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ANNEX 14

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DIAGNOSTIC PROTOCOLS INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DP 14: *Xanthomonas fragariae*

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 14: *Xanthomonas fragariae*

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1. Pest Information

Xanthomonas fragariae Kennedy and King, 1962 is the causal agent of bacterial angular leaf spot disease of strawberry. The disease is prevalent mainly in North America and was first reported in the United States in 1962 (Kennedy and King, 1962; Hildebrand *et al*., 1967; Maas *et al*., 1995), but it has been subsequently reported in many strawberry growing areas around the world, including South America and Europe (CABI). *Fragaria* × *ananassa*, the predominant cultivated strawberry, is the primary host of *X. fragariae*. Commercial cultivars vary in susceptibility, and other *Fragaria* species, including *F. chiloensis*, *F. virginiana* and *F. vesca*, as well as *Potentilla fruticosa* and *P. glandulosa*, are also susceptible. Among *Fragaria* species only *F. moschata* is immune (Kennedy and King, 1962; Kennedy, 1965; Maas, 1998).

X. fragariae is readily transmitted via asymptomatic planting stock with latent infection. Inoculum sources for primary infection are infected but visually asymptomatic daughter plants that develop on runners from infected nursery plants and that are used for planting in fruit production fields. Although *X. fragariae* is not free-living in the soil, it can overwinter in the soil in association with previously infected plant material and persist there for long periods of time (Maas, 1998). Residues of infected leaves and crown infections on runners used for planting are also sources of inoculum for primary infection.

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). Nevertheless, there is a high degree of similarity among pathogenic strains of this phytopathogen, and there has been no correlation between genotypes or phenotypes and geographic origin of the strains. Currently known *X. fragariae* strains around the world are thus likely to represent a clonal population. Early detection of *X. fragariae* in infected but asymptomatic strawberry planting stock is critical for avoiding dissemination of the pathogen and disease development.

2. Taxonomic Information

Name: *Xanthomonas fragariae* Kennedy and King, 1962

Synonyms: None

Taxonomic position: Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae

Common names: Bacterial angular leaf spot

Note: *Xanthomonas fragariae* Kennedy and King, 1962 is a member of the gamma subdivision of the Proteobacteria (Stackebrandt *et al.*, 1988), Phenon 3 of Van den Mooter and Swings (1990), DNA-DNA homology Group 1 of Rademaker *et al.* (2000) and DNA Group 1 of Rademaker *et al.* (2005).

3. Detection

Diagnosis of bacterial angular leaf spot disease of strawberry caused by *X. fragariae* is based on inspection for diagnostic symptoms, direct or indirect isolation of the pathogen, serological tests (e.g. indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA)) and molecular methods. Several polymerase chain reaction (PCR) detection tests, each targeting different loci in the *X. fragariae* genome, have been developed (Roberts *et al.*, 1996; Zimmerman *et al.*, 2004; Weller *et al.*, 2007; Vandroemme *et al.*, 2008; Turechek *et al.*, 2008; Vermunt and van Beuningen, 2008). These tests can be used to confirm the presence of *X. fragariae* in symptomatic plant material, and several of them have also been used for the detection of latent *X. fragariae* infection (Mahuku and Goodwin, 1997; Zimmerman *et al.*, 2004; Moltman and Zimmerman, 2005). A detached leaf assay (Civerolo *et al.*, 1997a) is useful for presumptive diagnosis of *X. fragariae* in cases where direct isolation is very slow or inhibited. The methods described in this diagnostic protocol, with the exception of the nested PCR,

have been validated in a test performance study funded by the European Union (SMT-4-CT98-2252) (López *et al.*, 2005).

Direct isolation of *X. fragariae* is difficult, even in the presence of characteristic symptoms and bacterial exudates, because the bacterium grows very slowly on artificial nutrient media and is readily overgrown by saprophytic bacteria (Hazel and Civerolo 1980; López, *et al.*, 1985; Schaad *et al.*, 2001; Saddler and Bradbury, 2005). Specific procedures for direct isolation of *X. fragariae* are given in López *et al.* (2005). Selective enrichment of the pathogen *in planta* by inoculating detached strawberry leaves with aqueous extracts of diseased or suspected infected tissue can facilitate isolation of *X. fragariae in vitro* (Civerolo *et al*., 1997a).

Procedures for the detection of *X. fragariae* in symptomatic and asymptomatic plants are presented below.

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

3.1 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. The lesions enlarge and coalesce, eventually appearing on the upper leaf surface as angular water-soaked spots that become reddish brown (Figure 1). 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 (Figure 2). The exudates become dry scale-like masses that are opaque and whitish or silvery at first, then turn brown (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 (Kennedy and King, 1962; EPPO, 1997; Rat, 1993; Maas, 1998).

In contrast to angular leaf spot disease of strawberry, bacterial leaf blight of strawberry caused by *X. 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; 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). Also, 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 (*Mycosphaerella fragariae*) and leaf scorch (*Diplocarpon earliana*) (Janse *et al.*, 2001).

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. 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 (Figure 3). Fruit tissue near severely infected calyx tissue may also become water-soaked.

X. fragariae can move systemically into the roots, crowns and runners without exhibiting obvious symptoms (Stefani *et al.*, 1989; Milholland *et al.*, 1996; Mahuku and Goodwin, 1997). This infection

may result in the appearance of water-soaked areas at the base of newly emerged leaves followed shortly by sudden plant collapse and death. This type of infection is not usually seen.

3.2 Sampling

For plants with symptoms, leaves with initial water-soaked spots are preferred as samples for the diagnosis of bacterial angular leaf spot as they facilitate successful isolation of *X. fragariae*. Alternatively, leaves with dry spots and with or without exudates can be used. Crown tissue should also be examined.

X. fragariae is a very slow growing bacterium and plating and serological tests are not suitable for detecting small numbers of bacteria in symptomless plants. For symptomless plants, it is recommended that several entire plants be selected and small amounts of tissue be excised from their leaves, petioles and crowns (EPPO, 2006). These tissues can be used directly for PCR-based analyses, as described in section 3.9.

Samples should not be left in a wet condition after collection. Preferably, samples should be partially dried, wrapped in paper, placed in polythene bags and kept cool. Samples should be transported in a well-insulated container, stored at 4 °C upon arrival at their destination and processed as soon as possible.

3.3 Sample preparation

For symptomatic plants, the surfaces of leaf and stem plant tissue can be disinfested 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. Rinse the sample in tap water to remove excess soil and then disinfest by immersing for 1 min in 70% ethanol followed by rinsing three times in sterile distilled water. Add approximately 0.1 g of leaf or crown and petiole tissue per sample to 9 ml phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4, distilled water to 1 litre; pH 7.2). Homogenize the plant tissue and incubate it at room temperature for 15 min.

For asymptomatic plants, collect a 30 g sample at random, place it in 150 ml PBS and shake it for 30 min. Either use the washing liquid directly for detection, or centrifuge it at 10 000 g for 10 min then resuspend the pellet in sterile distilled water to obtain a final volume of 5 ml. Leave it to settle for 15 min then collect the upper clarified part and prepare dilutions (1:10 and 1:100) in sterile distilled water (EPPO, 2006). These sample tissue macerates are then used in ELISA, immunofluorescence and PCR.

3.4 Rapid screening tests

Rapid screening tests facilitate the detection of *X. fragariae*. As the bacterium is very difficult to isolate, three tests (ELISA, immunofluorescence and PCR) should be positive to confirm *X. fragariae* detection. The detached leaf assay is a supplemental test for confirming the presence of viable *X. fragariae*. The correlation among ELISA, PCR and detached leaf assay is usually high (Civerolo *et al.*, 1997b).

3.5 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. Two media are recommended for isolation. Isolation is more successful on Wilbrink's medium with nitrate (Wilbrink-N) (10 g sucrose, 5 g proteose peptone (L85; Oxoid[1]), 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.25 g $NaNO_3$, 15 g purified agar, distilled water to 1 litre; pH 7.0–7.2) (Koike, 1965). Isolation on YPGA medium (5 g yeast extract, 5 g Bacto[1] peptone, 10 g glucose, 15 g purified

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agar, distilled water to 1 litre; adjust pH to 7.0–7.2; add 5 ml filter-sterilized cycloheximide (stock solution: 5 g cycloheximide per 100 ml absolute ethanol) after autoclaving) is less successful but still recommended. A third medium, SPA (20 g sucrose, 5 g Bacto[1] peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 15 g purified agar, distilled water to 1 litre; pH 7.2–7.4), may be useful for fastidious bacteria (Hayward, 1960). The use of purified agar (Oxoid[1] or Difco[1]) is recommended for all media as impurities in other commercial agars can inhibit the growth of *X. fragariae*.

3.5.1 Isolation method 1

For plants with symptoms, select leaves with initial lesions and disinfest the surface by wiping it with 70% ethanol. Isolations should be made from initial water-soaked lesions or from the margins of older lesions by excising a small piece of tissue (0.5–1.0 cm^2) with a sharp sterile scalpel.

Homogenize the tissue in a few millilitres of sterile distilled water or PBS and incubate it at room temperature (20–25 °C) for 10–15 min. Plate out aliquots (50–100 µl) of lesion tissue macerates as well as dilutions (1:10, 1:100, 1:1 000 and 1:10 000) onto the surface of Wilbrink-N, YPGA and/or SPA media. Similar aliquots of *X. fragariae* cell suspensions (10^4, 10^5 and 10^6 colony-forming units (cfu)/ml should also be plated out in order to verify the quality of the media and to compare the cultural characteristics of any bacterial colonies that develop. Incubate the plates at 25–27 °C for seven days, but mark the colonies appearing after two to three days as these will not be *X. fragariae*. Perform final readings after seven to ten days of incubation at 25–27 °C.

*X. fragari*ae colonies on Wilbrink-N medium are initially off-white, becoming pale yellow, circular, slightly convex, smooth and mucoid after four to six days. On YPGA and SPA media, the colonies are similar in morphology to those on Wilbrink-N, but they have a more intense yellow colour.

3.5.2 Isolation method 2

Excise pieces of leaf tissue with distinct water-soaked angular lesions and wash them in 50 ml tap water and a few drops of Tween 20. Incubate the leaf pieces at room temperature for 10 min, then rinse them in distilled water and blot dry. The surfaces of the leaf pieces can be disinfested in 70% ethanol for 5 s and blot-dried. Cut the leaf pieces into smaller pieces (1–4 mm^2) and place them in 5 ml of 0.1 M PBS. Mix and incubate at room temperature for 30 min to release any *X. fragariae* into the supernatant. Prepare a 1:100 dilution of the supernatant in 0.1 M PBS and add 20 µl aliquots of the undiluted sample and 1:100 dilution to separate wells of a multiwell microscope slide. Fix the bacterial cells to the slide by flaming for later immunofluorescence analysis (section 3.8). Place 200 µl undiluted supernatant in a microtube for later PCR analysis (section 3.9) and another 1 ml undiluted supernatant in a second microtube, adding glycerol to obtain a final concentration of at least 20 %, and store it at –20 °C or –80 °C for reference purposes. The remaining supernatant can be used for isolation by dilution plating as described above and for inoculation of detached strawberry leaves (section 3.6).

In addition to isolation methods 1 and 2 described above, isolation of *X. fragariae* from tissue may be performed from aliquots of fresh exudates from lesions directly onto Wilbrink-N, YPGA, SPA or other commonly used media.

3.5.3 Interpretation of isolation results

The isolation is negative if no bacterial colonies with morphology characteristic of *X. fragariae* colonies are observed after seven 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.

The isolation is 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 the ELISA, immunofluorescence and PCR tests are positive, the sample should be considered as presumptively positive for *X. fragariae*, pending final identification (section 4). The best isolation results are expected when using freshly

prepared sample extracts from young lesions. Isolation onto media can also be achieved by *in planta* enrichment, as described in section 3.6.

3.6 Detached leaf assay and biological enrichment

3.6.1 Detached leaf assay

Tissue sample preparations (section 3.3) can be used for inoculating detached strawberry leaves as soon as they are prepared in extraction buffer or distilled water (Civerolo *et al*., 1997a). Use young (7–14 day old) leaves of a cultivar susceptible to *X. fragariae* (e.g. Camarosa, Pajaro, Seascape, Selva, Korona) from greenhouse-grown, *X. fragariae*-free plants. The quality of the leaves and their age are essential considerations for a successful test.

Aseptically remove three leaves (each one with three leaflets) from the greenhouse-grown plants, cut off the basal portion of the petioles and immediately place the petioles in glass tubes containing sterile water.

Prepare a cell suspension of a reference *X. fragariae* strain (table 3) containing 10^5–10^6 cfu/ml in PBS or distilled water as a positive control. PBS or distilled water is used as a negative control. Infiltrate four sites on the abaxial surface of each leaflet (two on each side of the main vein) using a needleless syringe (3 cc plastic disposal BD[1], 2 mm orifice).

Rinse off the excess inoculum with sterile water 1 h after inoculation. Place the leaves with their petioles in the tubes in a humid chamber (relative humidity 95–100%) and incubate at 18–20 °C with a 12 h photoperiod for up to 21 days. The specified temperature and illumination during incubation is essential for avoiding false negative results. 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. Record symptoms every two days for 14–21 days.

3.6.2 Interpretation of detached leaf assay results

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*., 1997a).

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. 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*., 1997a).

3.6.3 Enrichment in planta isolation

Select one leaf per sample from those inoculated in the detached leaf assay 48 h after inoculation for isolation onto nutrient media. Excise 10–12 small discs, 0.5 cm in diameter, from each inoculated site per inoculated detached leaf and crush it in 4.5 ml PBS. Prepare dilutions as for direct isolation (section 3.5) in PBS and streak 50 µl of each dilution onto the surface of Wilbrink-N medium in triplicate. Incubate the plates at 25–27 °C and check for *X. fragariae*-like colonies after five to seven days.

3.6.4 Enrichment in vitro-PCR from detached leaf assay

Use the Wilbrink-N medium plates streaked with extracts prepared for isolation following enrichment *in planta* as described in section 3.6.3 after incubation at 25–27 °C for four days. Wash bacterial colonies off the surface of the medium in 3–5 ml PBS and use them for PCR analysis (section 3.9). This is a modification of the bio-enrichment PCR described by Schaad *et al.* (1995).

3.7 ELISA

The specificity of ELISA with two commercially available polyclonal anti-*X. fragariae* sera has been validated (López *et al*., 2005). Rowhani *et al.* (1994) showed that ELISA using polyclonal antibodies could specifically detect 34 strains of *X. fragariae* and the antibodies did not cross-react with other closely related pathovars or other bacteria isolated from strawberry plants. A test sensitivity of 10^5 cfu/ml has been reported for ELISA detection of *X. fragariae* (Rowhani *et al.*, 1994; Civerolo *et al.*, 1997b).

Use cell suspensions prepared from pure cultures of *X. fragariae* and a non-*X. fragariae* strain as positive and negative controls in each microtiter plate. It is recommended that the appropriate working dilution of each polyclonal antiserum be determined.

3.7.1 Indirect ELISA

Mix 210 µl of each test sample, the positive *X. fragariae* cell suspension (approximately 10^9 cfu/ml), the negative non-*X. fragariae* cell suspension (approximately 10^9 cfu/ml) and the negative control (suspension of healthy strawberry material, see below) with 210 µl coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, distilled water to 1 litre) and add 200 µl of the sample and buffer mixture to each of two wells of a microtiter plate (PolySorp (Nunc[1]) or equivalent). For the negative plant material control, crush approximately 0.1 g healthy strawberry leaf, petiole or crown tissue in 0.9 ml PBS and add 0.9 ml coating buffer.

Incubate the plate at 4 °C overnight. Wash the plate three times with PBS containing 0.05% Tween 20 (PBS-T) (8 g NaCl, 0.2 g KCl, 0.2 g $Na_2HPO_4 \cdot 12H_2O$, 2.9 g KH_2PO_4, 500 µl Tween 20, distilled water to 1 litre). After washing add 200 µl blocking buffer (PBS containing 1% bovine serum albumin (BSA) or non-fat milk powder) to each of the test wells and incubate at 37 °C for 1 h. Wash the plate three times with PBS-T.

Prepare the appropriate working dilution, according to the manufacturer's instructions, of the anti-*X. fragariae* serum in PBS and add 200 µl to each test well. Incubate at 37 °C for 2 h and then wash the plate three times with PBS-T. Add 200 µl of the antibody–enzyme conjugate at the appropriate dilution in PBS containing 0.2% BSA to each well. Incubate at 37 °C for 1 h and then wash the plate four times with PBS-T. Add 200 µl freshly prepared substrate (1 mg p-nitrophenylphosphate/ml substrate buffer, pH 9.8) to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min, and read the absorbance at 405 nm.

3.7.2 DAS-ELISA

For double antibody sandwich (DAS)-ELISA, add 200 µl of an appropriate dilution of anti-*X. fragariae* serum in the coating buffer to each well of two microtiter plates (PolySorp (Nunc[1]) or equivalent). Incubate at 37 °C for 4 h and wash the wells three times with PBS-T. Add 200 µl of each tissue macerate sample, and a positive and a negative control, as described for indirect ELISA (section 3.7.1), to each of two wells of each plate and incubate at 4 °C overnight. After washing the plates three times with PBS-T, add 200 µl of an appropriate dilution of the enzyme–antibody conjugate in PBS containing 0.2% BSA to each well. Incubate at 37 °C for 3 h. After washing the plates four times with PBS-T add 200 µl of freshly prepared substrate (1 mg ρ-nitrophenylphosphate/ml substrate buffer, pH 9.8) to each test well. Incubate in the dark at room temperature for 15, 30 and 60 min, and read the absorbance at 405 nm.

3.7.3 Interpretation of ELISA results

The ELISA is negative if the average absorbance reading of duplicate wells containing tissue macerate is <2× the average absorbance reading of the negative control wells containing healthy strawberry tissue macerate.

The ELISA is positive if (1) the average absorbance reading of duplicate sample wells is >2× the average absorbance reading of the negative control wells containing healthy strawberry tissue macerate, and (2) the average absorbance reading of the positive control wells is >2× that of the average absorbance reading of the negative control wells.

Negative ELISA results for positive control wells indicate that the test was not performed correctly and/or the reagents have degraded or expired.

Positive ELISA results for negative control wells indicate that cross-contamination or non-specific antibody binding has occurred. The test should be repeated with fresh tissue or another test based on a different principle should be performed.

3.8 Immunofluorescence

Immunofluorescence procedures for identifying phytopathogenic bacteria are given in De Boer (1990) and EPPO (2009). Three commercially available polyclonal anti-*X. fragariae* sera (Table 1) have 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).

Test samples consist of dilutions of tissue macerates (1:10, 1:100 and 1:1 000) and cell suspensions (10^6 cfu/ml) of a positive *X. fragariae* and a negative non-*X. fragariae* bacterial strain in PBS or distilled water. Negative controls should consist of healthy plant tissue extracts.

Add aliquots (20 µl) of test samples and positive and negative control suspensions to separate wells of a multiwell microscope slide. Air-dry the preparations and fix them by flaming or by soaking the slides in acetone for 10 min followed by air-drying. Slides can be stored at –20 °C until required. Dilute the primary *X. fragariae* antibody in PBS containing 10% skim milk powder. Select the lowest antibody concentration that gives good staining when there are up to 100 positive cells per microscope field. It is advisable that two dilutions of the antibody are used to detect cross-reactions with other bacteria. Apply 20 µl of the primary antibody to each well and incubate the slides in a moist chamber at room temperature or at 37 °C for 30–60 min. Rinse the slides in PBS and wash them by submerging them in the same buffer for 10 min. Dilute the FITC-conjugated secondary antibody in PBS (optimum dilutions usually vary between 1:20 and 1:200). Cover the wells of the slides with the secondary antibody and incubate in a moist chamber at room temperature or at 37 °C for 30–60 min. Repeat the washing step then air-dry the slides. Mount coverslips on the slides with mounting fluid (90 ml glycerol, 10 ml PBS) containing 1 mg ρ-phenylenediamine/ml and view the slides under oil immersion at 500–1 000× magnification. Count the cells that fluoresce and have a similar size to the cells of the reference *X. fragariae* strain (López *et al*., 2005).

3.8.1 Interpretation of immunofluorescence results

The immunofluorescence test is negative if green-fluorescing cells with characteristic morphology of *X. fragariae* are observed in positive control wells but not in test sample or negative control wells.

The immunofluorescence test is positive if green-fluorescing cells with characteristic morphology of *X. fragariae* are observed in positive control and test sample wells but not in negative control wells.

As a population of 10^3 cells/ml is considered the limit of reliable detection by immunofluorescence, samples with $>10^3$ cells/ml are considered positive (De Boer, 1990). The immunofluorescence test may be considered to be inconclusive for samples with $<10^3$ cells/ml. In this case, further testing or re-sampling should be performed. Samples with large numbers of incompletely or weakly fluorescing cells

compared with the positive control need further testing with different dilutions of antibody or another source of antibody.

Table 1. Polyclonal antibodies to *Xanthomonas fragariae* currently recommended for use in serological tests

Source	Recommended uses†
Neogen Europe[1]	Detection using immunofluorescence or double antibody sandwich-enzyme-linked immunosorbent assay
Plant Research International, Wageningen UR	Detection using immunofluorescence
Bioreba AG[1]	Detection using double antibody sandwich-enzyme-linked immunosorbent assay

† Validated in a test performance study in a European Union-funded project (SMT-4-CT98-2252) (López *et al.*, 2005).

3.9 PCR

The PCR methods described in this diagnostic protocol, with the exception of the nested PCR developed by Zimmerman *et al.* (2004), have been validated in a a test performance study funded by the European Union (SMT-4-CT98-2252) (López *et al.*, 2005). Nested PCR protocols have been reported to increase sensitivity up to 100 times compared with conventional PCR protocols (Roberts *et al.*, 1996; Zimmerman *et al.*, 2004).

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[1]) has also been reported to be appropriate for DNA extraction before amplification for testing large numbers of samples of asymptomatic leaves (Stöger and 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).

Two sensitive real-time PCR tests have been described for the detection of *X. fragariae* (Weller *et al.*, 2007; Vandroemme *et al.*, 2008) in strawberry tissue. The real-time PCR test developed by Weller *et al.* (2007) will also differentiate between *X. fragariae* and *X. arboricola* pv. *fragariae*. The real-time PCR described by Weller *et al.* (2007) is based on primers designed within regions of the *gyrB* gene unique to *X. fragariae* and the *pep* gene unique to *X. arboricola* pv. *fragariae*. The real-time PCR developed by Vandroemme *et al.* (2008), yielding a 41 base pair (bp) amplicon, is based on primers designed from the 550 bp amplicon from the PCR described by Pooler *et al.* (1996). These methods are potentially useful for detecting low levels of *X. fragariae* in asymptomatic or latent infections.

3.9.1 DNA extraction

The DNeasy Plant Mini Kit (Qiagen[1]), as modified for mycoplasmalike organism (MLO) DNA extraction (Lopez *et al.*, 2005), provided the best results during the European Union ring test (SMT-4-CT98-2252).

For DNA extraction use 250 µl test sample tissue macerate(s); similarly prepared healthy strawberry plant material and sterile PBS or ultrapure water as negative controls; and a cell suspension of a pure culture of *X. fragariae* as a positive control. Add 250 µl cetyl trimethylammonium bromide (CTAB) extraction buffer (50 ml of 1 M Tris-HCI, 50 ml of 5 M ethylenediaminetetraacetic acid (EDTA), 40.9 g NaCl, 5 g polyvinylpyrrolidone (PVP)-40, 12.5 g CTAB, distilled water to 500 ml) and 4 µl RNase A (100 mg/ml), mix by inverting gently five times, and incubate at 65 °C for 10 min with occasional mixing by inversion. Then follow the manufacturer's instructions until the DNA elution step.

To elute the DNA, add 100 µl of 10 mM Tris-HCI, pH 9 (preheated to 65 °C) to the column and centrifuge at ≥6 000 *g* for 1 min. Add an additional 100 µl Tris-HCI and repeat the centrifugation step. Adjust the DNA solution to a total volume of 300 µl with Tris-EDTA (TE) buffer and add 200 µl of 5 M ammonium acetate and 1 ml absolute ethanol. Mix well and incubate at –20 °C for 1 h to overnight. After incubation, centrifuge at 17 000 *g* for 10 min. Discard the supernatant and wash the DNA pellet in 1 ml absolute ethanol and centrifuge at 16 000 *g* for 5 min. Discard the supernatant and wash the

DNA pellet in 500 µl of 80% ethanol and centrifuge at 16 000 *g* for 5 min. Discard the supernatant. After the pellet has dried, resuspend it in 50 µl sterile distilled water.

3.9.2 Multiplex PCR

3.9.2.1 Protocol of Hartung and Pooler (1997)

Specificity for this protocol was confirmed in a study with 30 isolates of *X. fragariae*, 36 isolates of *X. campestris* (representing 19 pathovars) and 62 isolates of epiphytic bacteria commonly isolated from strawberry. Only *X. fragariae* was detected (in all isolates). This multiplex PCR enabled detection to 10^3 cfucfu/ml in plant tissue (Pooler *et al.*, 1996; Hartung and Pooler 1997).

The three sets of primers described by Pooler *et al*. (1996) are:

241A: 5′-GCCCGACGCGAGTTGAATC-3′

241B: 5′-GCCCGACGCGCTACAGAC TC-3′

245A: 5′-CGCGTGCCAGTGGAGATCC-3′

245B: 5′-CGCGTGCCAGAACTAGCAG-3′

295A: 5′-CGT TCC TGGCCGATT AATAG-3′

295B: 5′-CGCGTTCCT GCG TTTTTT CG-3′

PCR is carried out in 25 µl reaction mixtures containing 2.5 µl buffer (PerkinElmer[1]) (containing 15 mM $MgCl_2$), 5.0 µl deoxyribonucleotide triphosphate (dNTP) (1 mM), 2.0 µl (0.4 µM) of each of the six primers, 0.5 µl Taq DNA polymerase and 5.0 µl sample DNA. The cycling parameters are an initial activation step of 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. PCR products are analysed by 1.5% agarose gel electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer (EPPO, 2006).

Specific PCR amplicons for *X. fragariae* are 300, 550 and 615 bp, as previously described (Pooler *et al.*, 1996; Hartung and Pooler, 1997). 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.

The primers 245A and 245B can be used in a conventional PCR, using the procedure described above, and will produce an amplicon of 300 bp.

3.9.3 Nested PCR

The nested PCR described by Moltmann and Zimmerman (2005) using primers developed by Pooler *et al*. (1996) and Zimmerman *et al.* (2004) is recommended for diagnosing *X. fragariae* in symptomatic strawberry plants as well as for testing asymptomatic strawberry plants (frigo and green plants). The nested PCR described by Roberts *et al.* (1996) offers an alternative method for confirmation.

3.9.3.1 Protocol of Moltmann and Zimmerman (2005)

Specificity for this protocol was confirmed in a study with 14 isolates of *X. fragariae*, 30 isolates of *X. campestris* (representing 14 pathovars) and 17 isolates of unidentified bacteria associated with strawberry leaves. In addition, the specificity of the external primer set was verified by Hartung and Pooler (1997) (section 3.9.2.1). No cross-reactions were observed with the isolates tested. This PCR has been successfully applied to testing of samples collected during a survey of strawberry plants and imported plants (Moltmann and Zimmerman, 2005). It enabled detection to 200 fg DNA per reaction and was 100 times more sensitive than conventional PCR (Zimmerman *et al.*, 2004).

Incubate leaf, petiole and crown tissue (30–70 g) in 10–20 ml of 0.01 M sodium phosphate buffer (pH 7.2) per gram of tissue at room temperature overnight. Extract DNA and analyse by single and nested PCR as described by Zimmerman *et al.* (2004).

The primers are:

245A: 5′-CGCGTGCCAGTGGAGATCC-3′

245B: 5′-CGCGTGCCAGAACTAGCAG-3′
245.5: 5′-GGTCCAGTGGAGATCCTGTG-3′
245.267: 5′-GTTTTCGTTACGCTGAGTACTG-3′

PCR is carried out in 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The cycling parameters are an initial denaturation step of 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. For nested PCR, after amplification of DNA with the first round primers (245A and 245B), 1 µl of the first PCR product is used as template in a second PCR with the internal primers 245.5 and 245.267. The same cycling parameters are used except the annealing temperature is 62 °C for the internal primers 245.5 and 245.267. PCR products are analysed by 1.2% agarose gel electrophoresis in 0.5× TAE buffer.

Specific PCR amplicons for *X. fragariae* are 300 bp in the first round PCR using the 245A and 245B primers, and 286 bp in the nested PCR using the internal primers 245.5 and 245.267. With high template concentrations, a second fragment of approximately 650 bp is sometimes amplified.

3.9.3.2 Protocol of Roberts *et al.* (1996)

Specificity for this protocol was confirmed in a study with 30 isolates of *X. fragariae*, 17 isolates of *X. campestris* (representing 16 pathovars) and 9 isolates of non-pathogenic xanthomonads isolated from strawberry. No cross-reactions were observed with the isolates tested. This nested PCR enabled detection to approximately 18 *X. fragariae* cells in plant tissue (Roberts *et al.*, 1996).

The semi-nested primers, as described by Roberts *et al.* (1996), are:

XF9: 5′-TGGGCCATGCCGGTGGAACTGTGTGG-3′
XF11: 5′-TACCCAGCCGTCGCAGACGACCGG-3′
XF12: 5′-TCCCAGCAACCCAGATCCG-3′

PCR is carried out in 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The cycling parameters are an initial denaturation step of 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 45 s; and a final extension step of 72 °C for 5 min. For the semi-nested PCR, after amplification of DNA with the first round primers (XF9 and XF11), 3 µl of the first PCR product is used as template in a second PCR with the primers XF9 and XF12. The same cycling parameters as described for the first round are performed except that the annealing temperature is 58 °C. PCR products are analysed by 1.5% agarose gel electrophoresis in 0.5× TAE buffer.

Specific PCR amplicons for *X. fragariae* are 537 bp in the first round PCR using the XF9 and XF11 primers, and 458 bp in the semi-nested PCR using the XF9 and XF12 primers.

3.9.4 Real-time PCR

3.9.4.1 Protocol of Weller *et al.* (2007)

Specificity for this protocol was confirmed in a study with 10 isolates of *X. fragariae* and 24 *Xanthomonas* isolates (representing 12 species and 17 pathovars). Only *X. fragariae* was detected (in all isolates). This real-time PCR enabled detection to 10^3 cfu per leaf disc (Weller *et al.*, 2007). This protocol has been further validated by a laboratory in the Netherlands; the validation data are available on the EPPO database on diagnostic expertise ().

The primers, based on sequences of the *gyrB* gene, and TaqMan probe, covalently labelled at the 5′ end with the reporter dye JOE and at the 3′ end with the quencher dye TAMRA, are:

Xf gyrB-F: 5'-CCG CAG CGA CGC TGA TC -3'
Xf gyrB-R: 5'-ACG CCC ATT GGC AAC ACT TGA-3'
Xf gyrB-P: 5'-TCC GCA GGC ACA TGG GCG AAG AAT TC-3'

PCR is carried out by adding 4 µl template DNA to a reaction mixture containing 1× TaqMan Buffer A (Applied Biosystems[1]), 5.5 mM $MgCl_2$, 200 µM dNTPs (Promega[1]), 300 nM each primer, 100 nM probe and 0.63 U AmpliTaq Gold DNA polymerase (Applied Biosystems[1]). The cycling parameters are an initial activation step of 2 min at 50 °C then 15 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C.

3.9.5 Interpretation of PCR results

3.9.5.1 Conventional PCR

The PCR test is negative if none of the *X. fragariae*-specific amplicons of expected size is detected for samples and negative controls but the amplicons are detected for all positive controls.

The PCR test is positive if at least one of the *X. fragariae*-specific amplicons of expected size is detected, providing that it is not amplified from any of the negative controls.

Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control containing *X. fragariae* in water but negative results are obtained from positive controls with *X. fragariae* in plant extract. Repeating the PCR with 1:10, 1:100 and 1:1 000 dilutions of the extract or repeating the DNA extraction is recommended.

3.9.5.2 Real-time PCR

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

- the positive control produces an amplification curve with the pathogen-specific primers
- no amplification curve is seen (i.e. cycle threshold (Ct) value is 40) with the negative extraction control and the negative amplification control.

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

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

3.9.6 Controls for molecular tests

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

Positive controls should be prepared in a separate area than that in which the samples will be tested.

Positive nucleic acid control. This control is used to monitor the efficiency of PCR amplification. Pre-prepared (stored) nucleic acid, whole genome DNA or a synthetic control (e.g. cloned PCR product) may be used. For this protocol, a suspension of pure culture *X. fragariae* cells (10^4–10^6 cfu/ml) is recommended as a positive nucleic acid control.

Internal control. For conventional and real-time PCR, a plant housekeeping gene (HKG) such as *COX* (Weller *et al.*, 2000), 16S ribosomal (r)DNA (Weisberg *et al.*, 1991) or *GADPH* (Mafra *et al.*, 2012) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture.

PCR-grade water that was used to prepare the reaction mixture or sterile PBS is added at the amplification stage.

Positive extraction control. This control is used to ensure that nucleic acid from the target is of sufficient quality for PCR amplification. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target near the concentration considered the detection limit of the protocol.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For this protocol, *X. fragariae* tissue macerates spiked with 10^6 cfucfu/ml of a reference *X. fragariae* strain are recommended as positive extraction controls.

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

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified, or a tissue macerate sample extract previously tested negative for *X. fragariae*. Multiple controls are recommended to be included when large numbers of positive samples are expected.

4. Identification

The minimum requirements for identification are isolation of the bacterium and a positive result from each of the three detection techniques: (1) indirect ELISA, DAS-ELISA (section 3.7) or immunofluorescence (section 3.8) using polyclonal antibodies; (2) PCR (section 3.9); and (3) pathogenicity testing by inoculation of strawberry hosts to fulfil the requirements of Koch's postulates (sections 4.4 and 3.6). Additional tests (sections 4) may be done to further characterize the strain present. In all tests, positive and negative controls must be included.

In the case of latent infections or asymptomatic plants, after an initial screening test the pathogen should be isolated and its identity confirmed, including by pathogenicity testing with the pure culture and the fulfilment of Koch's postulates.

4.1 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. Nitrates not reduced, catalase test positive, and asparagine not used as a sole source of carbon and nitrogen (Bradbury, 1977; Bradbury, 1984; Schaad *et al*., 2001). Weak production of acids from carbohydrates. Colonies are mucoid, convex and shiny on YPGA and Wilbrink-N media (Dye, 1962; van den Mooter and Swings 1990; Swings *et al.*, 1993; Schaad *et al.*, 2001). *Xanthomonas* species are easily differentiated from the other genera of aerobic, Gram-negative rod-shaped and other yellow-pigmented bacteria by the characteristics shown in Table 2 as described by Schaad *et al*. (2001).

Table 2. Phenotypic characteristics for differentiating *Xanthomonas* from *Pseudomonas* and the yellow-pigmented bacteria *Flavobacterium* and *Pantoea* (Schaad et al. 2001)

Characteristic	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagellation	1, polar	>1, polar	None	Peritrichous
Xanthomonadin	Yes	No	No	No
Fluorescence	No	Variable	No	No
Levan from sucrose	Yes	Variable	No	No
H_2S from cysteine	Yes	No	No	No
Oxidase	Negative or weak	Variable	Positive	Negative
Fermentation	No	No	No	Yes
Growth on 0.1% triphenyltetrazolium chloride (TTC)	No	Yes	Yes	Yes

The reference *X. fragariae* strains available from different collections that are presented in Table 3 are recommended for use as positive controls in biochemical and physiological tests.

Table 3. Reference strains for *Xanthomonas fragariae*

Strain	Source
ATCC 33239	American Type Culture Collection, Manassas, VA, United States
CFBP 2510	Collection Française de Bactéries Phytopathogènes, INRA Station Phytobactériologie, Angers, France
ICMP 5715	International Collection of Microorganisms from Plants, Auckland, New Zealand
BCCM/LMG 708	Belgian Co-ordinated Collections of Micro-organisms / Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Ghent, Belgium
NCPPB 1469	National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; Culture Collection of the Plant Protection Service (PD), Wageningen, Netherlands
NCPPB 1822	National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; Culture Collection of the Plant Protection Service (PD), Wageningen, Netherlands

The most relevant or useful characteristics for distinguishing *X. fragariae* from other *Xanthomonas* species (Schaad *et al.*, 2001; Janse *et al.*, 2001; EPPO, 2006) are shown in Table 4.

Table 4. Diagnostic tests to distinguish *Xanthomonas fragariae* from the *"Xanthomonas campestris* group" and *Xanthomonas arboricola* pv. *fragariae*

Test	*X. fragariae*	*X. campestris*	*X. arboricola* pv. *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	–	+	+

ND, not determined; V, variable reaction.
Source: Janse et al. (2001) and EPPO (2006).

Biochemical characterization of isolated strains can be done using commercial systems and identification of *X. fragariae* can be obtained by specific profiling using API 20 NE and API 50 CH strips (BioMérieux[1]) (EPPO, 2006).

For the API 20 NE strips[1], follow the manufacturer's instructions for preparing suspensions from 48 h old test and reference strain cultures on Wilbrink-N medium and inoculate the strips. Incubate at 25–26 °C and read after 48 and 96 h. The readings after 48 h for enzymatic activity and 96 h for substrate utilization are compared with those characteristic of *X. fragariae* (Table 5).

Table 5. Reactions of *Xanthomonas fragariae* in API 20 NE strips

Test	Reaction (after 48 or 96 h)†
Glucose fermentation	–
Arginine	–
Urease	–
Esculin	+
Gelatin	+ (weakly)
Para-nitrophenyl-β-D-galactopyranosidase (PNPG)	+
Assimilation of:	
Glucose	+
Arabinose	–
Mannose	+
Mannitol	–
N-acetyl glucosamine	+
Maltose	–
Gluconate	–
Caprate	–
Adipate	–
Malate	+
Citrate	–
Phenyl acetate	–

† Common reactions from 90% of *X. fragariae* strains tested (López *et al.*, 2005).

For the API 50 CH strips[1], prepare bacterial cell suspensions of OD_{600nm} = 1.0 in PBS. Add 1 ml suspension to 20 ml modified medium C (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 5 g NaCl, 1 g yeast extract, 70 ml bromothymol blue (0.2%), distilled water to 1 litre; pH 6.8) (Dye, 1962). Follow the manufacturer's instructions for inoculation of the strips. Incubate at 25 °C under aerobic conditions and read after two, three and six days. Utilization of the different carbohydrates is indicated by a yellow colour in the wells after the incubation period (Table 6).

Table 6. Reactions of *Xanthomonas fragariae* in API 50 CH strips

Test†	Reaction (after six days)
D-Arabinose	Variable
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
N-acetyl glucosamine	+
Esculin	+
Sucrose	+
Trehalose	+
D-Lyxosa	+
L-Fucose	+

† The remaining sugars in the API 50 CH test strips are not utilized by *X. fragariae* (López *et al.*, 2005).

4.1.1 Fatty acid methyl ester profiling

Fatty acid methyl esters (FAMEs) associated with the cytoplasmic and outer membranes of Gram-negative bacteria are useful for bacterial identification (Sasser, 1990). Specific fatty acids that may be used to predict the genus of Gram-negative and Gram-positive bacteria are given by Dickstein *et al.* (2001). Identification is based on comparing the types and relative amounts of the fatty acids in a profile of an unknown strain with profiles from a wide variety of strains in a library database (e.g. TSBA40 library). It is critical that bacteria be grown under uniform conditions of time, temperature and nutrient media in order to obtain reproducible results. *X. fragariae* strains contain three major fatty acids: 16:1ω-7 *cis*, 15:0 *anteiso* and 15:0 *iso*. While some test strains give a good match to the library profile, other strains have differing fatty acid profiles that do not correspond well. Studies have shown that strains of *X. fragariae* show considerable diversity and fall into at least four distinct fatty acid groups (Roberts *et al.*, 1998). The method described by Roberts *et al*. (1998) is recommended for FAME profiling of *X. fragariae*. Test strains are grown on trypticase soy agar at 24 °C for 48 h, a fatty acid extraction procedure is applied and the extract is analysed using the Sherlock Microbial Identification System (MIDI) (Newark, DE, United States).

4.1.1.1 Interpretation of FAME profiling results

The FAME profiling test is positive if the profile of the test strain is identical to that of the *X. fragariae* positive control or reference strain(s). Fatty acid analysis is available from MIDI and the National Collection of Plant Pathogenic Bacteria (NCPPB) (Fera, York, United Kingdom). The composition and amounts of key FAMEs in *X. fragariae* and *X. arboricola* pv. *fragariae* are given in Janse *et al*. (2001).

4.2 Serological tests

4.2.1 Immunofluorescence

Immunofluorescence can be used for the identification of suspect *X. fragariae* strains. Prepare a suspension of approximately 10^6 cells/ml in PBS and apply the immunofluorescence procedure described in section 3.8. If performing only two identification tests for rapid diagnosis, do not use another serological test in addition to this one.

4.2.2 ELISA

Indirect ELISA or DAS-ELISA (described in sections 3.7.1 and 3.7.2, respectively) can be used for the identification of suspect *X. fragariae* strains isolated from plant material affected by suspected bacterial angular leaf spot. If performing only two identification tests for rapid diagnosis, do not use another serological test in addition to this one.

4.3 Molecular tests

4.3.1 PCR

Suspect *X. fragariae* cultures can be identified using the PCR protocols described in section 3.9.

4.3.2 REP-PCR

Specific repetitive extragenic palindromic (REP)-PCR protocols for the identification of *X. fragariae* strains are described by Opgenorth *et al*. (1996) and Pooler *et al*. (1996). Either one of these protocols can be used for the reliable identification of test strains as *X. fragariae*.

The PCR protocol described below is based on the reaction mixture and amplification conditions described by Opgenorth *et al*. (1996).

Bacterial strains to be analysed are taken from streaks or individual colonies on Pierce's disease modified medium (5.0 g sucrose, 2.5 g Phytone (BD BBL[1]), 10 g Phytagel (BD BBL[1]); distilled water to 1 litre, adjust pH to 7.5 with 2 N HCl before autoclaving;) (Opgenorth *et al.*, 1996). Different growth media can be used; however, these should be standardized before use.

The two sets of primers are:

REP1R-I: 5′-IIIICGICGICATCIGGC-3′

REP2-I: 5′-ICGICTTATCIGGCCTAC-3′

ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′

ERIC2: 5′-AAGTAAGTGACTGGGGTGAGC G-3′

The reaction buffer contains 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 µM EDTA, 30 mM 2-mercaptoethanol, 0.17 mg BSA/ml, 10% (v/v) dimethyl sulfoxide, 1.2 mM of each dNTP, 62 pmol each primer and 2 U Taq DNA polymerase. Bacteria from a representative colony of the test strain are transferred, using a sterile 10 µl pipette tip (or other suitable implement), to a PCR tube containing 25 µl of the reaction mixture. The cycling parameters are 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 and 65 °C for 8 min. The amplification cycles are followed by a final extension step of 68 °C for 16 min. The amplification products (5–10 µl) are electrophoresed in a 1.5% (w/v) agarose gel. Amplified DNA fragments are visualized after staining with ethidium bromide by ultraviolet transillumination.

4.3.2.1 Interpretation of REP-PCR results

Test bacterial strains are identified as *X. fragariae* if they have the same genomic fingerprints as those of the REP and ERIC genotypes of the reference strains (Pooler *et al*., 1996) amplified in the same PCR and run in the same gel. A small number of polymorphic bands may be obtained from different strains of *X. fragariae* owing to low levels of genomic variability.

4.3.3 Multilocus sequence analysis

A multilocus sequence analysis (MLSA) approach has been widely used for the specific identification of xanthomonads (Parkinson *et al.*, 2007; Almeida *et al.*, 2010; Hamza *et al.*, 2012) and could be used for the identification of *X. fragariae*, especially now that a draft genome sequence is available (Vandroemme *et al.*, 2013). However, it should be noted that this methodology has not yet been validated for the identification of *X. fragariae*. Housekeeping genes (e.g. *gyrB*, *rpoD*) are amplified using primers and conditions as described by Almeida *et al.* (2010) and Hamza *et al.* (2012). MLSA

consists of sequencing multiple loci (typically four to eight housekeeping genes) and comparing these sequences with reference sequences of *Xanthomonas* species deposited in nucleotide databases; for example, the Plant Associated and Environmental Microbes Database (PAMDB) (http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl) (Almeida *et al.*, 2010), MLVAbank for microbe genotyping http://mlva.u-psud.fr/mlvav4/genotyping/ and Q-bank Bacteria database (http://www.q-bank.eu/Bacteria/).

4.4 Pathogencity tests

The identity of bacterial strains suspected of being *X. fragariae* should be confirmed by a pathogenicity test, when required. Strains selected from isolation or enrichment plates should be inoculated into attached leaves of susceptible strawberry plants (or into detached leaves as described in section 3.6). Several procedures are available: Hazel and Civerolo (1980), Civerolo *et al*. (1997a) and Hildebrand *et al.* (2005).

4.4.1 General inoculation procedure

A recommended inoculation procedure is to use *X. fragariae*-free strawberry plants of a susceptible cultivar (e.g. Camarosa, Seascape, Selva, Korona, Pajaro). If possible, plants should be held 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.

Prepare bacterial cell suspensions (10^6 cfu/ml) in sterile distilled water or 10 mM PBS. Apply inoculum for each strain to 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[1]) 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, incubate plants in a chamber maintained at 20–25 °C with high humidity (>90%) and a 12–14 h photoperiod. Suspensions of cells of a reference *X. fragariae* strain (prepared in the same manner as the test strain) and sterile distilled water or 10 mM PBS serve as positive and negative controls, respectively, and should be inoculated in different trays. Evaluate lesion development weekly for three weeks (21 days) post-inoculation. Re-isolate the pathogen from such lesions, as described in section 3.5, and identify by ELISA, immunofluorescence or PCR.

4.4.1.1 Interpretation of pathogenicity test results

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. The same symptoms should appear on leaves inoculated with a reference *X. fragariae* strain (positive control).

Similar symptoms should not appear on the leaves inoculated with sterile distilled water or 10 mM PBS (negative control).

4.4.2 Hypersensitive reaction

A hypersensitive reaction (HR) in tobacco leaves can be an indication of the presence of *hrp* genes and a positive reaction is induced by many plant pathogenic bacteria. A positive control, for example a strain of *Pseudomonas syringae* pv. *syringae*, can be used. Use the tobacco cultivar Samsun or Xanthi plants with more than five leaves. Prepare bacterial suspensions of 10^9 cfu/ml (OD_{600nm} = 1.0) in sterile distilled water or 10 mM PBS and infiltrate the suspension into the intercellular spaces through the abaxial surfaces of adult leaves with a syringe equipped with a 25 gauge needle.

4.4.2.1 Interpretation of HR results

Complete collapse and necrosis of the infiltrated tissue within 24–48 h post-inoculation is recorded as a positive test result. Most *X. fragariae* strains are HR positive. However, some may be HR negative,

especially after being stored for some time. Similar reactions should not appear on leaves inoculated with sterile distilled water or 10 mM PBS as a negative control.

5. Records

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

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

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

United States Department of Agriculture (USDA) Agricultural Research Service (ARS) (formerly), (Edwin L. Civerolo; e-mail: emciv@comcast.net).

Plant and Environmental Bacteriology, Fera, Sand Hutton, York YO41 1LZ, United Kingdom (John Elphinstone; e-mail: john.elphinstone@fera.gsi.gov.uk).

Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López; e-mail: mlopez@ivia.es; tel.: +34 963 424000; fax: +34 963 424001).

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

7. Acknowledgements

The first draft of this protocol was written by E.L. Civerolo (USDA ARS (formerly), United States (see preceding section)) and revised by J. Elphinstone (Fera, United Kingdom (see preceding section)) and M.M. López (IVIA, Spain (see preceding section)).

8. References

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

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9. Figures

Figure 1. Symptoms of *Xanthomonas fragariae* on (A, left) an upper leaf surface and (B, right) a lower leaf surface. *Photo courtesy A.M.C. Schilder, Michigan State University, East Lansing, MI, United States.*

Figure 2. Bacterial ooze from *Xanthomonas fragariae* on a lower leaf surface.
Photo courtesy W.W. Turechek, United States Department of Agriculture Agricultural Research Service, Washington, DC, United States.

Figure 3. Symptoms of *Xanthomonas fragariae* on the calyx of fruit.
Photo courtesy A.M.C. Schilder, Michigan State University, East Lansing, MI, United States.

Publication history

This is not an official part of the standard.

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IPPC

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

Organization

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

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