures from individual suspect colonies of each sample (from each of the two media) by plating suspensions of the *Xanthomonas*-like colonies on Wilbrink-N medium. Avoid yellow colonies appearing after 2–3 days, which are not *X. fragariae*. Presumptive colonies of *X. fragariae* can be confirmed following the methods under *Confirmation*.

Method 2

Cut out leaf fragments bearing clear, preferably not rotten, leaf symptoms (water-soaked angular leaf spots), suspect for *Xanthomonas fragariae*, amounting if possible to 25 typical leaf spots. Place the dissected leaf fragments in a disposable plastic beaker containing 50 mL tap water and five drops (5 × 10 µL) Tween-20. Mix the leaf pieces thoroughly in the liquid and rub off air bubbles and dirt when present. Incubate for about 10 min. Mix and rub again. Discard the washing fluid (safely). Repeat three times with clean demineralized water. Blot leaf parts dry on tissue paper. Place the leaf fragments in a disposable beaker containing 35 mL alcohol (70%), ensuring that all are completely in contact with the alcohol. Stir and remove leaf fragments after 5 s. Immediately blot dry and place on another dry tissue paper to allow alcohol to evaporate completely. Cut leaf fragments into very small pieces (1–4 mm^2) and place them in a tube with 5 mL 0.01 M PBS. Label with sample number. Mix and incubate for 30 min at RT to extract the bacterium from the plant tissue. Prepare a 1 : 100 dilution of the original extract in 0.01 M PB. Use extract for dilution plating on nutrient media, as for Method 1.

The extract can also be used for IF and PCR. In that case, from both the undiluted and a 1 : 100 dilution of extract, spread 150 µL onto a 10-well IF slide. The slides are dried and the bacterial cells are fixed to the slide by heating in a flame. Place 200 µL sample extract in an Eppendorf vial for PCR. Place 1 mL extract in another Eppendorf vial and add 5–10% glycerol (1 droplet) and store at –20 or –80°C.

Appendix 4 Detached leaf assay

Use young (7–14 days old) leaves of a cultivar susceptible to *X. fragariae* (e.g. 'Camarosa', 'Seascape', 'Selva', 'Korona') from glasshouse-grown plants in the absence of *X. fragariae*. The quality of the leaves and their age is essential for a successful assay. Aseptically remove 3–5 leaves (each with 3 leaflets) per sample and immediately place the petioles in glass tubes (13 mm × 100 mm) containing sterile water. Aseptically, cut off the basal portion of the petioles and replace the petioles in the tubes under running water. Use a 10^9 cfu mL^{-1} suspension of a reference strain of *X. fragariae* as a positive control and PBS (Appendix 7) as a negative control. Infiltrate with a needle-less syringe (3 mL plastic disposal B-D; 2 mm orifice) at 4 sites on the abaxial surface of each leaflet (two on each side of the main vein).

Rinse off excess inoculum with sterile water 1 h after inoculation and, after allowing the water soaking resulting from inoculum infiltration to dissipate, place leaves in their tubes in racks in plastic boxes (with transparent cover or glass) and with moist paper towels on the bottom to maintain humidity. Incubate the inoculated leaves at 18–20°C with a 12 h photoperiod provided by standard fluorescent lights for 21 days. These temperature and ilumination conditions are critical, to avoid false negative results. The inoculated leaves should not show visible injury, and the 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. Replace water in the tubes every 2–3 days or when necessary. Record symptoms every 2 days for 21 days.

Appendix 5 Alternative PCR method for confirmation

Add 5 µL of sample suspension to 20 µL of the following PCR mix (Roberts *et al*., 1996): H_2O 5.65 µL; Buffer 10 × 2.5 µL; $MgCl_2$ (50 mM) 0.75 µL; dNTPs (10 mM) 0.5 µL; Primer XF9 (100 pmol µL^{-1}) 0.25 µL; Primer XF11 (100 pmol µL^{-1}) 0.25 µL; *Taq* Polymerase 0.2 µL. The sequences of the primers are as follows:

XF9 5′-TGGGCCATGCCGGTGGAACTGTGTGG-3′
XF11 5′-TACCCAGCCGTCGCAGACGACCGG-3′

The amplification conditions are: 95°C 2 min; 40 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min; 72°C 10 min.

Prepare a 1.5% agarose gel in TAE buffer 0.5 X (Appendix 7). Place 3-µL droplets of loading buffer (Appendix 7) on parafilm, mix 20 µL of PCR product by gentle aspiration with the pipette before loading. Load the wells of gel and include positive and negative controls. Include DNA marker 100 bp in the first well of the gel. Run the gel for 20 min at 100 V (medium gel tray:

15 × 10 cm). Soak the gel in ethidium bromide solution for 20 min. Visualize the amplified DNA fragments by UV *trans*-illumination. The specific fragment of 537 bp is described by Roberts *et al*. (1996).

Interpretation of the PCR results

The PCR test is negative if the *X. fragariae*-specific amplicon of expected size is not detected for the sample in question but is detected for all positive control samples.

The PCR test is positive if the *X. fragariae*-specific amplicon of expected size is detected, providing that it is not amplified from any of the negative control samples. Inhibition of PCR can occur if concentrated suspensions are used. In that case, dilute 1 : 10, 1 : 100 or 1 : 1000 the DNA extracted and perform a new amplification or a new DNA extraction of the sample.

Appendix 6 Alternative inoculation methods

Prepare 10^9 cfu mL^{-1} suspension in PBS 10 mM (see Appendix 7) of the suspected colonies after growing for 48–72 h in Wilbrink-N medium (Appendix 7). Inoculate strawberry plants of susceptible cultivars (e.g. 'Camarosa', 'Seascape', 'Selva', 'Korona', 'Pájaro') by atomizing bacterial suspension on the underside of the leaves with a Sigma Preval sprayer or similar, after wounding the leaves (by puncturing with a needle). Use 5–10 leaves for each test colony.

Use a 10^9 cfu mL^{-1} suspension of a reference strain as positive control in at least one plant. Use PBS as negative control in the same way. Plants inoculated with the positive and negative controls should be inoculated and kept apart in such a manner as to preclude cross contamination. Keep the plant in closed plastic bags for 72 h to maintain high RH, and then incubate at 20°C for 4 weeks at 80–100% RH. Record symptoms every week. Initial symptoms are water-soaked lesions on the leaf blade. Later these develop into necrotic spots surrounded by a yellow halo or marginal necrosis. The bacterium can then be reisolated as in Appendix 3 and/or identified by PCR, IF or ELISA (Appendix 2).

The hypersensitive (HR) reaction in tobacco leaves can give an indication of the presence of the *hrp* genes but is also positive for many plant-pathogenic bacteria. Prepare bacterial suspensions of 10^9 cfu mL^{-1} (OD = 1.0) and inject them into the intercellular space of adult tobacco leaves with a 25-gauge needle and syringe. Use tobacco plants of cvs. 'Samsun' or 'Xanthi' with more than 5–6 leaves. Complete collapse of the infiltrated tissue after 24–48 h is recorded as positive. Most *X. fragariae* strains are HR-positive but some can be negative, specially after being conserved for some time.

Appendix 7 Buffers and media

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 1 L

Carbonate buffer pH 9.6: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, distilled water 1 L.

Washing buffer (PBS, pH 7.2–7.4 supplemented with 0.05% Tween 20): NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, Tween 20 500 µL, distilled water 1 L.

Colorimetric substrate buffer for alkaline phosphatase: diethanolamine 97 mL, dilute in 800 mL of distilled water, adjust pH 9.8 with concentrated HCl, adjust at 1000 mL with distilled water.

CTAB extraction buffer: 1 M Tris-HCI 50 mL, 5 M EDTA 50 mL, NaCl 40.9 g, PVP 40.5 g, CTAB 12.5 g, distilled water 500 mL.

TE Buffer, pH 8.0: 5 M EDTA pH 8, 0.1 mL; 1 M Tris-HCl pH 8, 0.5 mL; distilled water 500 mL.

50X TAE buffer: Tris 242 g, 0.5 M Na_2EDTA pH 8.0 100 mL, glacial acetic acid 57.1 mL, distilled water to 1 L.

Loading buffer: bromophenol blue 0.025 g, glycerol in H_2O 3 g, distilled water 10 mL.

Wilbrink-N medium (Koike, 1965; with nitrate): sucrose Sigma S-8501 10 g; proteose peptone (L85 Oxoid) 5 g; K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.25 g; $NaNO_3$ 0.25 g; purified agar (L28 Oxoid) 15 g; distilled water 1 L. Adjust pH to 7.0–7.2. Prepare this medium with cycloheximide if fungal presence is suspected. After autoclaving, add 0.25 g cycloheximide per L (prepare a stock solution of 2.5 g per 33.33 mL of absolute ethanol and keep at −20°).

YPGA medium: yeast extract (L21 Oxoid) 5 g; bactopeptone (L37 Oxoid) 5 g; glucose Sigma G-7520 10 g; purified agar (L28 Oxoid) 15 g; distilled water 1 L. Adjust pH to 7.0–7.2. If necessary, prepare this medium with cycloheximide as for Wilbrink-N medium.

Medium C (Dye, 1962) modified: $NH_4H_2PO_4$ 0.5 g; K_2HPO_4 0.5 g; $MgSO_4$ $7H_2O$ 0.2 g; NaCl 5 g; yeast extract 1 g; bromothymol blue (0.2%) 70 mL; distilled water1 L. Adjust pH to 6.8.

Appendix 8 Commercially available standardized reference material

The following bacterial isolates of *X. fragariae* are recommended for use as positive controls: NCPPB 1469, NCPPB 1822, CFBP 2510. *X. fragariae* strains are commercially available from: National Collection of Plant Pathogenic Bacteria (NCPPB), Central Science Laboratory, York (GB); Culture Collection of the Plant Protection Service (PD), Wageningen (NL); Collection Française de Bactéries Phytopathogènes (CFBP), INRA Station Phytobactériologie, Angers (FR). Authenticity of the strains can be guaranteed only if obtained from one of the indicated culture collections.

Polyclonal antibodies to *X. fragariae* currently recommended for use in detection and identification tests may be obtained from Adgen, Ayr (GB) (recommended for IF and DAS-ELISA); PRI, Wageningen (NL) (for IF) and Bioreba AG (CH) (for DAS-ELISA). The DNA extraction kit currently recommended for use before PCR amplification comes from Qiagen Dneasy Plant Kit (Qiagen, USA) and REDExtract-N-Amp Plant PCR-Kit (Sigma).