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DIAGNOSTICS

PM 7/064 (2) *Xanthomonas arboricola* pv. *pruni*

Specific scope: This Standard describes a diagnostic protocol for *Xanthomonas arboricola* pv. *pruni*.[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment: Approved in 2005–09. Revised in 2021–03.

1 | INTRODUCTION

Bacterial spot of stone fruits is a disease caused by *Xanthomonas arboricola* pv. *pruni* that was described for the first time in the USA (Michigan) in 1903 on *Prunus salicina* (Japanese plum) (EPPO/CABI, 1997). The main hosts of economic importance are *P. persica* (peach), *P. persica* var. *nucipersica* (nectarine), *P. domestica* (plum), *P. armeniaca* (apricot) and *P. dulcis* (almond) (Young, 1977; Stefani *et al*., 1989; Scortichini & Simeone, 1997; Palacio-Bielsa *et al*., 2010). Species of the Sino-Japanese group (*P. salicina*) are generally more susceptible than European plums (Bazzi & Mazzucchi, 1980, 1984; Topp *et al*., 1989; Bazzi *et al*., 1990a,b; Simeone, 1990). Other hosts are *P. avium* and *P. cerasus* (sweet and sour cherries), *P. mume* (Japanese apricot), *P. davidiana* (Chinese wild peach), *P. buergeriana*, *P. crassipes* and *P. donarium*. Ornamental species such as *P. laurocerasus* (cherry laurel) are also affected (Marchi *et al*., 2011; Tjou *et al*., 2012). In the absence of conditions conducive to

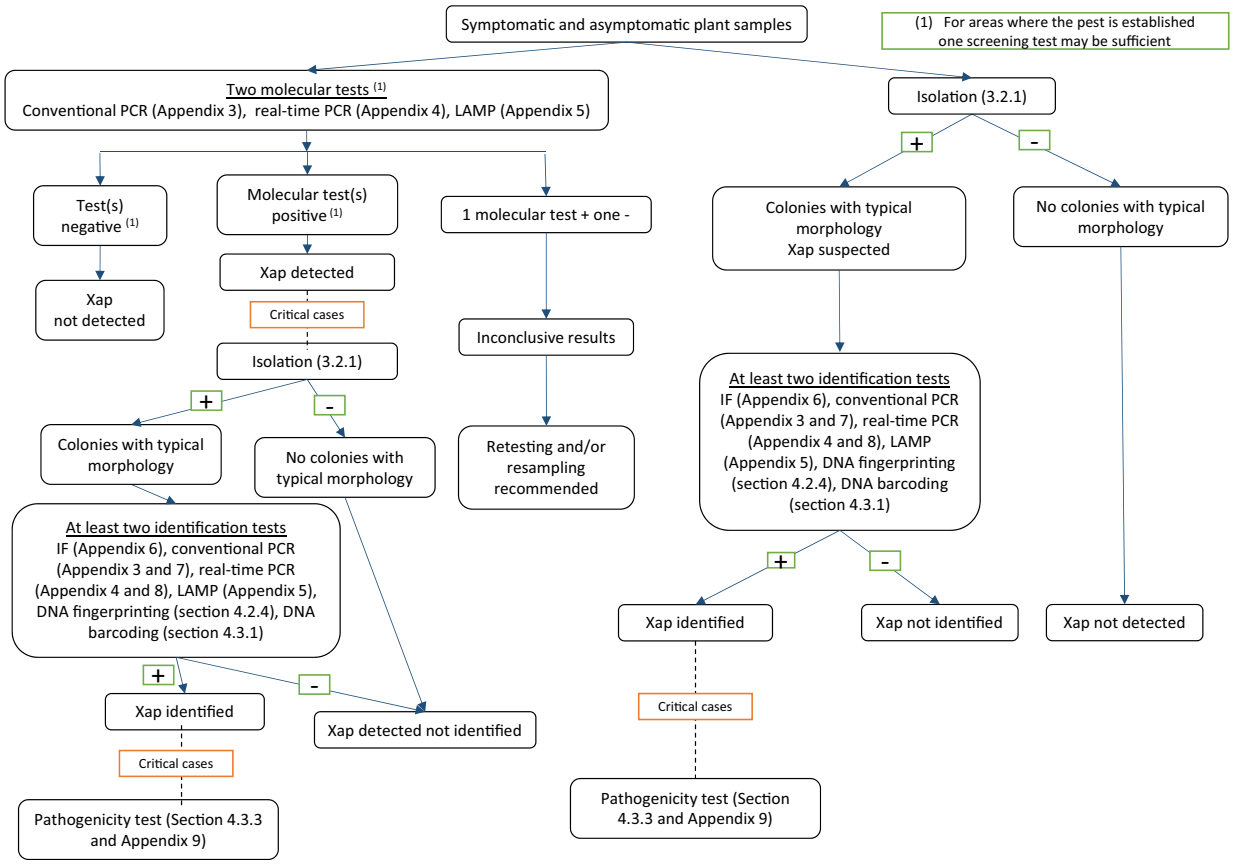

FIGURE 1 Flow-diagram for testing symptomatic and asymptomatic plant samples for the diagnosis of *X. arboricola* pv. *pruni*

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

infection, the bacteria have the capacity to survive for extended periods in protected sites on the trees. During cold periods and dormancy, the bacteria may persist in buds, axils and twig lesions (Zaccardelli *et al*., 1995; Battilani *et al*., 1999). *X. arboricola* pv. *pruni* may survive as an epiphyte on *Prunus* hosts in orchards or nurseries, associated with buds and leaf scars. From these, it can enter the host before full healing of the leaf scar, or through stomata. Further details on geographic distribution can be found in EPPO Global Database (EPPO, 2020). Further information on the biology is available in the EPPO data sheet on *X. arboricola* pv. *pruni* (EPPO/CABI, 1997).

This Standard describes different methodologies for the diagnosis of bacterial spot in symptomatic and asymptomatic plants of *Prunus* species. A flow diagram describing the diagnostic procedure for *X. arboricola* pv. *pruni* in symptomatic and asymptomatic *Prunus* species is presented in Figure 1.

2 | IDENTITY

Preferred name: *Xanthomonas arboricola* pv. *pruni* (Smith, 1903) Vauterin, Hoste, Kersters & Swing 1995.
Other scientific names: *Pseudomonas pruni* (Smith), *Xanthomonas campestris* pv. *pruni* (Smith, 1903) Dye, *Xanthomonas pruni* (Smith) Dowson.
Taxonomic position: Bacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae.
EPPO Code: XANTPR.
Phytosanitary categorization: EPPO A2 list no. 62, EU RNQP (Annex IV), EU PZ Quarantine pest (Annex III).

FIGURE 2 Plantlets of *P. persica* infected by *X. arboricola* pv. *pruni* showing chlorosis of the leaves and heavy defoliation. Courtesy of Plant Health Service, Valencian Government (ES)

3 | DETECTION

3.1 | Disease symptoms

Symptoms of bacterial spot of stone fruits can be observed on leaves, young and fully developed fruits, twigs, branches and trunks (EPPO, 2003, 2006). In orchards, although affected trees can develop symptoms on different parts, they may appear healthy if not inspected closely. However, in nursery plantlets, heavy infections can be detected easily, mainly on *P. persica* (peach and nectarine), because of heavy defoliation and chlorosis of the leaves (Figure 2). Some cultivars belonging to *P. salicina* appear

FIGURE 3 First stage of leaf infection by *X. arboricola* pv. *pruni* as small, water-soaked lesions on *P. persica*. Courtesy of Plant Health Service. Valencian Government (ES)

FIGURE 4 Typical bacterial spot lesions on peach leaves, cv. 'Zee Lady' infected by *X. arboricola* pv. *pruni*. Courtesy of Emilio Stefani, UNIMORE (IT)

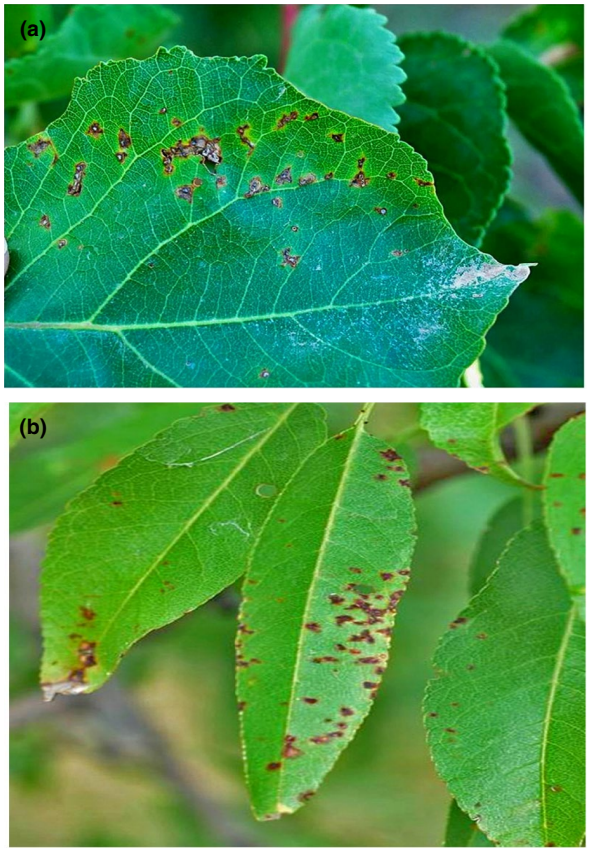

FIGURE 5 (a, b) Angular and dark lesions with the chlorotic surrounding tissue. (a) On apricot leaf infected by *X. arboricola* pv. *pruni*. (b) On almond leaf infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 6 Typical three-band colour (green-yellow-brown) symptoms on leaves of *P. persica* infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

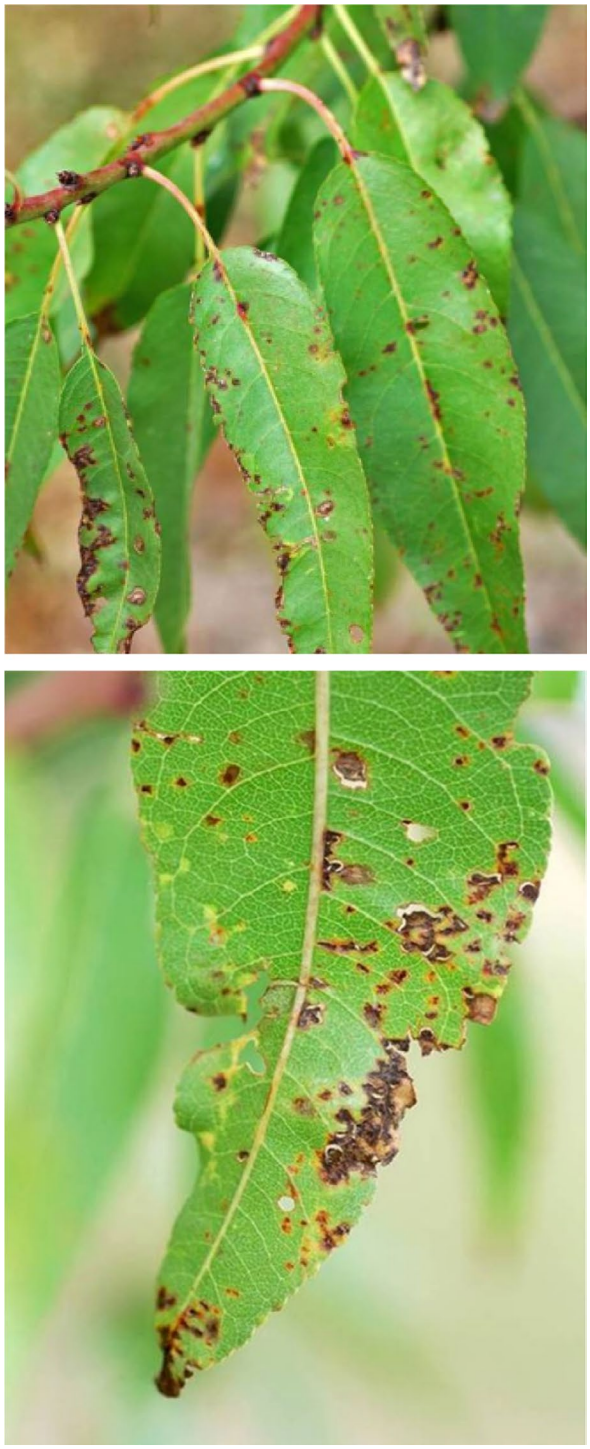

FIGURE 7 Tatter symptoms on almond leaves. The almond tree was suspected to be co-infected with fungal pathogens. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

to be very sensitive and they may develop severe cankers and die within a few years (Bazzi & Mazzucchi, 1980). The symptoms of bacterial spot become more obvious during warm seasons with temperatures of 19–28°C, frequent rain, wind and heavy dew, which are all favourable conditions for severe infection (Fahy & Persley, 1983; Bradbury, 1986; Du Plessis, 1988; Zehr & Shepard, 1996).

3.1.1 | Symptoms on leaves

On *P. persica* (peach and nectarine), *P. domestica*, *P. salicina*, *P. dulcis*, *P. armeniaca*, *P. avium* and *P. cerasus* (cherry), infection is first apparent on the lower surface of the leaves as small, water-soaked lesions (Figure 3) which develop into pale-green and later yellow circular or irregular areas (spots or lesions) with a light-tan centre. These spots soon become evident on the upper surface as they enlarge and turn angular and dark to deep-purple, brown or black. The immediate surrounding tissue may become yellow (Figures 4 and 5). On *P. persica* (peach and nectarine), symptoms on leaves can show a typical three-band colour (green-yellow-brown) (Figure 6), followed by heavy defoliation.

FIGURE 8 Necrotic lesions caused by *X. arboricola* pv. *pruni* as they appear on Japanese plum leaves, cv. 'Fortune'. Courtesy of Emilio Stefani, UNIMORE (IT)

Both young and old infected leaves exhibit leaf spot and tatter symptoms, but it is believed that most infection is initiated in young leaves. The lesions are circular, angular or irregularly shaped, and become reddish to dark in colour. As the lesions dry out they drop and shot-holes (Figure 7), often surrounded by a dark ring, are formed on the leaf. Spots are usually concentrated towards the leaf tip, sheltered parts of the leaf blades and along the midrib because the bacteria multiply in this region in droplets of rain or dew. Bacterial ooze may be associated with the spots.

On *P. domestica* and *P. salicina* (Japanese-plum) leaves, initial symptoms are angular water-soaked spots, rapidly turning reddish-brown, then dark-brown and necrotic, whereas chlorosis is minimal and less apparent than on peach leaves (Figure 8). The necrotic spots frequently perforate, so that the shot-hole effect can be pronounced. Affected plum leaves usually do not fall.

On the ornamental *P. laurocerasus* (cherry laurel), the leaf spots are often chlorotic, most having a necrotic brown centre with a distinct round margin, and the spots can readily abscise, resulting in a shot-hole appearance of the leaf (Figure 9).

Possible confusion

The bacterial spot leaf symptoms are easily confused with those caused by other pathogens, the fungal disease

FIGURE 9 Bacterial spot lesions on cherry laurel (upper and lower surface) caused by *X. arboricola* pv. *pruni*. Courtesy of Maria Bergsma-Vlami, NVWA (NL)

'shot-hole' caused by *Wilsonomyces carpophilus* and *Venturia carpophila*, and even with the first stages of the infections caused by *Polystigma fulvum* and *Tranzschelia pruni-spinosae*; however, the later stages of these fungal infections evolve very differently. Spots caused by *Apple chlorotic leaf spot virus* (ACLSV) and *Prunus necrotic ring spot virus* (PNRSV) can also be easily confused with *X. arboricola* pv. *pruni*. Confusion is also possible with the bacterial diseases caused by *Pseudomonas syringae* pv. *morsprunorum* or *Pseudomonas syringae* pv. *syringae*, but leaves may fall prematurely in infections caused by Pseudomonads. It should be noted that *P. syringae* pv. *morsprunorum* often produces more cankers and less foliar spots than *X. arboricola* pv. *pruni*. *X. prunicola* (López *et al*., 2018) can cause necrotic lesions on leaves of *Prunus persica*, similar to those caused by *X. arboricola* pv. *pruni*, as well as cankers and gummosis (3.1.3). Finally, some abiotic factors can lead to confusion such as copper phytotoxicity, but copper lesions are larger (2–6 mm in diameter) and often round in shape (Figure 10a) unlike bacterial angular spots caused by *X. arboricola* pv. *pruni* (Figure 10b). Frequently, copper lesions also show a reddish halo, which is not observed around bacterial spots.

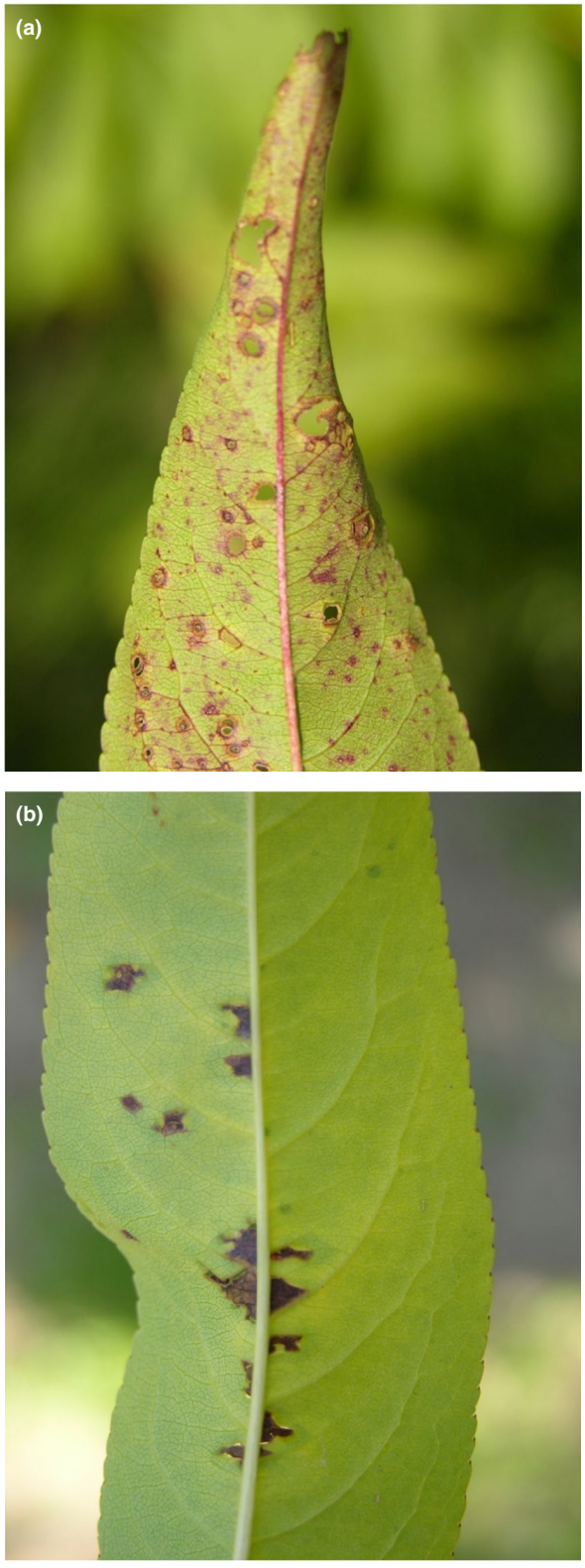

FIGURE 10 (a, b) Possible confusion. (a) Copper phytotoxicity lesions and (b) bacterial angular spots caused by *X. arboricola* pv. *pruni* as they appear on nectarine leaves, cv. 'Diamond Ray'. Courtesy of Emilio Stefani, UNIMORE (IT)

3.1.2 | Symptoms on fruits

Symptoms on fruits usually appear 3–5 weeks after petal fall and develop until the skin colour changes, when the ripening process begins and some physiochemical parameters change. Symptoms often occur after hail damage. These bacterial symptoms can be confused with those caused by *Wilsonomyces carpophilus* and *Venturia carpophila*.

On *P. persica* (peach and nectarine) and *P. armeniaca* (apricot) fruits, small circular brown spots appear on the surface. Lesions become sunken, the margins are frequently water-soaked and often light-green halos give a mottled appearance to the fruit. As a result of natural enlargement of the fruit, pitting and cracking occur in the vicinity of the spots (Figures 11 and 12). These cracks are often very small and difficult to see, but when heavy infection occurs on young fruits they can be wide and easily visible on the fruit surface (Figures 13–15). Gum flow, particularly after rain, may occur from bacterial wounds (Figure 16); this may be easily confused with insect damage.

On *P. salicina* (Japanese plum) fruits, symptoms may be quite different; large, sunken black lesions (Figure 17) are common on some cultivars, while, on others only small pit-like lesions occur. This depends on different cultivar susceptibility. On *P. domestica* (European plum) fruits, symptoms are not common: they may appear as small water-soaked lesions, later necrotizing and cracking (Figure 18).

On *P. avium* and *P. cerasus* (sweet and sour cherries), early fruit infection results in distorted fruit and bacteria may be found from the epidermis to the stone.

Symptoms on *P. dulcis* (almond) fruits are quite specific and different from those observed on other stone fruits. Infected fruits initially display sunken, corky

FIGURE 11 Pitting and cracking occur in the vicinity of the spots on fruits of *P. persica* infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 12 Symptoms on fruits of *P. armeniaca* infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 13 Severe necrotic lesions as they appear on peaches, cv. 'Zee Lady' infected by *X. arboricola* pv. *pruni*. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 14 Necrotic spots and cracks caused by *X. arboricola* pv. *pruni* on nectarines, cv. 'Big Haven'. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 15 Very severe necrotic lesions caused by *X. arboricola* pv. *pruni* on immature apricots, cv. 'Lady Cot'. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 16 Gum flow from bacterial wounds on fruits of *P. persica* infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 17 Large necrotic cracking spots caused by *X. arboricola* pv. *pruni* on immature fruits of Japanese plum cv. 'Golden plum' with water-soaked tissue surrounding the necrotic area. Courtesy of Remedios Santiago Merino, LSV-Junta de Extremadura (ES)

FIGURE 18 A few lesions caused by *X. arboricola* pv. *pruni* on a European plum, cv. 'Čačanska Rana'. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 19 Sunken, corky lesions, with oozing gum that streams or clumps on almond fruits infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 20 Raised sunken lesions on almond fruits infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 21 Circular dark spots on the endocarp of almond fruits infected by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 22 Almond mummies caused by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

lesions, oozing gum that streams or clumps (Figure 19). Later, when the mesocarp dehydrates, the sunken lesions become raised (Figure 20). In some cases, circular dark spots are observed on the endocarp, which can even affect the nut (Figure 21). Infected fruits either drop

prematurely or mummify and remain on the trees after harvest (Figure 22). These mummies contain viable bacteria, therefore serving as inoculum source thereafter.

3.1.3 | Symptoms on twigs

Twig lesions (black tip) are not observed as commonly as the leaf and fruit/nut symptoms.

On *P. persica* (peach and nectarine) twigs, spring cankers occur on the top portion of overwintering twigs and on water sprouts before green shoots are produced; initially small, water-soaked, slightly darkened, superficial blisters extend 1–10 cm parallel to the long axis of the twig and may even girdle it. In this case, the tip of the twig may die, while the tissue immediately below the necrotic area, in which the bacteria are present, is characteristically dark ('black tip' injury) (Figure 23). Twig infections later in the season result in summer cankers which appear as water-soaked, dark-purplish spots surrounding lenticels. These later dry out and become limited, dark, sunken, circular to elliptical lesions with a water-soaked margin. *X. prunicola* (López *et al*., 2018) can also cause cankers and gummosis on *P. persica*. When affecting buds, symptoms can be confused with those resulting from *Fusicoccum amygdali* (synonym *Phomopsis amygdali*) infections.

On *P. salicina* (Japanese plum) and *P. armeniaca* (apricot) twigs and branches, cankers are perennial, in contrast to peach, and continue developing in branches 2 and 3 years old. The inner bark is penetrated, resulting in deep-seated cankers which deform and kill twigs (Figures 24 and 25). Cankers on trunks caused by fungal pathogens as *Leucostoma cincta* or *Eutypa lata* can be confused with those caused by *X. arboricola* pv. *pruni*. On *P. domestica* cankers on twigs and branches are extremely rare.

FIGURE 23 Tip and twig lesions on young peach shoots caused by *X. arboricola* pv. *pruni*. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 24 Young canker and gummosis on European plum, cv. 'Anne Gold' caused by *X. arboricola* pv. *pruni*. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 25 Severe cankers and gummosis on 2–3-year-old branches of a European plum, cv. 'Golden Plum' caused by *X. arboricola* pv. *pruni*. Courtesy of Emilio Stefani, UNIMORE (IT)

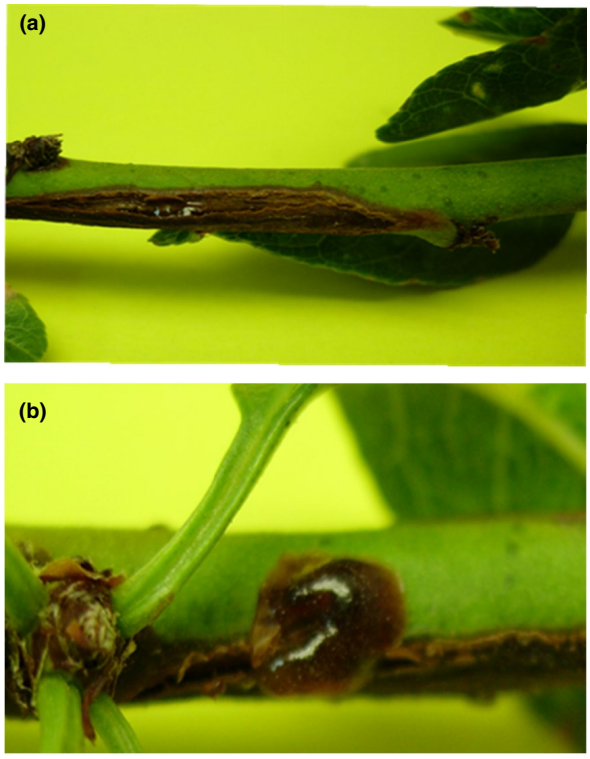

FIGURE 26 (a) Twig cankers with gummosis on *P. dulcis* cv. 'Marta' caused by *X. arboricola* pv. *pruni*. (b) Detail. Courtesy of Montserrat Roselló Pérez, LDF-Valencian Government (ES)

On *P. dulcis* (almond) the twig lesions on the current season's wood are dark and elongated along the length of the twig, slightly depressed and often have a shiny, greasy appearance with a water-soaked margin (Figure 26). If the lesion expands it may girdle the twig and dieback will occur. Cankers can also appear on branches.

On *P. laurocerasus* (cherry laurel) no lesions or cankers on twigs and branches have been observed.

For more detailed information on symptoms, see Dunegan (1932), Anderson (1956), Hayward and Waterston (1965), McIver (1973), Moffett (1973), Gasperini *et al*. (1984), Du Plessis (1988), Goodman and Hattingh (1988), Shepard (1994), Ritchie (1995) and Roselló *et al*. (2012).

3.2 | Detection on symptomatic plant material

Isolation or two molecular tests (one test for areas where the pest is established) such as conventional PCR (Appendix 3), real-time PCR (Appendix 4) or LAMP (Appendix 5) are recommended for the detection of *X. arboricola* pv. *pruni* on symptomatic plant material. A critical review of the available tests is presented in Palacio-Bielsa *et al*. (2012).

3.2.1 | Isolation

In all *Prunus* species, *X. arboricola* pv. *pruni* can be isolated from symptomatic leaves or from immature fruits showing (water-soaked) angular spots, or from twigs and branches with cankers. Generally, isolation from ripening fruits is challenging and, as the ripening process continues, isolation of the pathogen becomes more difficult. However, isolation remains easy on almond fruits as they ripen.

A few small pieces of tissue (1–2 mm) are taken from the margin of the lesion after the plant material has been surface sterilized with 70% ethanol. Plant material is crushed in a mortar or comminuted, adding a few drops of sterile water or phosphate-buffered saline (PBS). After crushing, 3–5 mL of PBS (see Appendix 1) is added and the suspension is left to settle for up to 10 min (longer settling would cause oxidation of the sample, resulting in loss of bacterial cell viability). In the case of cherry laurel, the material is incubated for approximately 30 min at room temperature.

The dilution-plating method should be used to spread 20–50 µL of the suspension or aliquots of 10-fold and 100-fold dilutions in the same buffer onto Wilbrink, YPGA (yeast-peptone-glucose agar), GYCA (glucose-yeast extract-calcium carbonate agar) or YDC (yeast

FIGURE 27 Pure culture of *X. arboricola* pv. *pruni* grown on YPGA for 4–5 days at 23°C. Courtesy of Remedios Santiago Merino, LSV – Junta de Extremadura (ES) and of Miguel Cambra Alvarez, CPV-Government of Aragón (ES)

extract-dextrose-calcium carbonate agar) (Appendix 1). The plates are incubated at approximately 28°C for 2–3 days. *X. arboricola* pv. *pruni* colonies are domed, smooth, mucoid and glistening: colour is bright, creamy yellow, with a tendency to darken a little, turning yellow-orange with age or depending on the media (Figures 27 and 28).

Another semi-selective medium mXCP1 (*Xanthomonas axonopodis* pv. *phaseoli* medium) can be used (Appendix 1). After incubation at approximately 28°C for 3–5 days, *X. arboricola* pv. *pruni* colonies on mXCP1 appear yellow and mucoid, and the hydrolysis of Tween 80 and soluble potato starch can be observed as a light halo around them (Figure 29). Typical colonies should be re-streaked onto Nutrient Agar (NA), YPGA, mXCP1 or YDC plates to obtain pure cultures for further identification.

Confusion with other species

Other bacterial species (*Pantoea* spp. and *Pseudomonas* spp.), living in association with stone fruit crops, may produce yellow colonies on the above-described media, although their colony colour might be slightly different in brilliance and intensity than that of *X. arboricola* pv. *pruni*. Phytopathogenic isolates of the *Pseudomonas syringae* complex, causing the bacterial canker of stone fruits, may also easily grow on the above media; however, they fail to produce bright yellow colonies. Therefore, the production of a yellow pigment on isolation media may help the selection of presumptive *X. arboricola* pv. *pruni* colonies. Confirmation of their identity needs to be performed.

When isolated on King's B medium, several phytopathogenic pseudomonads produce a fluorescent pigment, whereas *X. arboricola* pv. *pruni* does not produce this pigment and has a round-flat morphology and bright yellow colour in this medium (Figure 30). However, some *P. syringae*

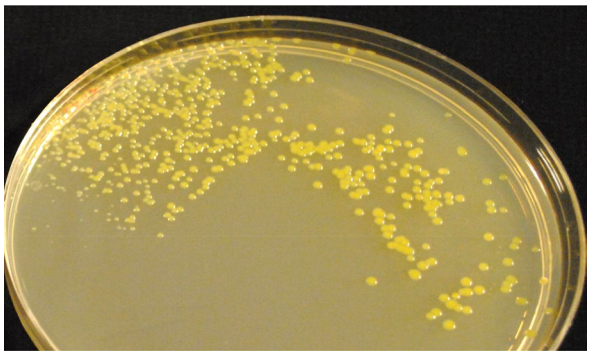

FIGURE 30 Typical colonies of *X. arboricola* pv. *pruni* on King's B medium after 3 days at 25°C. Courtesy of Miguel Cambra Álvarez, CPV-Government of Aragón (ES)

FIGURE 28 A 4 days pure culture of *X. arboricola* pv. *pruni* grown on GYCA. Courtesy of Emilio Stefani, UNIMORE (IT)

FIGURE 31 Typical colonies of *X. arboricola* pv. *pruni* (on the right) and *X. prunicola* (on the left) on YPGA medium. Courtesy of Ester Marco-Noales, IVIA (ES)

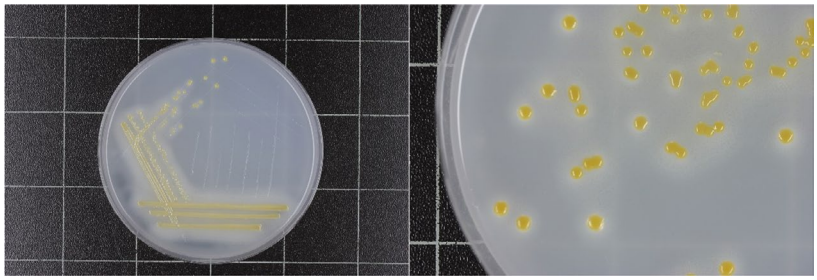

FIGURE 29 Typical colonies of *X. arboricola* pv. *pruni* on mXCP1 medium after 5 days incubation. Courtesy of Daniel Bakker/Harrie Koenraadt, Naktuinbouw (NL)

isolates and most of saprophytes growing on this media do not produce fluorescence either. *X. prunicola* colonies have the same morphology as *X. arboricola* pv. *pruni* (Figure 31).

3.2.2 | Rapid screening tests

These tests facilitate presumptive diagnosis on plants. Conventional PCR (Pothier *et al*., 2011a), real-time PCR (Palacio-Bielsa *et al*., 2011) and LAMP (Bühlmann *et al*., 2013) screening tests are described in Appendices 3–5. A few laboratories in the region experience problems with the LAMP test from Bühlmann *et al*. (2013) and in particular with inclusivity and exclusivity. Cross-reactions with species commonly found in *Prunus* is possible with this test (see Appendix 5). At least two molecular tests based on different DNA sequence targets in the genome need to be included in the screening. For areas where the pest is established, one screening test may be sufficient.

3.3 | Detection on asymptomatic plant material

Two molecular tests (one test for areas where the pest is established) such as conventional PCR (Appendix 3), real-time PCR (Appendix 4) or LAMP (Appendix 5) or isolation are recommended for the detection of *X. arboricola* pv. *pruni* on asymptomatic plant material.

3.3.1 | Test sample requirements

Samples of nursery material should consist of 100 dormant budchips, while samples from orchards in late winter should consist of 100 twigs (1 year old). One budchip for each tree or twig should be cut and the 100 budchips collected in a Stomacher bag. If single large trees (used to obtain scions or budchips for grafting) have to be tested, 30 twigs from each tree should be cut and 100 budchips taken from them.

3.3.2 | Extraction from plant material

The sample prepared according to section 3.3.1 is put into a Stomacher bag and crushed for 3 min at room temperature. After adding 10 mL of PBS (10 mM), the material is incubated for approximately 30 min at room temperature under agitation (100 rpm). From this non-concentrated extract, 3 mL is used directly for testing and the remaining extract is concentrated and tested to increase the probability of detection compared to the non-concentrated sample. The sample is concentrated by centrifugation for 20 min at 10 000*g*. The supernatant is discarded and the pellet is resuspended in 1.5 mL of Phosphate buffer (10 mM, pH 7.2) and used for testing. The part of the extract not used for testing of both non-concentrated and concentrated extract is kept at –20°C after addition of 15%–20% sterile glycerol.

Alternatively, 30 mL of 0.05 M sterile K-phosphate buffer, pH 7.0 is added to the Stomacher bag and crushed for 3 min at room temperature. The suspension is filtered through sterile gauze into a 50 mL centrifuge tube and spin for 5 min at 480 *g*. The supernatant is poured into a new tube and centrifuged again at 12 000 *g* for 10 min. The supernatant is discarded and the pellet resuspended with 1–2 mL of PBS to obtain the final concentrate. A portion of the final concentrate (0.5–1 mL) is kept at approximately –20°C after addition of 15–20% sterile glycerol.

3.3.3 | Direct isolation

The dilution-plate method should be used to spread 20–50 µL of the non-concentrated and concentrated extract or aliquots of 10-fold and 100-fold dilutions in the same buffer onto Wilbrink, YPGA, GYCA or YDC (Appendix 1). The plates are incubated at approximately 28°C for 2–3 days and colonies resembling *X. arboricola* pv. *pruni* are selected and purified.

3.3.4 | Molecular tests

Conventional PCR (Pothier *et al*., 2011a), real-time PCR (Palacio-Bielsa *et al*., 2011) and LAMP (Bühlmann *et al*., 2013) are described in Appendix 3–5. It is recommended that the screening is performed using at least two molecular tests based on different DNA sequence targets. A few laboratories in the region experience problems with the LAMP test from Bühlmann *et al*., 2013 and in particular with inclusivity and exclusivity. Cross-reactions with species commonly found in *Prunus* are possible with this test (see Appendix 5).

4 | IDENTIFICATION

Pure cultures of presumptive *X. arboricola* pv. *pruni* should be identified with at least two tests based on different biological principles (e.g. combinations of serological or molecular tests) or two molecular tests based on different DNA sequence targets in the genome. Known *X. arboricola* pv. *pruni* reference strains should be included in each test.

4.1 | Serological methods

Depending on the availability of validated antibodies, suspensions of presumptive isolates (containing

approximately 10^6 cfu/mL) may be identified using immunofluorescence according to EPPO diagnostic protocols PM 7/97 (1) *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Recommended polyclonal antisera are PRI-I-731-BCD-08 and X10-713-BCD-08. Both have been validated in the Netherlands for the identification of pure cultures by IF (Appendix 6).

4.2 | Molecular tests

4.2.1 | Conventional PCR

Appropriate PCR procedures (Pothier *et al*., 2011a,b) should be used, following Appendices 3 and 7.

4.2.2 | Real-time PCR

A real-time PCR by Palacio-Bielsa *et al*. (2011) is described in Appendix 4.

A real-time PCR by Garita-Cambronero *et al*. (2017) is described in Appendix 8.

For routine detection and identification of *X. arboricola* pv. *pruni* (Xap), it is recommended to use in conjuction the real-time from Palacio-Bielsa *et al*. (2011) with the real-time PCR amplification protocol based in xopE3 gene (Garita-Cambronero *et al*. 2017). If both tests result positive, the bacterial isolate could be identified as Xap and, on the other hand, if the isolated bacterium shows positive results only for the real-time PCR from Palacio-Bielsa *et al*. (2011) it could be designated as a member of the Xap-look-a-like group.

4.2.3 | LAMP

A LAMP test (Bühlmann *et al*., 2013) is described in Appendix 5.

4.2.4 | DNA fingerprinting methods

The comparison of genomic DNA between isolates and type strains by means of rep-PCR fingerprinting can be a useful identification test [see EPPO Standard PM 7/100 (1) *Rep-PCR tests for identification of bacteria* (EPPO, 2010, 2014)].

4.3 | Other tests

The following tests provide additional information to support pest identification.

4.3.1 | DNA barcoding methods

Procedures are described in the EPPO Standard PM 7/129 on *DNA barcoding as an identification tool for plant pests*. Single locus sequence typing of the partial *gyrB* gene can be used to support identification of *X. arboricola* from the most closely related species (Parkinson *et al*., 2009).

4.3.2 | Biochemical tests

X. arboricola pv. *pruni* is a Gram-negative bacterium, rod-shaped, motile by one flagellum, measuring 0.2–0.4 × 0.8–1.0 µm. It is a strict aerobe with an optimum growth temperature range of 24–29°C. Different biochemical characteristics can be used for the identification of *X. arboricola* pv. *pruni* as described in Fahy and Persley (1983) and Schaad *et al*. (2001).

4.3.3 | Hypersensitivity and pathogenicity tests

The hypersensitivity reaction (HR) on tobacco (e.g. cvs 'Samsun' or 'Xanthi') or tomato (e.g. cvs 'Moneymaker' or 'Roma') leaves is performed with a bacterial suspension from a 48 h culture at a concentration of about 10^9 cfu/mL in sterile distilled water or PBS (Klement, 1963; Klement *et al*., 1964). Typical HR in tomato leaves is observed after 24–48 h, but in tobacco usually only after 2–3 days. In some cases, an atypical HR in tobacco can be observed as a loss of turgidity in the infiltrated area after 24 h, which becomes chlorotic after 48–72 h. Five days after inoculation, infiltrated sites show a collapse of the tissue surrounded by a chlorotic area (Figure 32).

FIGURE 32 Atypical HR in tobacco cv. 'Xanthi' 5 days after inoculation: infiltrated sites show a collapse of the leaf tissue surrounded by a chlorotic area. Courtesy of Montserrat Roselló Pérez, LDF-Valencian Government (ES)

In critical cases (see PM 7/76) or when additional confidence in the outcome of the diagnosis is required, final confirmation requires a pathogenicity test on leaves or on 1-year grafted rootstocks of known susceptible peach or plum cultivars or rootstocks (see Appendix 9). When the pathogenicity test is positive, the virulence of the isolate is confirmed. When the pathogenicity test is negative, the virulence of the isolate is not confirmed and DNA barcoding is recommended to confirm identification (i.e. to exclude possible false-positive results) see section 4.3.1.

5 | REFERENCE MATERIAL

The following *X. arboricola* pv. *pruni* isolate is recommended for use as positive control: CFBP 2535 ^{T} = ATCC 19316 = ICMP 51 = LMG 852 = NCPPB 416.

The following collections can provide this *X. arboricola* pv. *pruni* reference strain:

Centre International de Ressources Microbiennes-Collection Française de Bactéries associées aux Plantes, Angers, France (CIRM-CFBP);
American Type Culture Collection, Rockville, Md, USA (ATCC);
International Collection of Micro-organisms from Plants, Lincoln, New Zealand (ICMP);
Laboratorium voor Microbiologie Bacterial Collection, Universiteit Gent, Belgium (LMG);
National Collection of Plant Pathogenic Bacteria, Sand Hutton, York, United Kingdom (NCPPB).

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Bergsma-Vlami M., National Plant Protection Organisation, PO Box 9102, 6700 HC, Wageningen (Netherlands). E-mail: maria.vlami@nvwa.nl
Palacio-Bielsa A., Centro de Investigación y Tecnología Agroalimentaria de Aragón, Zaragoza (Spain). E-mail: apalaciob@aragon.es
Cubero J., Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ctra. de la Coruña km 7,5, 28040 Madrid (Spain). E-mail: cubero@inia.es
Gottsberger R., Austrian Agency for Health and Food Safety (AGES) (Austria). E-mail: richard.gottsberger@ages.at
Stefani E., Department of Life Sciences, University of Modena & Reggio Emilia, Modena (Italy). E-mail: emilio.stefani@unimore.it

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When *errata* and *corrigenda* are in press this will also be marked on the website.

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APPENDIX 1 BUFFERS AND MEDIA

All media are sterilized by autoclaving at approximately 121°C for 15 min, except when stated otherwise.

Buffers

Phosphate buffered saline (PBS) 10 mM	
NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L
Adjust pH to 7.2	

PBS may be stored at approximately 4–8°C for several days.

K-phosphate buffer 50 mM, pH = 7.0	
K_2HPO_4	8.71 g
KH_2PO_4	6.81 g
Distilled water to	1 L

Extraction buffer **(Llop *et al.*, 1999)**	
Tris HCl, pH 7.5	24.2 g
NaCl	14.6 g
EDTA	9.3 g
SDS	5 g
Polyvinylpyrrolidone (PVP-10)	20 g
Distilled water	1 L
Sterilize by filtration	

Extraction buffer should be freshly prepared prior to its use. Do not expose to temperatures below 15°C since SDS may crystallise.

Phosphate buffer (10 mM PB, pH 7.2) for resuspension of pelleted extracts	
$Na_2HPO_4.12H_2O$	2.70 g
$NaH_2PO_4.2H_2O$	0.40 g
Distilled water	1.0 L

Phosphate buffer may be stored at approx. 4–8°C for several days.

Media

In general, fungal contamination is not a problem during the isolation of *X. arboricola* pv. *pruni* on agar media. If problems with fungal contamination occur, it is recommended to add antifungal compounds (such as cycloheximide 50–150 mg/L or nystatin 35 mg/L) to the media.

Agar plates may be stored at 4–8°C for a maximum of 30 days.

Nutrient Agar (NA) **(Lelliott and Stead, 1987)**	
Peptone	5.0 g
Yeast extract	3.0 g
NaCl	0.5 g
Microbiological grade agar	15.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

Yeast-peptone-glucose agar medium (YPGA) **(Ridé, 1969; Lelliott & Stead, 1987)**	
Yeast extract	5.0 g
Bacteriological peptone	5.0 g
Glucose	10.0 g
Microbiological grade agar	20.0 g
Distilled water to	1 L
Adjust pH 7.0	
Autoclave	
Cool to about 50°C	

Yeast extract-dextrose-calcium carbonate agar medium (YDC) **(Stolp & Starr, 1964)**	
Yeast extract	10.0 g
Dextrose (glucose)	20.0 g
$CaCO_3$ (fine powder)	20.0 g
Microbiological grade agar	15.0 g
Distilled water to	1 L
Adjust to pH 7.0	
The autoclaved medium should be cooled to 50°C in a water bath, and $CaCO_3$ suspended by swirling before pouring the plates.	

Wilbrink agar medium **(Koike, 1964; Sands *et al.*, 1986)**	
Peptone special (Oxoid/LP0072)	5.0 g
K_2HPO_4 (Merck/1.05101)	0.5 g
$MgSO_4{\cdot}7H_2O$ (Merck/1.05886)	0.25 g
Sucrose (Fluka/84100)	10.0 g
Agar technical no.3 (Oxoid/LP0013)	18.0 g
Distilled water to	1 L
Adjust to final pH 7.0 (±0.2) with KOH/HCl before adding Agar	

Validation of the isolation of *X. arboricola* pv. *pruni* on Wilbrink agar medium (NVWA)

Analytical sensitivity
4.6×10^3 cfu/mL
Analytical specificity
50%; many different Xanthomonads grow equally well on the Wilbrink medium, however, this is not a problem

since identification tests on the pure cultures will reveal if *X. arboricola* pv. *pruni* is present among the other Xanthomonads.

mXCP1 medium **(Duchefa): (Bergsma-Vlami *et al.*, 2012) modified from McGuire *et al.* (1986)**	
Peptone	10.0 g
KBr	10.0 g
$CaCl_2$ anhydrous	0.25 g
Soluble potato starch	10.0 g
Distilled water to	1 L
Agar	15.0 g

Add the following ingredients/antibiotics sterile filtered (0.45 µm filter) after autoclaving (at 45°C):

0.15 mL crystal violet
35 mg/L nystatin (200 000 U)
10 mg/L cephalexin
3 mg/L 5-fluorouracil
0.112 mg/L tobramycin sulphate
10 mL/L sterile Tween 80 (10%)

Keep plates for 4 days at 4°C to get better visibility of starch hydrolysis.

Glucose yeast extract calcium carbonate agar (GYCA) **(Dye, 1962)**	
D(+) glucose	10.00 g
Yeast extract	5.0 g
$CaCO_3$ (Sigma-Aldrich 12010)	30.0 g
Agar	20.0 g
Distilled water to	1 L

It is recommended to use very pure, precipitated and anhydrous $CaCO_3$.

Validation data from Loreti *et al.* (2015)

Samples set: peach crude extract spiked with 10^7 (three replicates), 10^6 (three replicates), 10^5 (three replicates), three healthy peach extracts.

Diagnostic sensitivity = 70%
Diagnostic specificity = 100%
Accuracy = 82%

APPENDIX 2 DNA EXTRACTION

Plant material

Nucleic acid extraction from asymptomatic plant material according to Llop *et al.* (1999)

Use 1 mL of each macerate prepared according to section 3.3.2. Centrifuge the macerates at 10 000*g* for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 500 µL of extraction buffer (Llop *et al.*, 1999) and shake for 1 h at room temperature. Centrifuge at 4000*g* for 5 min. Take 450 µL of the supernatant and add the same volume of isopropanol, invert and leave for 1 h at room temperature. Centrifuge at 10 000*g* for 5 min, discard the supernatant and dry. If there is still a coloured precipitate (brown or green) at the bottom of the tubes, carefully take it while discarding the supernatant to obtain a cleaner DNA. Resuspend the pellet in 200 µL of water. Use for PCR reaction or store at approximately −20°C.

Commercial kits (plant material)

DNeasy® Plant Mini Kit (Qiagen)

Extraction and concentration of the bacterium should be performed as described in section 3.3.2.

500 µL of concentrate should be used; the bacterial cells lysis and DNA purification should be performed according to the manufacturer instructions.

High Pure PCR Template Preparation Kit (Roche)

Manufacturer instructions should be followed.

QIAamp DNA minikit (Qiagen)

Manufacturer's instructions should be followed.

Bacterial colonies

Nucleic acid extraction from bacterial colonies

Bacterial colonies should be resuspended in 500 µL of molecular-grade water and heat-treated (95°C for 10 min). Alternatively, a loopful of 48 h pure bacterial culture is suspended in sterile distilled water to prepare a bacterial suspension at 10^6 cfu/mL. Alkaline lysis can be performed by adding 10 µL of NaOH 0.5 M to 90 µL of bacterial suspension; the suspension should be mixed by inversion several times, incubated at 95–100°C for 4 min and then placed on ice. Cold suspensions are then centrifuged at 10 000 rpm for 1 min. Extracts of total nucleic acids can be stored at approximately 4°C for use within 1 week or at approximately −20°C for longer periods.

APPENDIX 3 CONVENTIONAL PCR ADAPTED FROM PAGANI (2004) AND POTHIER *ET AL.* (2011a)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. This PCR is suitable for the detection and identification of *X. arboricola* pv. *pruni* on plant extracts or pure cultures.

1.2. The test is adapted from Pagani (2004).

1.3. The target sequence is located on an open reading frame (ORF) predicted to encode a putative protein of 243 amino acids that shares similarities to the ABC transporter family.

1.4. Oligonucleotides and average amplicon size:

Primers	**Sequence**	**Amplicon size**
Forward primer Y17CoF	5′-GAC GTG GTG ATC AGC GAG TCA TTC-3′	943 bp
Reverse primer Y17CoR	5′-GAC GTG GTG ATG ATG ATC TGC-3′	

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. Matrices: plant extracts or bacterial isolates.

2.1.2. The protocol for DNA extraction from plant material or bacterial isolates is described in Appendix 2.

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

2.2. Conventional PCR.

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	29.5	NA
Go Taq Flexi buffer (Promega)	5×	10.0	1×
$MgCl_2$ (Promega)	25 mM	3.0	1.5 mM
dNTPs (Promega)	5 mM	1.0	0.1 mM
Forward primer Y17CoF	10 µM	0.5	0.1 µM
Reverse primer Y17CoR	10 µM	0.5	0.1 µM
GoTaq Flexi DNA polymerase (Promega)	5 U/µL	0.5	2.5 U
Subtotal		45	
Total DNA		5	
Total		50	

2.2.2 PCR conditions: 5 min at 93°C, 45 cycles of 1 min at 93°C, 1 min at 55°C and 2 min at 72°C and a final step of 72°C for 10 min.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. Include DNA extracted from a *X. arboricola* pv. *pruni* isolate (see section 5, *Reference material*), total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results: to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC: a band of the expected size [943 bp] should be visualized.

When these conditions are met

- A test will be considered positive if a band of the expected size [943 bp] is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from Pothier *et al.* (2011a) and Bergsma-Vlami *et al.* (2012).

4.1. Analytical sensitivity

The analytical sensitivity was 50 cells per PCR reaction after DNA extraction from apricot leaf tissue.

4.2. Analytical specificity

Inclusivity was evaluated with six strains of *X. arboricola* pv. *pruni*. All strains were detected.

Exclusivity was evaluated using 79 *Xanthomonas* genus strains representing 26 species and 14 non-*Xanthomonas* strains. Amplification was obtained with *X. arboricola* strains and with all tested (10) strains of *X. arboricola* pv. *corylina* (a pathogen of hazelnut not

reported from *Prunus*), one of three *X. arboricola* pv. *celebensis* (the pathotype strain, a pathogen from banana not reported from *Prunus*) and two of four *X. arboricola* pv. *poinsettiicola* type C strains (a pathogen from poinsettia only reported from New Zealand and not from *Prunus*). When a blind collection of 68 saprophytic and epiphytic isolates associated with *Prunus* plants was tested, no cross-reactions were observed (Pothier *et al*., 2011a).

In routine diagnostics, cross-reactions can be occasionally observed with very closely related *Xanthomonas* spp. isolated from symptomatic leaves of *P. laurocerasus* in the Netherlands, which show the typical yellow, shiny colony phenotype on both Wilbrink and mXCP1 media (Bergsma-Vlami *et al*., 2012).

Cross-reaction may also occur with some strains of *X. arboricola* pv. *corylina* and *X. arboricola* pv. *juglandis* but these two pathogens are not observed in *Prunus* species (Stefani, pers. comm. 2020). However, López *et al.* (2012) observed good correlation among this PCR and isolation and real-time PCR results in the analysis of symptomatic samples of several hosts.

4.3. Data on repeatability
None.

4.4. Data on reproducibility
None.

Validation data from Loreti *et al*., 2015.

4.1. Analytical specificity data

PCR on bacterial isolates:
Inclusivity: 96%. In the TPS, false negative results were obtained by two (out of four) laboratories with colonies of *X. arboricola* pv. *pruni* (DLS 360).
Exclusivity: 74%
Cross-reaction observed with *X. arboricola* pv. *celebensis* (NCPPB1832), *X. arboricola* pv. *corylina* (NCPPB 935), *X. arboricola* pv. *juglandis* (NCPPB 411, LMG 745), *X. campestris* pv. *campestris* (ISPaVe 1032), *X. arboricola* pv. *populi* (NCPPB2987).

4.2. Diagnostic sensitivity
PCR on plant material: 69%

4.3. Diagnostic specificity
PCR on plant material: 100%

4.4. Repeatability: 100%

4.5. Reproducibility: 80%

APPENDIX 4 REAL-TIME PCR ADAPTED FROM PALACIO-BIELSA *ET AL*. (2011)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. This PCR is suitable for the detection and identification of *X. arboricola* pv. *pruni* in plant material and bacterial colonies.
1.2. The test is based on Palacio-Bielsa *et al.* (2011).
1.3. The target sequence is located on an open reading frame (ORF) predicted to encode a putative protein of 243 amino acids that shares similarities to the ABC transporter family.
1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer Xap-2F	5′-TGG CTT CCT GAC TGT TTG CA-3′
Reverse primer Xap-2R	5′-TCG TGG GTT CGC TTG ATG A-3′
Probe Xap-2P	5′-FAM-TCA ATA TCT GTG CGTTGC TGT TCT CAC GA – TAMRA-3′

1.5. Real-time PCR system: Smartcycler (Cepheid Inc.)

2. METHODS

2.1. Nucleic acid extraction and purification.
2.1.1. Matrices: naturally infected symptomatic and asymptomatic plant material and bacterial colonies.
2.1.2. The protocol for DNA extraction from plant material or bacterial isolates is described in Appendix 2.
2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. Real-time polymerase chain reaction (real-time PCR).
2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	8.625	NA
QuantiMix Easy Probes kit (Biotools)	2×	12.5	1×
Forward primer Xap-2F	20 µM	0.5	0.4 µM
Reverse primer Xap-2R	20 µM	0.5	0.4 µM

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Probe Xap-2P	10 µM	0.375	150 nM
Subtotal		22.5	
DNA		2.5	
Total		25	

2.2.2 PCR cycling conditions: 5 min at 95°C, 45 cycles of 1 min at 95°C and 1 min at 59°C.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on colonies, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

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

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from Palacio-Bielsa *et al.* (2011).

4.1. Analytical sensitivity data

For bacterial suspensions the analytical sensitivity is 10^2–10^3 cfu/mL.

For peach, almond and GF-677 rootstock washed tissues without DNA extraction the analytical sensitivity is 10^2 cfu/mL. However, the analytical sensitivity is only 10^5 cfu/mL for Japanese plum washed tissues.

For different plant extracts after DNA extraction following Llop *et al.* (1999), the analytical sensitivity is 10^2 cfu/mL.

4.2. Analytical specificity data

Inclusivity: 159 strains, from a variety of geographical locations, all positive.

Exclusivity: 12 bacterial strains representative of other closely related *Xanthomonas* species, 34 non-*Xanthomonas* phytopathogenic strains and two strains of saprophytic bacteria. Positive PCR resulted only from *X. citri* subsp. *citri* or *X. arboricola* pv. *corylina*.

Samples infected with *X. prunicola* give negative results with this test according to Lopez *et al.* (2018).

4.3. Data on repeatability
None.

4.4. Data on reproducibility
None.

APPENDIX 5 LAMP TEST ACCORDING TO BÜHLMANN *ET AL.* (2013)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. This test is suitable for the detection and identification of *X. arboricola* pv. *pruni* in plant material and bacterial colonies.

1.2. The test is based on Bühlmann *et al.*, (2013).

1.3. The target sequence is located on a gene coding for the hypothetical protein XAP3806.

1.4. Oligonucleotides:

Primers	Sequence
External forward primer F3	5′-CAC TGC GGA TTG TTA CAC GT-3′
External reverse primer B3	5′-TGA TGC CCC TCA AGA GAG G-3′
Inner forward primer FIP	5′-TGC GTG GGT CGA ATA GGT ACC AGG GTG TGG AGT TGG TCG T-3′
Inner reverse primer BIP	5′-TAC GGG ATC GAG ACA CCT TGG TCG GTG CAT GGT AGA TCA CAT-3′
Forward loop primer LoopF	5′-AGC ATG CAG AAT CTG CCA GCA C-3′
Reverse loop primer LoopR	5′-TGC CGG GGA CGC AAT GTA ATG C-3′

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. Matrices: plant material and bacterial colonies.

2.1.2. Nucleic acid extraction from plant material: for experimentally infected plants at 45–50 days post-infection 0.1 g of leaf, twig or woody tissue samples with symptoms were collected, ground in 1 mL PBS and boiled at 99°C for 20 min. Aliquots of 1 µL of boiled tissue extract were used directly as a template for LAMP.

2.1.3. Nucleic acid extraction from bacterial colonies: DNA is extracted following the protocol of Sambrook et a. (1989). Alternatively, colonies are picked from the agar, added to dH_2O and boiled at 99°C for 20 min. It is recommended to use at least 100 cells per reaction. Alternatively, alkaline lysis can be performed following the protocol in Appendix 2.

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

2.2. LAMP

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	0.2	NA
Isothermal Master Mix (*Optigene Ltd*)	1.6×	7.2	1× ca.
External forward primer F3	3.2 µM	0.6	0.16 µM
External reverse primer B3	3.2 µM	0.6	0.16 µM
Inner forward primer FIP	3.2 µM	0.6	0.16 µM
Inner reverse primer BIP	3.2 µM	0.6	0.16 µM
Forward loop primer LoopF	1.6 µM	0.6	0.08 µM
Reverse loop primer LoopR	1.6 µM	0.6	0.08 µM
Subtotal		11	
Total DNA		1	
Total		12	

2.2.2 PCR conditions: 70°C for 45 min with a specific melting temperature observed at 88 ± 0.2°C.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on colonies, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no turbidity/colour change or no fluorescence
- PIC, PAC should produce:

  The expected turbidity/colour change. The test based on turbidometry can be performed with Bst DNA polymerase, but it cannot be used with the polymerases from Optigene because they are engineered not to produce turbidity.

  Fluorescence. For end point measurement a positive reaction is defined by FU and/or T_m (°C± known variation). For real-time measurement a positive reaction is defined by time of positivity (minutes) and/or T_m (°C± known variation) as given by the manufacturer.

When these conditions are met

- A test will be considered positive if it produces a positive reaction as defined for PIC and PAC (see above).
- A test will be considered negative if it produces no turbidity/colour change or no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from Bühlmann *et al*. (2013).

4.1. Analytical sensitivity data

Boiled samples: the LAMP reactions showed a dynamic range with quantitative amplification over six orders of magnitude from 2.4×10^9 to 2.4×10^4 cfu/mL. Additionally, the LAMP assay was shown to detect bacterial concentrations as low as 2.4×10^3 cfu/mL, at which the detection limit of the assay was reached. At concentrations below 2.4×10^4 cfu/mL the amplification became nonlinear and quantification difficult to reliably interpret.

Extracted samples: a range from 1 ng/μL to 0.1 fg/μL was tested, which, extrapolated to a *X. arboricola* pv. *pruni* genome of 5.07 Mbp, would result in an equivalent range of 2×10^9–2×10^2 cfu/mL. The detection limit was at 1 fg/μL, equivalent to 2×10^3 cfu/mL, confirming the values obtained with boiled cells.

4.2. Analytical specificity

Inclusivity: 100%, evaluated on 28 genotypically representative *X. arboricola* pv. *pruni* strains.

Exclusivity: 100%, evaluated on 51 non-bacterial target strains including *X. arboricola* pv. *celebensis*, (13) *X. arboricola* pv. *corylina*, *X. arboricola* pv. *fragariae*, *X. arboricola* pv. *juglandis*, *X. arboricola* pv. *poinsettiicola*, *X. arboricola* pv. *populi*, *Xalbilineans*, *X alfalfae* subsp. *alfalfae*, *X. alfalfae* subsp. *citrumelonis*, *X axonopodis* pv. *axonopodis*, *X. axonopodis* pv. *citri*, *X bromi*, *X campestris* pv. *campestris*, *X cassavae*, *X codiaei*, *X cucurbitae*, *X citri* pv. *citri*, *X cynarae*, *X fragariae*, *X fuscans*, *X gardneri*, *X hortorum* pv. *hederae*, *X hyacinthi*, *X melonis*, *X oryzae* pv. *oryzae*, *X perforans*, *X pisi*, *X populi*, *X sacchari*, *X theicola*, *X translucens* pv. *translucens*, *X vasicola* pv. *holcicola*, *X vesicatoria*, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Erwinia rhapontici*, *Pantoea vagans*, *Pseudomonas syringae* pv. *persicae*, *Stenotrophomonas maltophilia*.

4.3. Data on repeatability

None.

4.4. Data on reproducibility

None.

Data from ANSES

4.1. Analytical specificity

None.

4.2. Analytical specificity

Cross-reactions with *Pseudomonas syringae pv. morsprunorum*, *X. arboricola* pv *populi*, *X. phaseoli* pv *phaseoli*, *X. arboricola* pv. *corylina*, *Xylella fastidiosa*. No cross-reactions with *Xylophilus ampelinus*, *Pseudomonas viridiflava*.

4.3. Data on repeatability
None.

4.4. Data on reproducibility
None.

APPENDIX 6 IMMUNOFLUORESCENCE TEST

For general instructions on how to perform the immunofluorescence (IF) test see EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Only specific features are presented below. Always use validated antisera.

Performance characteristics available for antiserum number: X10-713-BCD-08 (data from NVWA).

The performance characteristics were obtained during the validation for identification of isolates.

1. Diagnostic sensitivity: 90% (nine *X. arboricola* pv. *pruni* strains gave positive results with IF out of 10 *X. arboricola* pv. *pruni* strains used).
2. Analytical specificity:

Number of *X. arboricola* pv. *pruni* strains tested: 10.
Number of non-target organisms tested: 10.
Cross-reactions were observed with *X. arboricola* pv. *corylina* from *Corylus maxima* and with *X. axonopodis* pv. *manihotis* from *Manihot esculenta*.

3. Diagnostic specificity: 80%

Comparison with the true status.
Eight negative IF results out of 10 true negative samples.

Performance characteristics available for antiserum number: PRI-I-731-BCD-08 (data from NVWA).

The performance characteristics were obtained during the validation for identification of isolates.

1. Analytical sensitivity: 1.2×10^4 cfu/mL.
2. Diagnostic sensitivity: 91.3% (21 *X. arboricola* pv. *pruni* strains gave positive results with IF out of 23 *X. arboricola* pv. *pruni* strains used).
3. Analytical specificity:

Number of *X. arboricola* pv. *pruni* strains tested: 23.
Number of non-target organisms tested: 21.

Cross-reactions were observed with *X. arboricola* pv. *corylina* and a number of closely related *Xanthomonas* sp. from symptomatic cherry laurel plants.

4. Diagnostic specificity: 95.2%

Comparison with the true status.
Twenty true negative IF results out of 21 true negative samples.
Validation data from Loreti *et al.* (2015).
Analytical specificity
Inclusivity: 95% with ADGEN kit.
Exclusivity: 83% with ADGEN kit.
Cross-reaction observed with *X. arboricola* pv. *celebensis* (NCPPB1832), *X. arboricola* pv. *corylina* (NCPPB 935), *X. arboricola* pv. *fragariae* (ISF1G), *X. campestris* pv. *campestris* (ISPaVe 1032), *X. arboricola* pv. *populi* (NCPPB2987).
False negative results were obtained with *X. arboricola* pv. *pruni* (UniMoRe 360).
Diagnostic sensitivity data.
52% with ADGEN kit.
Diagnostic specificity data.
100% with ADGEN kit.
Repeatability: 83%
Reproducibility: 82%

APPENDIX 7 CONVENTIONAL PCR TEST ADAPTED FROM POTHIER *ET AL.* (2011b)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. This PCR is suitable for the identification of *X. arboricola* pv *pruni* from bacterial colonies.
1.2. The test is adapted from Pothier *et al.* (2011b).
1.3. The target sequence is located on the gene *repA1*, located at the pXap41 plasmid and involved in its replication and mobilization.
1.4. Oligonucleotides and average amplicon size:

Primers	Sequence	Amplicon size
pXap41repA1-F repA1	5′-GCG AGG ACA TGG CTT TCA C-3′	343 bp
pXap41repA1-R repA1	5′-GCG GCC AAG GCG TGC ATC TGC-3′	

2. METHODS

2.1. Nucleic acid extraction and purification.
2.1.1. Matrix: bacteria isolates.
2.1.2. The protocol for DNA extraction from bacterial isolates is described in Appendix 2.

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

2.2. Conventional PCR.
2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	18.25	NA
Supertaq PCR buffer (SpheroQ)	10×	2.5	1×
dNTPs	10 mM	0.5	200 µM
Forward primer pXap41repA1-F repA1	10 µM	0.5	0.2 µM
Reverse primer pXap41repA1-R repA1	10 µM	0.5	0.2 µM
Supertaq polymerase (SpheroQ)	5 U/µL	0.25	1.25 U
Subtotal		22.5	
Total DNA		2.5	
Total		25	

2.2.2 PCR conditions: 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; 7 min at 72°C and cooling at 15°C.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: For the NIC 100 µL of molecular-grade water is used.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated. For the PIC, a suspension of 10^9 cfu/mL and dilutions series of *X. arboricola* pv. *pruni* could be used.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification controls (PACs) to monitor the efficiency of the amplification of nucleic acid of each of the target organism: 10^9 cfu/mL and dilution series.

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

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC: a band of the expected size [343 bp] should be visualized.

When these conditions are met

- A test will be considered positive if a band of the expected size (343 bp) is visualized.
- A test will be considered negative if no band of the expected size (343 bp) or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

4.1. Analytical sensitivity data

None.

4.2. Analytical specificity data

Inclusivity: The presence of this pXap41-associated gene was tested on a geographically and genetically representative collection of 35 *X. arboricola* pv. *pruni* isolates. Amplification with the primer set designed for plasmid pXap41 was obtained with DNA from all *X. arboricola* pv. *pruni* isolates.

Exclusivity was evaluated with six other *X. arboricola* pathovars (two strains each), including *X. arboricola* pv. *juglandis*, *X. arboricola* pv. *corylina*, *X. arboricola* pv. *poinsettiicola*, *X. arboricola* pv. *fragariae*, *X. arboricola* pv. *celebensis* and *X. arboricola* pv. *populi*. No amplification was obtained for all other *X. arboricola* pathovars, indicating the pathovar-level discriminatory power of this PCR method.

4.3. Data on repeatability

None.

4.4. Data on reproducibility

None.

APPENDIX 8 REAL-TIME PCR ACCORDING TO GARITA-CAMBRONERO *ET AL*. (2017)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. This PCR is suitable for the identification of *X. arboricola* pv. *pruni* from bacterial colonies.

1.2. The test is based on Garita-Cambronero *et al*. (2017).

1.3. The target sequence is located on the *xopE3* gene coding for the *Xanthomonas* outer protein E3, Type III effector.

1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer xopE3F	5′-TCA GCG ATC ACG CAT CCA-3′
Reverse primer xopE3R	5′-CGC ACC AGA TCG ACA AAC AC-3′
Probe xopE3P	5′-FAM - CATG CGC AGG CCG CAC AT-TAMRA-3′

1.5. Real-time PCR system: Applied Biosystems

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. Matrix: bacterial isolates.

2.1.2. The protocol for DNA extraction from bacterial isolates is described in Appendix 2.

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

2.2. Real-time polymerase chain reaction (real-time PCR).

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	5.1	NA
GoTaq probe qPCR MasterMix (*Promega*)	2×	12.5	1×
Forward primer (*xopE3F*)	10 µM	1	0.4 µM
Reverse primer (*xopE3R*)	10 µM	1	0.4 µM
Probe 1 (*xopE3P*)	10 µM	0.4	160 nM
Subtotal		20	
DNA dilution		5	
Total		25	

2.2.1 PCR conditions: pre-incubation at 95°C for 5 min, followed by 45 cycles of (95°C for 1 min and 59°C for 1 min).

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on colonies, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

4.1. Analytical sensitivity data

The sensitivity of the real-time PCR test was 10 cfu/mL or 100 pg/µL of DNA with a PCR efficiency of 2.2 ± 0.22 or 1.8 ± 0.03 for bacterial cells or purified DNA, respectively.

4.2. Analytical specificity data

Inclusivity was evaluated with 54 strains of *X. arboricola* pv. *pruni*. Amplification was observed for all strains.

Exclusivity was evaluated with seven strains of *X. arboricola* pv. *pruni* look-a-like (CITA 14, CITA 42, CITA 44, CITA 49, CITA 51, CITA 124, CITA 149), 10 strains from other pathovars of *X. arboricola* (pv. *corylina*, *fragariae*, *juglandis*, *populi*), 11 strains from other species of Xanthomonas (*X. campestris*, *X. citri* subsp. *citri*, *X. fuscans* subsp. *fuscans*, *X. hortorum* pv. *pelargonii*, *X. vesicatoria*), eight strains from other genera of phytopathogenic bacteria (*Agrobacterium tumefaciens*, *Agrobacterium* spp., *Pantoea* spp., *Pseudomonas syringae* pv. *syringae*), and nine strains from the natural microbiota of *Prunus* spp. (*Curtobacterium* spp, *Microbacterium* spp, *Pantoea* spp, *Pseudoclavibacter* spp, *Pseudomonas* spp, *Sphingomonas* spp, *Terrabacter* spp). From these, amplification was observed for *X. campestris* (IVIA 2734-1), *X. citri* subsp. *citri* (strains 306, IVIA 2889-1 and IVIA 3026-1), *X. hortorum* pv. *pelargonii* (CITA Xp-2), *X. fuscans* subsp. *fuscans* (NCPPB 381 and IVIA 151835DA) and *X. vesicatoria* (IVIA 3619-1).

Samples infected with *X. prunicola* give negative results with this test, according to López *et al*. (2018).

4.3. Data on repeatability

None.

4.4. Data on reproducibility

None.

APPENDIX 9 PATHOGENICITY TEST

Strains of *X. arboricola* pv. *pruni* can show differential virulence on peach, plum, apricot, almond and cherry. Cross-infections between different hosts species are common, but not always observed (Du Plessis, 1988; Scortichini *et al*., 1996). Their pathogenicity can be tested in a detached leaf assay and/or in whole plants.

FIGURE 33 Inoculation on *Prunus dulcis* with *X. arboricola* pv. *pruni* following the procedure described by Randhawa and Civerolo (1985). Infiltrated sites show a collapse of the leaf tissue surrounded by a chlorotic area. Courtesy of Montserrat Roselló Pérez, LDF-Valencian Government (ES)

FIGURE 34 (a, b) Sequence of symptoms after inoculation on *P. persica* with *X. arboricola* pv. *pruni* following the procedure described by Randhawa and Civerolo (1985). Infiltrated sites show first a water-soaked appearance that continues in necrosis at the points of infiltration. The complete leaf becomes chlorotic and curls up, and finally drops. (a) Infiltrated sites show a water-soaked appearance. (b) Necrosis at the points of infiltration. The complete leaf becomes chlorotic and curls up, and finally drops. Courtesy of Montserrat Roselló Pérez, LDF-Valencian Government (ES)

For the confirmation of presumptive *X. arboricola* pv. *pruni* from almond through a pathogenicity test, a susceptible almond cultivar should be used such as

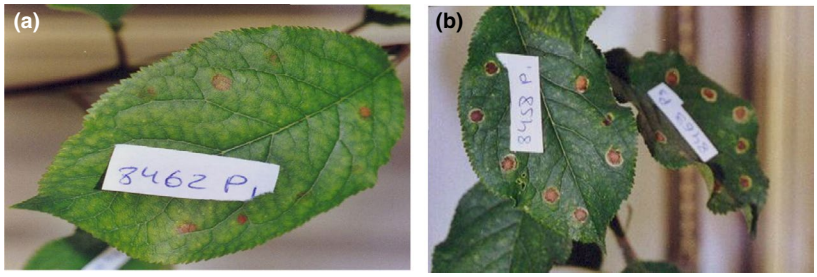

FIGURE 35 (a, b) Sequence of symptoms after the inoculation on *P. salicina* with *X. arboricola* pv. *pruni* following the procedure described by Randhawa and Civerolo (1985). Infiltrated sites show first necrosis at the points of infiltration. After several days, the necrotic tissue drops showing a shot-hole appearance. (a) Necrosis at the points of infiltration. (b) Necrotic tissue drops showing a shot-hole appearance. Courtesy of Montserrat Roselló Pérez, LDF-Valencian Government (ES)

cv. 'Marta', cv. 'Guara' and cv. 'Marcona'. For the confirmation of presumptive *X. arboricola* pv. *pruni* from cherry laurel, a susceptible cherry laurel variety should be used, such as cv. 'Novita' or cv. 'Rotundifolia'. For isolates of *X. arboricola* pv. *pruni* from cherry laurel, the use of other stone fruit species (e.g. cherry) is not recommended.

Section 4.3.3 provides guidance on how to proceed when the pathogenicity test gives negative results.

Detached leaf bioassay

Following the method of Randhawa and Civerolo (1985), young fully expanded leaves (e.g. third to sixth leaf from the shoot tip) are detached from peach seedlings cv. 'Sunhigh', 'Red Haven, 'Suncrest', 'Angeleno', 'Fortune' or other known susceptible peach or plum cultivar (Randhawa & Civerolo, 1985; Ritchie *et al*., 1993) or *P. laurocerasus* cv 'Novita' or cv 'Rotundifolia' grown in the glasshouse. The leaves are briefly washed under running tap water to remove dirt and disinfected for 40–60 s with 70% ethanol. They are rinsed repeatedly in sterile water and immediately used for inoculation. Bacterial suspension of 10^7 cfu/mL is prepared. Leaves or parts of them, abaxial side upward, are placed on several layers of sterile blotter. Inoculum is infiltrated by using a syringe without needle and by applying gentle and steady pressure while holding the open end of the syringe against the leaf until a 2- to 4-mm diameter area of mesophyll tissue is water-soaked. Sic sites on each leaf are inoculated approximately 1 cm apart. The leaves are lightly blotted to remove any excess of inoculum. In the same way, negative controls are prepared using sterile water (instead of bacterial suspension), and positive controls using a suspension of a known strain of *X. arboricola* pv. *pruni* at 10^7 cfu/mL. All inoculated leaves (test sample, negative control, positive control) are placed on 0.5% water agar and incubated for 2 weeks at 25°C under fluorescent light (60–75 $\mu E \times s^{-1} \times m^{-2}$) timed to a 16-h photoperiod. It is recommended to use a minimum of 5 leaves for each isolate to be tested, plus an additional 5 + 5 leaves for positive and negative controls.

The test is positive if after 6–9 days inoculated sites exhibit confluent water soaking, becoming dark brown and brittle necrotic spots often surrounded by a greyish white or purple margin. Bacterial ooze occurs frequently on older lesions. The maximum time period for the observation is 15 days.

Inoculation of whole plants with *X. arboricola* pv. *pruni*

One-year micropropagated plants or one-year grafted rootstocks of known susceptible peach or plum cultivars or rootstocks (peach cvs. 'Barrier', 'Catherine', 'Parade', 'Royal Glory' 'Rich Lady' or Sunhigh; plum cvs. 'Angeleno', 'Black Star', 'Black Amber', 'TC Sun', 'Golden Plum', 'Fortune,' 'Anne Gold') can be inoculated by two methods. Following Randhawa and Civerolo (1985), young leaves on young shoots are inoculated using a plastic syringe without a needle, applying gentle and steady pressure while holding the open end of the syringe against the leaf until the mesophyll tissue is water-soaked. Following Du Plessis (1988), plants are maintained at 25–27°C and 95–100% relative humidity for 8 h before inoculation and the first young but fully expanded leaves from the tip of the shoots are spray-inoculated on the abaxial side with a spray gun connected to a compressed air supply. Both protocols use bacterial suspensions of 10^7 cfu/mL. In the same way, negative controls are prepared using sterile water, and positive controls using a suspension of a known strain of *X. arboricola* pv. *pruni* at 10^7 cfu/mL. The plants should be maintained under glasshouse conditions at about 25°C and high humidity. Lesions can be recorded 1–4 weeks after inoculation. Symptoms are as follows: for syringe inoculated leaves, inoculation sites develop confluent water soaking, becoming dark brown and turning into necrotic spots, often surrounded by a greyish white or purple margin; for spray-inoculated leaves, small water-soaked spots develop, frequently with angular shape, later necrotizing (see Figures 33–35). *X. arboricola* pv. *pruni*-like colonies should be re-isolated from inoculated material showing typical symptoms and their identity confirmed.