DRAFT ANNEX to ISPM 27 – *Xanthomonas fragariae* (2004-012)

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Contents

To be added later.

Adoption

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The annex is a prescriptive part of ISPM 27 (*Diagnostic protocols for regulated pests*).

1. Pest Information

*Xanthomonas fragariae* Kennedy and King, 1962a 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, 1962a; Hildebrand *et al*., 1967; Maas, 1995), but it has been subsequently reported in many strawberry growing areas around the world, including South America, Africa and Europe (CABI, 2015). *Fragaria × ananassa*, the predominant cultivated strawberry, is the primary host of *X. fragariae*. However, 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, 1962a; Kennedy, 1965; Maas, 1998).

*X. fragariae* is readily transmitted via asymptomatic planting stock with latent infection. Inoculum sources for primary infection are infected but clinically 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. 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). However, 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, 1962a

**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 analyses (e.g. indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA)) and molecular methods, including polymerase chain reaction (PCR)-based techniques (López *et al*., 1985; Roberts *et al*., 1996; Civerolo *et al*., 1997a, 1997b; Hartung and Pooler, 1997; Zimmerman *et al.,* 2004; López *et al*., 2005). A detached leaf bioassay (Civerolo *et al.*, 1997a) is useful for direct presumptive diagnosis of *X. fragariae*. Analyses of field-collected or clinical samples are generally based on leaves with young water-soaked spots if available, or leaves with older lesions with or without dried bacterial exudates. If systemic infection is suspected, analysis of crown tissue is necessary (López *et al.*, 2005). The methods indicated, with the exception of the nested PCR, have been validated in a ring test project 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 (Hazel and Civerolo 1980, López, *et al.*, 1985; Schaad *et al.*, 2001; Saddler and Bradbury, 2005) and is readily overgrown by saprophytic bacteria. 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 (Civerolo *et al*., 1997a) with aqueous extracts of diseased or suspected infected tissue can facilitate isolation of *X. fragariae* *in vitro*.

Procedures for the detection of *X. fragariae* in plants with symptoms 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. (This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named.). 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 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. Viscous bacterial exudates that are white, milky, cream or yellow in colour develop from lesions under wet conditions or when the relative humidity is high. The exudates become dry scale-like masses that are opaque or brown. 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, 1962a; EPPO, 1992; Rat, 1993; Maas, 1998).

In contrast to angular leaf spot disease of strawberry, bacterial leaf blight of strawberry caused by *Xanthomonas arboricola* pv. *fragariae* is characterized by small reddish-brown lesions on the lower leaf surface that are neither water-soaked nor translucent; reddish spots on the upper leaf surface; 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*).

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.

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 (Gubler *et al.*, 1999). 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 type of infection can result in the appearance of water-soaked areas at the base of newly emerged leaves followed shortly by sudden plant collapse and death.

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 and is neccessary for successful isolation of *X. fragariae*. Alternatively, leaves with dry spots and with or without exudates can be used. When systemic infection is suspected it is necessary to analyse crown tissue from affected 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 can be used directly for PCR-based analyses as described in section 3.9. A reference *X. fragariae* strain should be included in all tests as a positive control.

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 and on arrival at their destination stored at 4 °C.

3.3 Sample preparation

The surfaces of 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. The sample is rinsed in tap water to remove excess soil and then disinfested by immersing for 1 min in 70% ethanol followed by rinsing three times in sterile distilled water. Approximatley 0.1 g of leaf or crown and petiole tissue per sample is added to 9 ml phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g Na2HPO4.12H2O, 0.2 g KH2PO4, distilled water to 1 litre; pH 7.2). The plant material is crushed and incubated at room temperature for 15 min. These sample tissue macerates are then used in ELISA, Immunofluorescence and PCR tests as decribed in the following sections.

3.4 Rapid screening tests

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

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 secondary 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), 0.5 g K2HPO4, 0.25 g MgSO4.7H20, 0.25 g NaNO3, 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 peptone, 10 g glucose, 15 g purified 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. SPA medium (20 g sucrose, 5 g peptone, 0.5 g K2HPO4, 0.25 g MgSO4.7H2O, 15 g purified agar, distilled water to 1 litre; pH 7.2–7.4) may be useful for fastidious bacteria (Hayward, 1960); however, the reliability of this medium for isolating *X. fragariae* has not been validated (López *et al.*, 2005). The use of purified agar (Oxoid or Difco)[[1]](#footnote-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 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 cm2) with a sharp sterile scalpel.

Tissue is crushed in a few mls of sterile distilled water or PBS and incubated at room temperature for 10–15 min. Aliquots (50 – 100 µl) of lesion tissue macerates as well as dilutions (1:10, 1:100, 1:1 000 and 1:1 0000) are plated out onto the surface of Wilbrink-N and YPGA media. Similar aliquots of *X. fragariae* cell suspensions (104, 105 and 106 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 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 at 25–27 °C for 7 days.

*X. fragari*ae colonies on Wilbrink-N medium are initially off-white, becoming pale yellow, circular, slightly convex, smooth and mucoid after 4–6 days. On YPGA medium, the colonies are similar in morphology to those on Wilbrink-N, 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* *fragariae*-like colonies on Wilbrink-N medium.

3.5.2 Isolation method 2

Excise pieces of leaf tissue with distinct water-soaked angular lesions and wash in 50 ml tap water and a few drops of Tween-20 and incubate at room temperature for 10 min. Rinse the leaf pieces in distilled water and blot dry. The surfaces of the leaf pieces can then be disinfected in 70% ethanol for 5 s and blot dried. Place the leaf pieces 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 supernatant in 0.1 M PBS and add 20 µl aliquots of the undiluted sample and 1:100 dilution to separate wells of a multi-well 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 a drop of glycerol, and store at –20 oC or –80 oC 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).

An alternative to isolation of *X. fragariae* from tissue is to streak 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 similar to *X. fragariae* colonies are observed after 7 days in either of the two 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 serological tests and PCR analyses are positive, the sample should be considered as presumptively positive for *X. fragariae*, pending final identification (section 4)*.*

There is not always a good correlation between isolation, serological tests (i.e. immunofluorescence, ELISA) and/or PCR because isolation frequently fails. 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 days old) leaves of a cultivar susceptible to *X. fragariae* (e.g. Camarosa, Seascape, Selva, Korona) from greenhouse-grown, *X. fragariae-*free plants. The quality of the leaves and their age are essential considerations for a successful assay.

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

Prepare a cell suspension of a reference *X. fragariae* strain (section 4.1) containing approximately 105–106 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 B-D, 2 mm orifice).

Rinse off excess inoculum with sterile water 1 h after inoculation. Place leaves with their petioles in their tubes in a humid chamber 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 2 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, water-soaked when viewed with reflected light; translucent yellow when viewed with transmitted light) and/or chlorotic halos appear at some 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.

The detached leaf assay is positive when typical *X. fragariae* angular leaf spots (i.e. dark, 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.

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 media after *in planta* enrichment. Excise 10–12 small discs 0.5 cm in diameter from each inoculated site per inoculated detached leaf and crush 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 plates at 25–27 oC and record results for *X. fragariae*-like colonies after 5–7 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 oC for 4 days. Wash bacterial colonies off the surface of the medium in 3–5 ml PBS 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 two ELISA protocols with 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 105 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. Frequent cross-reactions among phytopathogenic or other bacteria can occur with polyclonal antibodies. It is recommended that the appropriate working dilution of each polyclonal antiserum is determined.

3.7.1 Indirect ELISA

Mix 210 µl of each test sample, the positive *X. fragariae* cell suspension (approximately 109 cfu/ml) and the negative non-*X. fragariae* cell suspension (approximately 109 cfu/ml) and the negative control (healthy strawberry material) with 210 µl coating buffer (Na2CO3, NaHCO3, 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)[[2]](#footnote-2) or equivalent). For the negative plant material control, crush about 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 + 0.05% Tween 20 (PBS-T) (8 g NaCl, 0.2 g KCl, 0.2 g Na2HPO4.12H2O, 2.9 g KH2PO4, 500 µl Tween 20, distilled water to 1 litre). After washing add 200 µl blocking buffer (PBS + 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 in 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 wash the plate four times in 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 a coating buffer to each well of two microtiter plates (PolySorp (Nunc)[[3]](#footnote-3) 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, a positive and a negative control, as described for indirect ELISA, 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 test is negative if the average absorbance readings of duplicate wells containing tissue macerate is <2× the average absorbance of the negative control wells containing healthy strawberry tissue macerate.

The ELISA is positive if (1) the average absorbance readings of duplicate sample wells is >2× the average absorbance readings of the negative control wells containing healthy strawberry tissue macerate, and (2) the average absorbance readings of the positive control wells is >2× that of the average 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). Two commercially available polyclonal anti-*X. fragariae* sera (Table 1) have been validated using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (López *et al.,* 2005).This method will permit detection of approximately 103–104 cfu/ml *X. fragariae* in strawberry tissue (Calzolari and Mazzucchi, 1989).

Test samples consist of dilutions of tissue macerate samples (1:10, 1:100 and 1:1 000) and cell suspensions (106 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.

Aliquots (20 μl) of test samples and positive and negative control suspensions are added to separate wells of a multi-well microscope slide. Preparations are air-dried and fixed by flaming or by soaking slides in acetone for 10 min followed by air-drying. Slides can be stored at –20 oC until required. Primary *X. fragariae* antibody is diluted in PBS + 10% skim milk powder. Select the lowest antibody concentration that gives good staining when there is up to 100 positive cells per microscope field. It is advised that two dilutions of the antiserum is 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. The slides are then rinsed in PBS and washed by submerging in the same buffer for 10 min. The FITC-conjugated secondary antibody is diluted in PBS (optimum dilutions usually vary between 1:20 and 1:200). The wells of all slides are then covered with the secondary antibody and incubated in a moist chamber at room temperature or at 37 oC for 30–60 min. The washing step is repeated and air-dried. The coverslips are mounted with mounting fluid (90 ml glycerol, 10 ml PBS) containing 1 mg ρ-phenylenediamine/ml) and slides viewed under oil immersion at 500–1 000× magnification. Count the cells that fluoresce and have a similar size to 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 wells with test samples or negative controls.

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 103 cells/ml is considered the limit of reliable detection by immunofluorescence, samples with >103 cells/ml are considered positive (De Boer, 1990). The immunofluorescence test may be considered to be inconclusive for samples with <103 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.** Antibodies to *Xanthomonas fragariae* currently recommended for use in detection and identification tests

|  |  |  |
| --- | --- | --- |
| **Antibody** | **Type** | **Source[[4]](#footnote-4)** |
| *X. fragariae* | Polyclonal1,2 | Neogen Europe Ltd |
| *X. fragariae* | Polyclonall | Plant Research International, Wageningen UR |
| *X fragariae* | Polyclonal2 | Bioreba AG |

1 Recommended for detection using immunofluorescence (validated in ring tests in a European Union-funded project (SMT-4-CT98-2252)) (López *et al.,* 2005).

2 Recommended for detection using double antibody sandwich-enzyme-linked immunosorbent assay (validated in ring tests 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 ring test funded by the European Union (SMT-4-CT98-2252) (López *et al.,* 2005). Nested PCR protocols were 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)[[5]](#footnote-5) 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 other primers (Roberts *et al.*, 1996) are available; however, these may not be as reliable (e.g. reduced sensitivity) and have not been validated for clinical applications (López *et al.*, 2005).

Two sensitive real-time PCR assays have been described for detection of *X. fragariae* (Weller *et al.*, 2007; Vandroemme *et al.*, 2008) as well as differentiation of *X. fragariae* and *X. arboricola* pv. *fragariae* (Weller *et al.*, 2007) in strawberry tissue. The real-time PCR assay described by Weller *et al*. (2007) is based on primers designed within regions of the *gyraseB* gene unique to *X. fragariae* and the *pep* gene unique to *X. arboricola* pv. *fragariae*. The real-time PCR assay developed by Vandroemme *et al*. (2008) is based on primers yielding a 41 base pair (bp) amplicon, designed from the 550 bp fragment amplicon described by Pooler *et al*. (1996). To date (March, 2015), neither of these methods has been verified or validated (e.g. in a ring test). However, 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)[[6]](#footnote-6), 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 extract from healthy strawberry plant material and sterile PBS or ultra-pure water as negative controls; and a suspension of a pure culture of *X. fragariae* cells as a positive control. Add 250 µl cetyl trimethylammonium bromide (CTAB) extraction buffer (50 ml 1 M Tris-HCI, 50 ml 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 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

**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 protocol enabled detection to 103 cfu.f.u/ml in plant tissue (Pooler *et al.,* 1996; Hartung and Pooler 1997).

PCR is carried out in 25 µl reaction mixtures containing 2.5 µl buffer (PerkinElmer)[[7]](#footnote-7) (containing 15 mM MgCl2), 5.0 µl deoxyribonucleotide triphosphate (dNTP) (1 mM), 2.0 µl (0.4 µM) of each of six primers, 0.5 µl Taq DNA polymerase and 5.0 µl sample DNA. 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 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 finally 72 °C for 7 min. PCR products are analysed by 1.5% agarose gel electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer.

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.

3.9.3 Nested PCR

The nested PCR protocol 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. This protocol is also useful for testing asymptomatic strawberry plants (frigo and green plants) (Moltmann and Zimmerman, 2005). The nested PCR protocol described by Roberts *et al.* (1996) also offers an alternative method for confirmation.

**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 Pooler *et al.* (1996) (see previous section 3.9.2). Only *X. fragariae* was detected (in all isolates). This method has been successfully applied to testing of samples collected during a survey of strawberry plants and imported plants (Moltmann and Zimmerman, 2005). This nested PCR protocol 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 0.1 M sodium phosphate butter (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 MgCl2), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The reaction conditions 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 of primers (245A and 245B), 1 µl of the reaction mixture is used as template in a second PCR with the internal primers 245.5 and 245.267. The same PCR cycling conditions 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 can sometimes be amplified.

**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. Only *X. fragariae* was detected (in all isolates). This nested PCR technique 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′-TGGGCCATGCCGGTGGAACTGTGTGTGG-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 MgCl2), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase. The reaction conditions are an initial denaturation step of 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s; and a final extension step of 72 °C for 5 min. For the nested PCR, after amplification of DNA with the first round of primers (XF9 and XF11), 3 µl of the reaction mixture is used as template in a second PCR with the primers XF9 and XF12.The same PCR condtions as described for the first round are performed. 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 primers XF9 and XF12.

3.9.4 Interpretation of PCR results

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 Controls for molecular testing

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 where 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. a cloned PCR product) may be used. For this protocol, a suspension of pure culture *X fragariae* cells (10**4** 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 PCR protocol as a control to eliminate the possibility of 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 at 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 104 and 106 cfu/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. If required, the positive control used in the laboratory should be sequenced so that the 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 cross-reaction with the host tissue. The control comprises of 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 when large numbers of positive samples are tested.

4. Identification

The minimum requirements for identification are isolation of the bacterium and a positive result from each of the three detection techniques: (1) DAS-ELISA, indirect ELISA or immunofluorescence using polyclonal antibodies (sections 3.7 and 3.8); (2) PCR (section 3.9); and (3) pathogenicity testing by inoculation of strawberry hosts to fulfil the requirements of Koch’s postulates (sections 4.3 and 3.5). Additional tests (sections 4.1 and 4.3.2) may be done to further characterize the strain present. In all tests, positive and negative controls must be included.

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

4.1 Biochemical and physiological tests

*X. fragariae* has the common characteristics of all xanthomonads. They are Gram-negative, aerobic rods, with a single polar flagellum. They do not reduce nitrates, they are catalase positive, and asparagine is not used by them as a sole source of carbon and nitrogen (Bradbury, 1977; Bradbury, 1984; Schaad *et al*., 2001). They are weak producers of acids from carbohydrates. Colonies are mucoid, convex and shiny on YPGA and Wilbrink-N media (Dye, 1962; van den Mooter *et al.,* 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 3 and described in Schaad *et al*. (2001).

The reference *X. fragariae* strains available from different collections that are presented in Table 2 are recommended for use as positive controls in biochemical and physiological tests.

**Table 2.** Reference *Xanthomonas fragariae* strains

|  |  |
| --- | --- |
| **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 |

**Table 3.** Phenotypic characteristics for differentiating *Xanthomonas* from *Pseudomonas* and other yellow-pigmented bacteria (*Flavobacterium* and *Pantoea)*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Characteristic** | ***Xanthomonas*** | ***Pseudomonas*** | ***Flavobacterium*** | ***Pantoea*** |
| Flagellation  Xanthomonadin  Fluorescence  Levan from sucrose  H2S from cysteine  Oxidase  Fermentation  Growth on 0.1% triphenyltetrazolium chloride (TTC) | 1, polar  Yes  No  Yes  Yes  Negative or weak  No  No | >1, polar  No  Variable  Variable  No  Variable  No  Yes | None  No  No  No  No  Positive  No  Yes | Peritrichous  No  No  No  No  Negative  Yes  Yes |

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

**Table 4.** Diagnostic tests to distinguish *Xanthomonas fragariae* from the *“X. campestris* group” and *X. arboricola* pv. *fragariae* (Janse *et al.,* 2001)

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

ND, not determined; V, variable reaction.

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)[[8]](#footnote-8) (EPPO, 2006).

For the API 20 NE strips[[9]](#footnote-9), 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 activities 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 tests

|  |  |
| --- | --- |
| **Test** | **Reaction (48 or 96 h)1** |
| Glucose fermentation  Arginine  Urease  Esculin  Gelatin  Para-NitroPhenyl-ßDGalactopyranosidase (PNPG)  Assimilation of:  Glucose  Arabinose  Mannose  Mannitol  N-acetyl-glucosamine  Maltose  Gluconate  Caprate  Adipate  Malate  Citrate  Phenyl-acetate | –  –  –  +  + (weakly)  +  +  –  +  –  +  –  –  –  –  +  –  – |

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

For the API 50 CH strips[[10]](#footnote-10), prepare bacterial cell suspensions of OD = 1.0 in PBS. Add 1 ml suspension to 20 ml modified medium C (0.5 g NH4H2PO4, 0.5 g K2HPO4, 0.2 g MgSO4,5 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 2, 3 and 6 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 tests

|  |  |
| --- | --- |
| **Test1** | **Reaction (six days)** |
| d-arabinose  Galactose  d-glucose  d-fructose  d-mannose  N-acetyl-glucosamine  Esculin  Sucrose  Trehalose  d-lyxosa  l-fucose | Variable  +  +  +  +  +  +  +  +  +  + |

1 The remaining sugars in the API 50 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 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).

***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 (Newark, DE, United States) 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 identification of suspect *X. fragariae* strains. Prepare a suspension of approximately 106 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) can be used for 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 protocols for identification of *X. fragariae* strains are given by Opgenorth *et al*. (1996) and Pooler *et al*. (1996). Either one of these repetitive extragenic palindromic (REP)-PCR protocols can be used for reliable identification of test strains as *X. fragariae*.

The protocol below is based on the PCR 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 (BBL Microbiology Systems, Baltimore, MD)[[11]](#footnote-11), 10 g Phytagel (BBL Microbiology Systems)[[12]](#footnote-12); adjust pH to 7.5 with 2 N HCl before autoclaving; distilled water to 1 litre) (Opgenorth *et al.,* 1996).

The two sets of PCR 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 (NH4)2SO4, 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 reaction tube containing 25 μl of the reaction mixture. 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 cycle of 68 °C for 16 min. The amplification products (5–10 μl) are electrophoresed in a 1.5% (w/v) agarose gel at room temperature for 4 h at 5 V/cm in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). 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 the same genomic fingerprints are obtained as those of the REP and ERIC genotypes of the reference strains (Pooler *et al*., 1996) amplified in the same test and run in the same gel.

4.3.3 Multilocus sequence analysis

A multilocus sequence analysis (MLSA) approach has been widely used for the specific identification of xanthomonads (Almeida *et al.,* 2010; Hamza *et al.*, 2012; Parkinson *et al.,* 2007) and could be used for identification of *X. fragariae* especially now that a draft genome sequence is now available (Vandroemee *et al.,* 2013). However, it should be noted this methodology has not yet been validated for identification of *X. fragariae*. Housekeeping genes are amplified using primers and PCR 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 Microbes Database (PAMDB) (<http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>) (Almeida *et al.*, 2010) and the MLVAbank for microbe genotyping (<https://bioinfo-prod.mpl.ird.fr/MLVA_bank/Genotyping/>).

5. Pathogencity tests

The identity of bacterial strains suspected of being *X. fragariae* should be confirmed by a pathogenicity test, when required. Strains selected from the 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)).

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

Prepare bacterial cell suspensions (108–109 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. DeVilbiss) so as not to induce water-soaking. Infection may be facilitated by wounding leaves (e.g. puncturing the abaxial surface with a needle) before applying inoculum, although it is not necessary to do this. After inoculation, 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.

5.1.1 Interpretation of pathogenicity test results

If the sample tissue extract inoculum 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 plants 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).

5.2 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 109 cfu/ml (OD600nm = 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.

5.2.1 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 mock-inoculated with sterile distilled water or 10 mM PBS as a negative control.

6. Records

Records and evidence should be retained as described in 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. photograph of gels, printout of ELISA results, PCR amplicons).

7. Contact Points for Further Information

Further information on this protocol can be obtained from:

USDA/ARS (formerly), San Joaquin Valley Agricultural Sciences Center, 9611 S. Riverbend Ave, Parlier, CA 93648, United States (Edwin L. Civerolo; e-mail: [emciv@comcast.net](mailto:emciv@comcast.net)).

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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](mailto:mlopez@ivia.es); tel.: +34 963424000; fax: +34 963424001).

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).

8. 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)).

9. References

The present standard also refers to other International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the IPP at <https://www.ippc.int/core-activities/standards-setting/ispms>

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1. 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. 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. [↑](#footnote-ref-1)
2. See footnote 1. [↑](#footnote-ref-2)
3. See footnote 1. [↑](#footnote-ref-3)
4. See footnote 1. [↑](#footnote-ref-4)
5. See footnote 1. [↑](#footnote-ref-5)
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