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EPPO STANDARD ON DIAGNOSTICS

PM 7/24 (5) *Xylella fastidiosa*

Specific scope: This Standard describes a Diagnostic Protocol for *Xylella fastidiosa*.[1]
It should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: First approved in 2004–09.
Revised in 2016–09, 2018–04, 2019–05 and 2023–02. The revisions of this protocol have been prepared based on the outcome of different EU funded projects (XF-ACTORS, PONTE) as well as Euphresco projects.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Xylella fastidiosa causes many important plant diseases worldwide such as Pierce's disease (PD) of grapevine, phony peach disease, plum leaf scald, citrus variegated chlorosis disease, olive scorch disease, as well as leaf scorch on almond and on shade trees in urban landscapes, for example *Ulmus* spp. (elm), *Quercus* spp. (oak), *Platanus sycamore* (American sycamore), *Morus* spp. (mulberry) and *Acer* spp. (maple). Based on current knowledge, *X. fastidiosa* occurs primarily on the American continent (Almeida & Nunney, 2015). *Xylella taiwanensis* (Su et al., 2016) is a distant relative which was found in Taiwan on nashi pears (Leu & Su, 1993). However, *X. fastidiosa* has also been confirmed on grapevine in Taiwan (Su et al., 2014). The presence of *X. fastidiosa* on almond and grapevine in Iran (Amanifar et al., 2014) was reported (based on isolation and pathogenicity tests), but so far strain(s) are not available. The reports from Turkey (EPPO, 2016; Guldur et al., 2005), Lebanon (Habib et al., 2016; Temsah et al., 2015) and Kosovo (Berisha et al., 1998; EPPO, 1998) are unconfirmed and are considered invalid. Since 2012, different European countries have reported interception of infected coffee plants from Latin America (Mexico, Ecuador, Costa Rica and Honduras; Bergsma-Vlami et al., 2015; Jacques et al., 2016; Legendre et al., 2014). The outbreak of *X. fastidiosa* in Italy (EPPO, 2019; Martelli et al., 2016; Saponari et al., 2013) and the common presence of the bacterium in plant species in several Mediterranean countries constituted an important change to its geographical distribution and also added new host plants. More information on the geographical distribution of *Xylella fastidiosa* is available from EPPO Global Database. The EFSA database (EFSA, 2023) includes 679 plant species reported to be infected by *X. fastidiosa*, for 423 of which the infection has been determined with at least two different detection tests or gave positive results with sequencing or isolation. These species cover hundreds of host plant genera in 88 botanical families (68 botanical families when considering only records with at least two different detection methods or which gave positive results with sequencing or isolation). The list of hosts in Europe is regularly updated with the results of surveys.

Xylella fastidiosa is a member of the family Lysobacteraceae (formerly Xanthomonadaceae) of the Gammaproteobacteria. The genus *Xylella* contains two species, *X. fastidiosa* and *X. taiwanensis*. There are three accepted subspecies of *X. fastidiosa*, i.e. *fastidiosa*, *pauca* and *multiplex* (Schaad et al., 2004), based on DNA–DNA hybridization data, although only two, *fastidiosa* and *multiplex*, are so far considered valid names by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB; Bull et al., 2012). Since that publication, several additional *X. fastidiosa* subspecies have been proposed based on multilocus sequence typing (MLST) analysis (Scally et al., 2005; Yuan et al., 2010), including subsp. *sandyi* (on *N. oleander*; Schuenzel et al., 2005), subsp. *tashke* (on *Chitalpa tashkentensis*; Randall et al., 2009) and subsp. *morus* (on mulberry; Nunney, Ortiz, et al., 2014, Nunney, Schuenzel, et al., 2014). However, based on comparative genomic analyses, strains of *X. fastidiosa* can be differentiated into three main subspecies: *fastidiosa*, *multiplex* and *pauca* (Denancé et al., 2019; Marceletti & Scortichini, 2016).

The bacterium colonizes two distinct habitats, i.e. the xylem network of plants and the foregut of insects belonging to the order Hemiptera, sub-order Auchenorrhyncha (Redak et al., 2004), that feed on xylem fluid (Chatterjee et al., 2008). Transmission of *X. fastidiosa* by insects does not require an incubation period in the vector and the bacterium is persistently transmitted (Almeida et al., 2005). Both nymphs and adults can acquire the bacterium by feeding on the xylem fluid of an infected plant and

[1]The use of names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable. Temperatures given for refrigeration, freezing, growth chambers etc. are usually approximate.

transmit the pathogen to healthy plants immediately after acquisition. *Xylella* cells are typically organized as single layer biofilm in the foregut, cibarium and precibarium (Backus & Morgan, 2011; Newman et al., 2003) and do not systemically colonize the insect body. Nymphs lose infectivity with every stage as the foregut is renewed with moulting. Newly emerged adults must feed on an infected plant to become infectious. The bacterium is not transmitted transovarially to the progeny of the vector (Freitag, 1951). However, once infected an insect can transmit the pathogen during its entire lifetime (Almeida et al., 2005). Winged adults are the major means for spread.

Flow diagrams describing the diagnostic procedure for *X. fastidiosa* are presented in Figures 1 and 2.

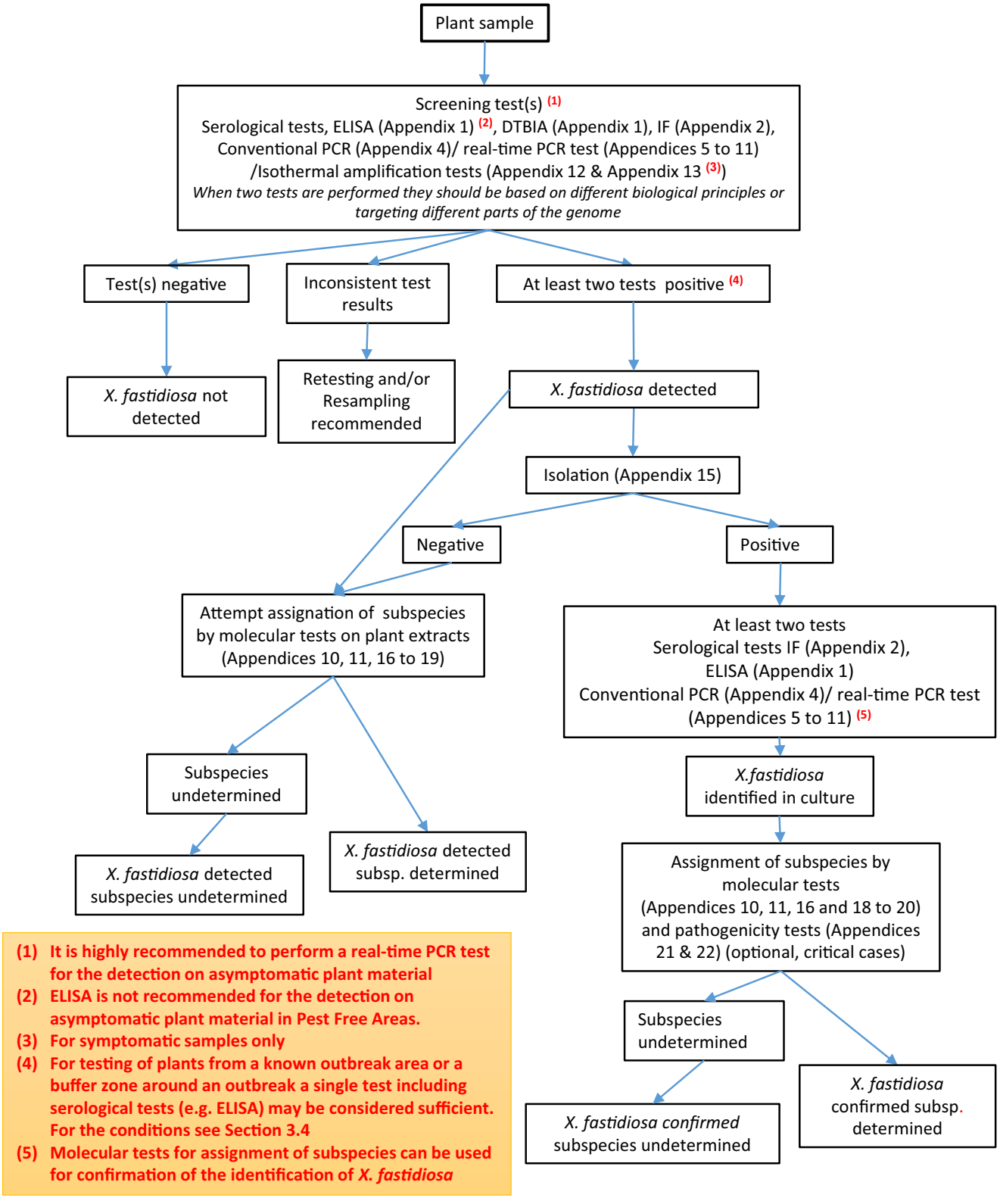

FIGURE 1 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* on plant material.

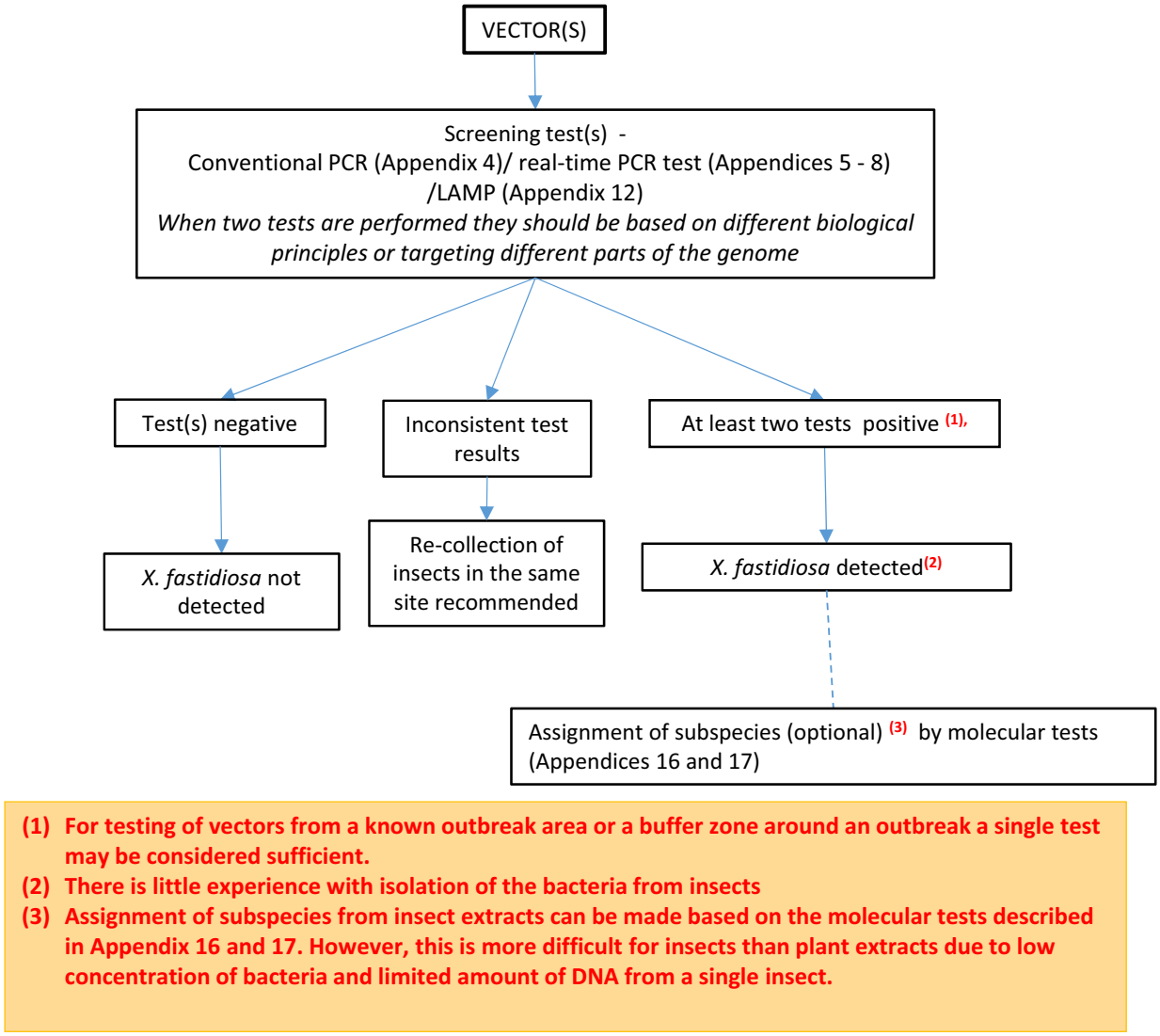

FIGURE 2 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* on vectors.

2 | IDENTITY

Name: *Xylella fastidiosa* Wells et al. (1987)
Taxonomic position: Bacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae.
EPPO Code: XYLEFA
Phytosanitary categorization: EPPO A2 List no. 166; EU Annex designation I/AII as *Xylella fastidiosa*, IIAI as citrus variegated chlorosis and IVAI as peach phony rickettsia.

Note on nomenclature. In Annex 25 of ISPM 27 (IPPC, 2018) the taxonomic position is referred to as Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae.

3 | DETECTION

As stated in the Introduction over 600 plant species are host to *X. fastidiosa*. However, the bacterium does not appear to cause disease in many of these plant species. Colonization in many hosts is frequently asymptomatic for a long time after inoculation and does not necessarily result in the development of disease (however, these plants can serve as a source of inoculum). There are also significant differences in susceptibility between hosts.

3.1 | Disease symptoms

Symptoms depend on the combination of host plant and *X. fastidiosa* strain. As the bacterium invades xylem vessels it blocks the movement of water and mineral nutrients. Generally, symptoms include leaf scorching, wilting of the foliage, defoliation, chlorosis or bronzing along the leaf margin and dwarfing. Bacterial infections can be so severe as to lead to the death of the infected plants. The bronzing may intensify before browning and drying (Janse & Obradovic, 2010). Symptoms usually appear on just a few branches but later spread to cover

the entire plant. Depending on the plant species or cultivar, the presence of yellow spots on leaves, chlorotic foliage, often together with pronounced yellow discoloration between healthy and necrotic tissues, irregular lignification of bark, stunting, premature leaf drop, reduction of production and dimension of fruits, fruit distortion, crown dieback or a combination of symptoms may occur. Symptoms can be confused with those caused by other biotic or abiotic factors (other pathogens, environmental stresses such as water deficiencies, salt, air pollutants, nutritional problems, sunburn etc.); illustrations of possible confusions can be downloaded following this link[2].

Symptoms on various hosts can be seen at https://gd.eppo.int/taxon/XYLEFA/photos as well as on https://www.ponteproject.eu/category/symptom-xylella/. Symptoms of diseases associated with *X. fastidiosa* in Europe and in the Americas are presented below (in alphabetical order of disease name).

3.1.1 | Alfalfa dwarf

The main symptom is stunted regrowth after cutting. This stunting may not be apparent for many months after initial infection. Leaflets on affected plants are smaller and often slightly darker (often with a bluish colour) compared with uninfected plants, but are not distorted, cupped, mottled or yellow. The taproot is of normal size, but the wood has an abnormally yellowish colour, with fine dark streaks of dead tissue scattered throughout. In recently infected plants the yellowing is mostly in a ring beginning under the bark, with a normal, white-coloured cylinder of tissue inside the yellowed outer layer of wood. Unlike in bacterial wilt caused by *Clavibacter insidiosus*, the inner bark is not discoloured, nor do large brown or yellow patches appear. Dwarf disease progressively worsens over 1–2 years after the first symptoms and eventually kills infected plants. Noticeable dwarfing requires 6–9 months after inoculation in the greenhouse, probably longer in the field (http://alfalfa.ucdavis.edu).

3.1.2 | Almond leaf scorch

The most characteristic symptom associated with *X. fastidiosa* on almond is leaf scorching followed by decreased productivity and general tree decline. Usually, a narrow band of yellow (chlorotic) tissue develops between the brown necrotic tissue and the green tissues of the leaves; however, when the sudden appearance of leaf scorch symptoms is prompted by hot weather the narrow chlorotic band may not develop. As the disease progresses, affected twigs on branches die back from the tip (Mircetich et al., 1976). Even highly susceptible varieties take many years to die, but nut production is severely reduced within a few years in most varieties.

FIGURE 3 Leaf scorch symptoms on almond. Courtesy D. Boscia, CNR – Institute for Sustainable Plant Protection (IT).

Leaf scorching symptoms have been also reported on almond in late summer/autumn in Italy (Figure 3).

3.1.3 | Bacterial leaf scorch of blueberry

The first symptom caused by the bacterium in blueberry is marginal leaf scorching (Figure 4). The scorched leaf area may be bordered by a darker band (Brannen et al., 2016). In the early stages of disease progression, symptoms may be localized, but over time symptoms can become uniformly distributed throughout the foliage. Newly developed shoots can be abnormally thin with a reduced number of flower buds. Leaf drop occurs, and twigs and stems have a distinct 'skeletal' yellow appearance (Figure 5). Following leaf drop the plant

FIGURE 4 Scorch symptoms of blueberry with distinct leaf burn surrounded by a dark line of demarcation between green and dead tissue. Courtesy P. M. Brennan, University of Georgia (US).

[2]https://agriculture.gouv.fr/telecharger/85855?token=9f22e2e6c496c32d8195cb9e164470bde14d654153cdc47f57cf04094ff14b4f

FIGURE 5 Infected blueberry plants with yellow stems and a 'skeletal' appearance. Courtesy P. M. Brennan, University of Georgia (US).

dies, typically during the second year after symptoms are observed (Chang et al., 2009).

3.1.4 | Bacterial leaf scorch of shade trees

Symptoms of bacterial leaf scorch are similar on different tree hosts such as *Acer* spp., *Cornus florida*, *Celtis occidentalis*, *Liquidambar styraciflua*, *Morus alba*, *Platanus* spp., *Quercus* spp. and *Ulmus americana* (Gould & Lashomb, 2007). In most cases the disease is identified by a characteristic marginal leaf scorch where affected leaves have marginal necrosis and may be surrounded by a chlorotic (yellow) or red halo. Generally, symptoms progress from older to younger leaves, and as the disease progresses branches die and the tree declines. Symptoms first appear in late summer to early autumn. Some plant species may be killed by the disease. More information and pictures of symptoms can be found in Gould and Lashomb (2007), which is available online.

3.1.5 | Citrus variegated chlorosis

The first symptoms of citrus variegated chlorosis to appear on leaves are small chlorotic spots on the upper surface that correspond to small gummy brown spots on the underside of the leaf. Symptoms are most obvious on developed leaves independently of plant age and mainly on sweet orange cultivars (Figures 6 and 7).

Affected trees show foliar interveinal chlorosis on the upper surface that resembles zinc deficiency.

FIGURE 6 Citrus variegated chlorosis (CVC): typical spots caused on sweet orange leaves. Courtesy M. Scortichini, Istituto Sperimentale per la Frutticoltura, Rome (IT).

FIGURE 7 Small raised lesions appear on the underside of the citrus leaves. © USDA and University of Florida (US).

FIGURE 8 Citrus variegated chlorosis (CVC): fruits are smaller and mature earlier (left side) than fruits from healthy trees (right side). Small, raised lesions appear on the underside of leaves. Courtesy M. M. Lopez, Instituto Valenciano de Investigaciones Agrarias, Valencia (ES).

Sectoring of symptoms in the canopy occurs on newly infected trees. However, citrus variegated chlorosis generally develops throughout the entire canopy on old, infected trees. Affected trees are stunted and the canopy has a thin appearance because of defoliation and dieback of twigs and branches. Blossom and fruit set occur at the same time on healthy and affected trees, but normal fruit thinning does not occur on affected trees and the fruits remain small (Figure 8), have a hard ring and ripen earlier. The plants do not usually die, but the yield and quality of the fruit are severely reduced (Donadio & Moreira, 1998). On affected trees of cv. Pera and other orange cultivars, fruits often occur in clusters of 4–10, resembling grape clusters. The growth rate of affected trees is greatly reduced, and twigs and branches may wilt. Trees in nurseries can show symptoms of variegated chlorosis, as do trees aged over 10 years. Young trees (1–3 years) become systemically colonized by *X. fastidiosa* faster than older trees. Trees more than 8–10 years old are usually not totally affected, but rather have symptoms on the extremities of branches.

3.1.6 | Coffee leaf scorch

Symptoms of coffee leaf scorch appear on new growth of field plants as large marginal and apical scorched areas on recently developed leaves (Figure 9). Affected leaves drop prematurely, shoot growth is stunted and apical leaves are small and chlorotic. Symptoms may progress to shoot dieback. Infection of coffee plants by *X. fastidiosa* can also lead to the 'crespera' disease which was reported from Costa Rica (Figure 10). Symptoms range from mild to severe curling of leaf margins, chlorosis and deformation of leaves, asymmetry (see Figure 10), stunting of plants and shortening of internodes (Montero-Astúa et al., 2008).

FIGURE 9 Leaf scorch symptoms on *Coffea* sp. Courtesy M. Bergsma-Vlami, NPPO (NL).

FIGURE 10 'Crespera' symptoms on *Coffea* sp. including curling of leaf margins, chlorosis and deformation (asymmetry). Courtesy M. Bergsma-Vlami, NPPO (NL).

3.1.7 | Olive leaf scorching and quick decline

Xylella fastidiosa infections in olive were first reported by Krugner et al. (2014) in trees exhibiting leaf scorch or branch dieback symptoms in California (US), where infections were found to be associated with *X. fastidiosa* subsp. *multiplex*. However, a poor correlation was found

FIGURE 11 Symptoms of olive quick decline syndrome. Courtesy D. Boscia, CNR – Institute for Sustainable Plant Protection (IT).

FIGURE 12 Symptoms of olive quick decline syndrome. Courtesy D. Boscia, CNR – Institute for Sustainable Plant Protection (IT).

between the symptoms and the presence of *X. fastidiosa*. Since then, *X. fastidiosa* subsp. *multiplex* has also been detected in olive in Spain.

More recently a new olive disorder, consisting of olive plants showing leaf scorching and desiccated branches (including partial defoliation and shoot death) and associated with the presence of *X. fastidiosa*, has been reported in Southern Italy (Giampetruzzi et al., 2015; Saponari et al., 2013), Argentina (Haelterman et al., 2015) and Brazil (Coletta-Filho et al., 2016). The *X. fastidiosa* strains in all these cases have been assigned to the subspecies *pauca* (Saponari et al., 2017).

In southern Italy, this new olive disorder has been termed 'olive quick decline syndrome'. Olive quick decline syndrome is characterized by leaf scorching and scattered desiccation of twigs and small branches which, in the early stages of the infection, are mainly observed on the upper part of the canopy. Leaf tips and margins turn dark yellow to brown, eventually leading to desiccation (Figure 11). Over time, symptoms become increasingly severe and extend to the rest of the crown, which acquires a blighted appearance (Figure 12). Desiccated leaves and mummified drupes remain attached to the shoots. Trunks, branches and twigs viewed in cross-section show irregular discoloration of the vascular elements, sapwood and vascular cambium (Nigro et al., 2013). Rapid dieback of shoots, twigs and branches may be followed by death of the entire tree. *Xylella fastidiosa* has also been detected in young olive trees with leaf scorching and quick decline.

There are limited data on *X. fastidiosa* infecting olive, but evidence indicates that different subspecies can infect olive (subspecies *pauca* and *multiplex*). While *X. fastidiosa* is associated with but does not cause disease in olive in the USA (Krugner et al., 2014), Koch's postulates have been fulfilled in Italy (Saponari et al., 2016); pathogenicity data are not available from Brazil, Argentina, or Spain. Nonetheless, a strong correlation between leaf scorching symptoms and the presence of *X. fastidiosa* has been observed in three distant regions around the world (southern Italy, Spain, Argentina and Brazil) (Coletta-Filho et al., 2016; Landa, 2017).

3.1.8 | Pierce's disease of grapes

On grapevine, the most characteristic symptom of primary infection is leaf scorch. An early sign of infection is a sudden drying of part of a green leaf, which then turns brown while adjacent tissues turn yellow or red (see Figure 13). The leaf symptoms can be confused with fungal diseases, in particular with Rotbrenner, a fungal disease of grapevines caused by *Pseudopezicula tracheiphila* (Figure 14). The desiccation spreads over the whole leaf causing it to shrivel and drop, leaving only the petiole attached (Figure 15). Diseased stems often

FIGURE 13 Yellowing and desiccation of grapevine leaves and wilting of bunches in the Napa Valley, California (US). Courtesy ENSA – Montpellier (FR).

FIGURE 14 Symptoms in grapevine leaves caused by *Pseudopezicula tracheiphila*. Courtesy H. Reisenzein, AGES (AT).

FIGURE 15 Pierce's disease of grapevine. Persistent petioles. Courtesy J. Clark and A. H. Purcell, University of California, Berkeley (US).

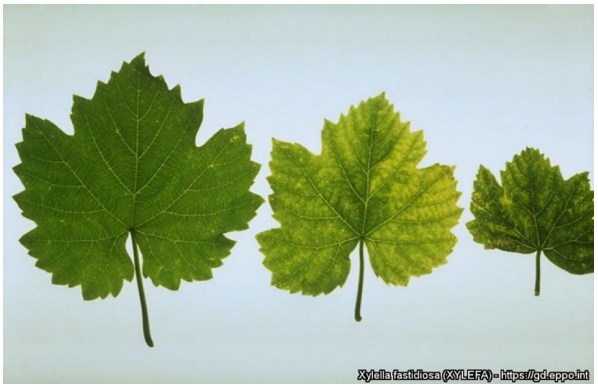

FIGURE 16 Pierce's disease of grapevine. Spring symptoms in cultivar Chardonnay (healthy leaf on the left). Courtesy A. H. Purcell, University of California, Berkeley (US).

FIGURE 17 Phony peach: typical 'phony peach' symptom on peach leaves caused by *Xylella fastidiosa*. Courtesy M. Scortichini, Instituto Sperimentale per la Frutticoltura, Rome (IT).

mature irregularly, with patches of brown and green tissue. Chronically infected plants may have small, distorted leaves with interveinal chlorosis (Figure 16) and shoots with shortened internodes. Fruit clusters shrivel. In later years, infected plants develop late and produce stunted chlorotic shoots. Symptoms involve a general loss of plant vigour followed by death of part of or the entire vine. Highly susceptible cultivars rarely survive for more than 2–3 years, although signs of recovery may be seen early in the second growing season. Young vines succumb more quickly than mature vines. More tolerant cultivars may survive chronic infection for more than 5 years.

3.1.9 | Phony peach disease and plum leaf scald

On infected peach trees, young shoots are stunted and bear greener, denser foliage than healthy trees (Figure 17). Lateral branches grow horizontally or droop, so that the tree seems uniform, compact and rounded. Leaves and flowers appear early and remain on the tree for longer than on healthy trees. Early in summer, because of shortened internodes, infected peach trees appear more compact, leafier and darker green than normal trees. Affected trees yield increasingly fewer and smaller fruits until, after 3–5 years, they become economically worthless. Fruits may also be more strongly coloured and will often ripen a few days earlier than normal. Infected peach and plum trees bloom several days earlier than healthy trees and tend to hold their leaves later into the autumn. The leaves of infected peach never display the typical leaf scorching seen on infected plum trees. Symptoms of plum leaf scald are a typical scorched and scalded appearance (Figure 18). Plum leaf scald also increases the susceptibility of the tree to other problems. Phony peach disease and plum leaf scald can limit the life of peach and plum orchards (Mizell et al., 2015).

FIGURE 18 Plum leaf scald: typical scorched symptom on plum leaf caused by *Xylella fastidiosa*. Reproduced from Mizell et al. (2015).

3.1.10 | Other hosts: Leaf scorching symptoms seen in other hosts in Europe

For a general description of symptoms see Section 3.1 above. Besides olive, *X. fastidiosa* has been detected in different hosts under natural conditions in the current European outbreak areas. Most of these findings refer to symptomatic plants, which display typical leaf scorching symptoms. A list of hosts in which *X. fastidiosa* has been

FIGURE 19 Marginal leaf scorch symptoms caused by *Xylella fastidiosa* subsp. *pauca* on oleander. Courtesy D. Boscia, CNR – Institute for Sustainable Plant Protection (IT).

FIGURE 20 Symptoms on *Polygala myrtifolia*. Courtesy B. Legendre, ANSES, Plant Health Laboratory (FR).

FIGURE 21 Infected *Polygala myrtifolia*. Courtesy B. Legendre, ANSES, Plant Health Laboratory (FR).

FIGURE 22 Leaf scorch symptoms caused by *Xylella fastidiosa* on cherry. Courtesy D. Boscia, CNR – Institute for Sustainable Plant Protection (IT).

detected in Europe is available and regularly updated at: http://ec.europa.eu/food/plant/plant_health_biosecurity/legislation/emergency_measures/xylella-fastidiosa/susceptible_en.

On oleander, necrosis typically develops on the leaf margins (Figure 19). As in olive, infections may lead to the death of infected plants.

Polygala myrtifolia is one of the major susceptible hosts in the current European outbreak. Infected plants show scorched leaves, with desiccation starting from the tip and progressing to the entire blade (see leaf tip desiccation in Figure 20). An illustration of an infected plant is given in Figure 21.

Leaf scorching symptoms have been also reported on cherry (Figure 22) in late summer/autumn in Italy.

3.2 | Sampling of plant material and sample preparation in the laboratory

3.2.1 | Sampling of plant material

3.2.1.1 | *Sampling period for symptomatic or asymptomatic plants*

The bacterial concentration in a plant depends upon environmental factors, strains and the host plant species or cultivars. In general, sampling should preferably be performed during the active growth period of the plant to maximize the likelihood of detection (Hopkins, 1981). For tropical plant species grown indoors, such as coffee plants, sampling may be performed all year round.

Experience gathered in Europe provides the following information on different host plants.

a. For *O. europaea* and *N. oleander*, observations conducted in Italy (Apulia region) indicated that:

- withering, desiccation and leaf scorching symptoms associated with *X. fastidiosa* infections are more strongly expressed in summer, although persistent during the entire year;
- in some cases, symptoms were also observed during winter, for example soon after frost periods (abiotic stress);

Nevertheless, sampling can be performed all year around with no decrease in the diagnostic sensitivity during the winter and spring seasons (evidence collected in the framework of the EU funded project XF-ACTORS). These observations are considered valid for areas with mild winters.

b. For *Polygala* spp., sampling can be performed from late spring to early autumn.
c. For deciduous plant species (e.g. *Prunus* spp.) in Italy (Apulia region) symptoms were consistently recorded, together with a detectable bacterium concentration, in leaves collected during summer. Asymptomatic leaves collected earlier in the vegetative period from the same trees tested negative whereas, as also shown in Spain (Alicante province) and more recently in Israel, in the same period detection has been possible on 1-year twigs of almond trees as well as during dormancy (Roselló, pers. comm. 2019; Zecharia et al., 2021). These observations are considered valid for areas with mild winters.
d. If necessary, dormant plants can be sampled by taking mature branches (e.g. woody cuttings), from which the xylem tissue is recovered and processed for detection of *X. fastidiosa*.

Experience in temperate areas in other parts of the world shows that in grapevine or deciduous trees, e.g. cherry and almond, that have been infected for some time, the bacterium is not detected into the new season's growth until the middle of summer, when symptoms may also become visible. For example, the most suitable time for searching for symptoms in grapevine is late summer to early autumn when weather conditions are predominantly hot and dry or when grape plants are exposed to drought stress (Galvez et al., 2010).

3.2.1.2 | *Sample collection*

This section applies to sampling in places of production and in consignments. Guidance on inspection is provided in PM 3/81 *Inspection of consignments for Xylella fastidiosa* (EPPO, 2022a) and PM 3/82 *Inspection of places of production for Xylella fastidiosa* (EPPO, 2022b). ISPM 31 (IPPC, 2008) provides useful information on the number of plants to be sampled.[3]

After samples are taken, they should be sent to the laboratory as soon as possible.

As *X. fastidiosa* is confined to the xylem tissue of its hosts, the petiole and midrib recovered from leaf samples are the best source for diagnosis as they contain a higher number of xylem vessels (Hopkins, 1981). However, other sources of tissue include small twigs and roots of peach (Aldrich et al., 1992), blueberry stem and roots (Holland et al., 2014) and citrus fruit peduncles (Rossetti et al., 1990).

Samples for the laboratory should be composed of branches/cuttings with attached leaves. The sample should include mature leaves. Young growing shoots should be avoided. Studies conducted in the EU funded project XF-ACTORS showed that in infected olive trees, the bacterium was more consistently detected in twigs than in leaves, especially when samples are collected from resistant olive cultivars (i.e. with a low bacterial population).

For small plants the entire plant can be sent to the laboratory.

For sclerotic leaves (e.g. *Coffea*) individual leaves and petioles can be sampled.

3.2.1.2.1 | *Symptomatic plants*.

The sample should consist of branches/cuttings representative of the symptoms seen on the plant(s) and containing at least 10–25 leaves depending on leaf size. Symptomatic plant material should preferably be collected from a single plant; however, a pooled sample may also be collected from several plants showing similar symptoms.

3.2.1.2.2 | *Asymptomatic plants*.

- General recommendations

For asymptomatic plants, the sample should be representative of the entire aerial part of the plant. Studies conducted in the EU funded project XF-ACTORS showed that in olive orchards, sampling in the upper part of the olive canopy is more reliable. It was also shown in this project that sampling plants along the first two boundary rows of a field is an effective method for detecting *X. fastidiosa* even in conditions of low prevalence of infection. As mentioned in 3.2.1.2 the bacterium was more consistently detected in twigs than in leaves.

For testing individual asymptomatic plants, the number of branches to be collected is at least 4–10, depending on the host and plant size.

- Sampling for testing composite plant samples

Evaluations performed in the framework of XF-ACTORS, aiming at verifying the minimum amount of tissue to be collected from a plant to get consistent and reliable detection, have provided detailed information regarding sampling procedures for many plants (Loconsole et al., 2021).

- Samples composed of small amounts of tissue

Details on sampling for testing samples composed of small amounts of tissue is presented in Table 1.

[3]ISPM 31 provides information on the number of units to be sampled, which is considered useful to determine sample sizes for both consignments and places of production.

TABLE 1 Indications both for sampling and laboratory testing of composite samples processed as individual samples (based on an extraction procedure with no bacterial concentration step).

Host species	Minimum nb of leaves/ twigs/stems per plant to be collected	Nb plants that can be pooled	Tissue to process	Volume of extraction buffer
Olea europaea (small-size plants, i.e. in nurseries/ consignment)	4 (leaves)	5	Leaf midribs or petioles or leaf basal part for each leaf	1:10 w:v
Olea europaea (trees of large size, i.e. in orchards)	4 (twigs)	7	One portion of 1.0–1.5 cm for each cutting should be excised	1:10 w:v
Nerium oleander	3 (leaves)	8	Leaf petioles for each leaf	1:10 w:v
Polygala myrtifolia	6 (twigs)	8	One portion of 1.5–2 cm for each twig should be excised	1:10 w:v
Prunus avium	4 (twigs)	5	0.1 g of xylem tissue to be recovered from each twig	1:10 w:v

- Samples composed of large amounts of tissue (e.g. composite samples from consignments/places of production of plants for planting)

Details on sampling for testing samples composed of large amount of tissue are presented in Table 2.

3.2.2 | Sample preparation in the laboratory for plant material

Samples should be processed as soon as possible after arrival at the laboratory.

If the plant samples originate from areas where infected vectors may occur, it is recommended to check whether insects are present in the sample before opening the bags. If any insects are present, samples should be stored in the refrigerator for approximately 12 h.

For isolation, samples may be kept refrigerated for up to 3 days. For other tests, samples may be stored refrigerated for up to 1 week or frozen before processing (Amsden et al., 2010).

Samples should be inspected for symptoms and, if present, symptomatic leaves (including their petioles) should be selected and processed (removing the necrotic and dead tissue). If no symptoms are noted, leaves should be representative of the entire sample received in the laboratory.

Dirty samples should be cleaned.

For isolation, samples should be surface disinfected (see Section 3.6).

3.2.2.1 | *Laboratory sample*

From the sample received, indications on the minimum amounts of plant material to be used and the approximate weight of the laboratory sample are given in Tables 1 and 2 for composite samples and Table 3 for individual plants.

The sample is processed according to the test to be used as described in this protocol (see Sections 3.4, 3.6 and 4).

3.3 | Sampling of vectors and sample preparation in the laboratory

Field-collected insects can be analysed to detect *X. fastidiosa* by molecular tests. Serological tests [enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF)] are not sensitive enough, as the bacterium only colonizes the insect foregut where, in spite of its multiplication, it is generally present at low levels (Purcell et al., 2014).

3.3.1 | Sample collection

Adult vectors should preferably be collected with sweeping nets (adults) or aspirators. Sticky traps are usually not as effective as active sampling for xylem feeders, but insects may be trapped accidentally, and specimens collected from sticky traps can be used for testing. A video on insect collection has been published by EFSA and is available at https://www.youtube.com/watch?v=Rjh7FFQCtg8. The EPPO Standard PM 7/141 Diagnostic Protocol for *Philaenus spumarius*, *Philaenus italosignus* and *Neophilaenus campestris* provides information on detection and identification of the currently known European vectors (EPPO, 2022c). A key to the species of most European Aphrophoridae and Cercopidae (except some Mediterranean species) is provided in Holzinger et al. (2003) and Biedermann and Niedringhaus (2004). Identification keys with pictures to distinguish suborder Cercopoidea from Delphacidae and Cicadellidae are available online (Purcell et al., 2014).

Vectors can be removed from the traps using small forceps/pincers and a suitable solvent such as vegetal xylene, Bio-Clear (Bio-Optica, Milano, IT), kerosene or regular fuel (Purcell et al., 2014). After removal from the traps, insects should be rinsed in ethanol/acetone (95%–99%). Traps should be serviced on a weekly basis.

Sampling for insects should preferably be done from late spring until early autumn to maximize the likelihood of detection of the bacterium.

TABLE 2 Guidance on sampling for lots of plants for selected species and tissue to be recovered when testing samples composed of large amounts of tissue[a] (e.g. composite samples from consignment/places of production of plants for planting). The extraction procedure performed includes a bacterial concentration step.

Host	Minimum no. of leaves/ twigs/stems to be collected per plant	Nb. of plants that can be pooled	Tissue to process	Volume of extraction buffer
Olea europaea[b]	4 (leaves)	Up to 225	Up to 20 g of leaf midribs/petioles/basal part can be processed as a single sample (about 800–900 leaf petioles), corresponding to up to 200–225[e] sampled plants	1:3 w:v
Nerium oleander	2 (leaves)	Up to 100	Up to 20 g of leaf petioles can be processed as a single sample (about 200 leaf petioles), corresponding to up to 100 sampled plants	1:3 w:v
Herbaceous plantlets	1 (plantlet)	Up to 200	Up to 40 g of stems/plants (pieces of 1.5–2 cm) can be processed as a single sample (this amount can be obtained by pooling the stem portions from approximately 200 sampled plants)	1:1 w:v or 1:1.5 w:v
Polygala myrtifolia[c]	2 (twigs)	Up to 125	Up to 20 g of twigs (pieces of 1.5–2 cm from each twig) can be processed as a single sample (this amount can be obtained by pooling pieces of twigs from approximately 125 sampled plants)	1:3 w:v
Lavandula spp.[d]	2 (stems)	Up to 100	Up to 20 g of stems (pieces of 2.5–3 cm from each stem) can be processed as a single sample (this amount can be obtained by pooling pieces of shoots from approximately 90–100[e] sampled plants)	1:3 w:v
Prunus dulcis/*Prunus avium*	2 (twigs)	Up to 100	Up to 20 g of xylem tissue recovered from each shoot corresponding to pooling pieces of twig from approximately 100 sampled plants	1:3 w:v
Coffea spp.	2 (leaves)	Up to 50	Up to 10 g of leaf midribs or petioles or leaf basal part (per sample or sub sample)	1:4 w:v
Helichrysum italicum	2 (stems)	Up to 50	5 g (2 pieces of stem 2–3 cm per plant)	1:3 w:v

[a]The indications contained in this table are based on the data published by Bergsma-Vlami et al. (2017), for coffee; ANSES, FR for *Helichrysum italicum*; National Institute of Biology, SI; and Loconsole et al. (2018) for other plants. Validation data is available in the EPPO Diagnostic Database.

[b]When sampling plants from a lot, at least four leaves per plant should be collected.

[c]Tests performed on leaves repeatedly failed to detect the bacterium.

[d]Leaves should be removed either by detaching them from the stem or by cutting out the leaf blade.

[e]The range is calculated based on minimum and maximum weight of the plant portions (petioles/midribs or stem pieces).

TABLE 3 Number of leaves (including their petioles) or other plant material to be used and approximate weight of the laboratory sample when testing individual plants.

Type of sample	Host plants/type of tissue	Minimum no. of leaves per laboratory sample	Approximate weight of the laboratory sample (g)
Samples from plants with large leaves such as *Coffea* spp., *Ficus* spp., *Vitis* spp., *Nerium oleander* (symptomatic or asymptomatic)	Basal parts of leaves	5	0.5–1
Samples from plant species with small leaves such as *Polygala myrtifolia* and *Olea* spp. (symptomatic or asymptomatic)	Basal parts of leaves	25	0.5–1
Samples from plants species without petioles or with small petioles and midribs (symptomatic or asymptomatic)	Basal parts of leaves and midrib	25	0.5–1
Dormant plants or dormant cuttings	Xylem tissue[a]	N.A.	0.5–1
Other cuttings	Stem	N.A.	1

[a]The superficial bark should be removed, and scrapings (see Figure 23) taken from the active tissues (youngest external ring).

FIGURE 23 Procedure to recover xylem tissue. (a) Initial twig; (b) Twig with bark removed; (c) scraping of the active tissue; (d) scrapings. Courtesy CNR, IT.

If insects cannot be processed immediately, they should be stored in 95%–99% ethanol or at approximately −20°C or −80°C. Sticky traps can also be stored at −20°C.

3.3.2 | Sample preparation in the laboratory for vectors

Since *X. fastidiosa* only colonizes the foregut and does not systemically spread into the body, only the head of the insect should be used for DNA extraction, thus avoiding the extraction of several contaminants that may inhibit the enzymatic reactions (Purcell et al., 2014). In the previous version of this Diagnostic Protocol a recommendation was made to remove the eyes as it could affect PCR sensitivity. An interlaboratory comparison was performed in 2017 where PCR tests were performed without removing the eyes of *Philaenus spumarius*. No difference in sensitivity was noted (Legendre, pers. comm. 2018). However, it is recommended that the eyes should be removed for larger vectors. (The report can be accessed at https://upload.eppo.int/download/268obc05d6355.)

Before DNA extraction, it is essential to remove the solvent (ethanol/acetone). To achieve this, the insects can be transferred for a few minutes to a dry filter paper and may be further dried in a SpeedVac centrifuge to

facilitate evaporation of the solvent. Total DNA can be extracted from single (or pooled) insect heads depending on the procedures (Appendix 3). Experience in Italy on *Philaenus spumarius* shows that up to five insects can be pooled to perform one test and experiments conducted in France have shown that up to 10 *Philaenus spumarius* can be pooled for detection of *X. fastidiosa* (Cunty et al., 2020).

For the loop-mediated isothermal amplification (LAMP) test, single captured insects are used (see Appendix 12).

3.4 | Screening tests

Unlike in other EPPO Protocols for bacteria, isolation is not recommended as a screening test because the bacterium in question is very difficult to isolate (see Figures 1 and 2).

Samples (including vectors) should be considered as 'samples with *X. fastidiosa* detected' when at least two screening tests are positive based on different biological principles or targeting different parts of the genome. Subspecies assignment by the molecular tests included in Section 4.2 and/or sequencing analysis should then be performed. Isolation should also be attempted. For areas where the pest is known to be present or in buffer zones (see below) one positive test is sufficient to consider a sample as 'sample with *X. fastidiosa* detected'. In the case of conflicting results between two tests, retesting and/or resampling are recommended.

- Symptomatic plant material

Serological and molecular tests are both suitable for screening of symptomatic plant material.

- Asymptomatic plant material

  - Testing asymptomatic plants in a pest-free area

Experimental data on testing asymptomatic olive plant material has been produced during the EU project XF-ACTORS (Section 3.2.1.2.2).

For other hosts, the recommendations given in this Protocol are derived from data on testing symptomatic material and test performance studies. In most situations, the concentration of *X. fastidiosa* in asymptomatic plant material is likely to be lower than in symptomatic plant material (Almeida & Nunney, 2015; Purcell & Saunders, 1999). Consequently, molecular test(s) should be performed for detection on asymptomatic plant material and ELISA is not recommended.

  - Testing asymptomatic plants in other areas

Testing for asymptomatic plants in an outbreak area or a buffer zone around an outbreak often implies that a large number of tests need to be performed. In such a situation, and given that the concentration of the bacterium is expected to be higher than in an area thought to be pest free, a single test including serological tests (e.g. ELISA) may be performed. When ELISA is used, it is recommended to check 5% of the negative samples using a molecular test. In certain cases (e.g for a new plant species, or to check the subspecies), when ELISA is positive for asymptomatic samples in a buffer zone or outbreak area, a second test needs to be performed to confirm, or provide more information on the positive result of the ELISA.

- Vectors

Molecular test(s) should be performed for detection in vectors.

3.4.1 | Serological tests

Serological tests developed over the years include ELISA (Sherald & Lei, 1991), membrane entrapment immunofluorescence (MEIF; Hartung et al., 1994), dot immunobinding assay (DIBA), Western blotting (Chang et al., 1993; Lee et al., 1992) and IF (Carbajal et al., 2004).

Direct tissue blot immunoassay (DTBIA) was reported as an alternative rapid screening test for the detection of *X. fastidiosa* in olive samples (Djelouah et al., 2014). Recommended kits and performance characteristics for DTBIA are given in Appendix 1.

Instructions for performing an ELISA (including tissue print, squash or dot ELISA) are provided in the EPPO Standard PM 7/101 ELISA tests for plant pathogenic bacteria (EPPO, 2010). Recommended antisera and validation data are given in Appendix 1.

Instructions for performing an IF are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). For the IF test, it should be noted that bacterial cells of *X. fastidiosa* might not be equally distributed on the window of the IF slide because the cells remain clearly attached to the vascular system of the plant material. This should be considered when the slide is examined under the microscope (PM 7/97, point 4.1). Recommended antisera and validation data are given in Appendix 2.

3.4.2 | Molecular tests

Several molecular tests have been developed for *X. fastidiosa*. Only those that are most commonly used in the EPPO region or included in the IPPC Protocol on *X. fastidiosa* (IPPC, 2018) are described in full. Molecular tests can be performed on plants and insects. Validation data are available for testing of plants. Some of these tests have been used for detection in insects, validation data has been produced in the framework of the Euphresco PROMODE project.

Although several PCR tests have been developed that effectively detect *X. fastidiosa* DNA in purified DNA

extract, a recurrent problem with some matrices is the presence of inhibitors (Modesti et al., 2017). These effects may be overcome by adequate DNA extraction protocols and dilutions of the extract.

The procedures for extracting DNA from plants and insects are described in Appendix 3.

The tests listed in this section allow the detection of *X. fastidiosa* regardless of the subspecies (tests specific for subspecies are presented in Section 4.2).

Because of the higher analytical sensitivity of real-time PCR compared to other molecular tests, its use is highly recommended in areas where *X. fastidiosa* is not present and for asymptomatic plants.

3.4.2.1 | *Conventional PCR*

The test based on Minsavage et al. (1994) is described in Appendix 4.

3.4.2.2 | *Real-time PCR*

Several real-time PCR tests are recommended and have been validated:

- a test based on Harper et al. (2010) (and erratum 2013) is described in Appendix 5 and can be used in simplex or duplex when testing plant material and duplex (Ioos et al., 2009) when testing insects
- two tests based on Francis et al. (2006) are described in Appendix 6
- the real-time PCR test of Ouyang et al. (2013) is described in Appendix 7
- the real-time PCR test of Li et al. (2013) is described in Appendix 8
- triplex real-time PCR of Bonants et al. (2019) is described in Appendix 9
- tetraplex real-time PCR of Dupas et al. (2019) is described in Appendix 10
- real-time PCR test of Hodgetts et al. (2021) is described in Appendix 11.

It should be noted that the TaqMan version of Francis et al. (2006) does not detect some American strains of *X. fastidiosa*; limited information is available for the other tests. The real-time test of Harper et al. (2010) is the most commonly used test in the EPPO region.

3.4.2.3 | *Isothermal amplification tests*

3.4.2.3.1 | *LAMP.* At the time of this revision LAMP is not widely used in the EPPO region but has been successfully used so far outside the EPPO region and in Italy to detect *X. fastidiosa* in different plant species (e.g. *Citrus* spp., *O. europaea*, *Prunus dulcis*, *Quercus rubra*, *Vitis vinifera* and *Vitis rotundifolia*) and insects using standardized extraction protocols (Harper et al., 2010, erratum 2013) or without prior extraction steps (Yaseen et al., 2015). In the EPPO region it is mainly used for the detection of *X. fastidiosa* in insects. It can also be used for plants after DNA extraction (see Appendix 3).

A test based on primers developed by Harper et al. (2010, erratum 2013) modified by Yaseen et al. (2015) is described in Appendix 12.

- Recombinase Polymerase Amplification (RPA)

A test developed for field application by Li et al. (2016) XfAmplifyRP can be used on plant extracts from symptomatic plant material and is described in Appendix 13. Currently this test is not widely used in the EPPO region but has been successfully used so far outside the EPPO region.

3.5 | Additional tests

Electron microscopy can be used to detect the bacterium in vessels in cross-sections of petioles (Cariddi et al., 2014).

3.6 | Isolation

Xylella fastidiosa is very difficult to isolate and grow in axenic culture, even from symptomatic plants. The bacterium does not grow on most common culture media and requires specific media: PD2 (Davis et al., 1980), BCYE (Wells et al., 1981) or PWG (modified after Hill & Purcell, 1995) are widely used for the isolation from different host species. Culture media are described in Appendix 14.

The use of at least two different media is recommended, in particular when isolation is attempted for new hosts or in the case of a first detection. Based on the experience of different laboratories, modified PWG is considered the best isolation medium for samples from most plants. Samples from olive plants are best isolated on BCYE.

It is very important to surface disinfect the sample to avoid growth of saprophytes because *X. fastidiosa* grows very slowly (the colonies can take up to 28 days to be visible) and can be readily overgrown by other microorganisms in the plates.

Procedures for isolation from plant material are presented in Appendix 15.

As a control, whenever possible a suspension of a *X. fastidiosa* strain (see Section 5) at a concentration of about 10^6–10^7 cfu/mL should be plated onto the same medium. Colonies are small, and depending on the subspecies the colony size is 1–1.5 mm in diameter after 1–3 weeks of incubation at approximately 28°C.

Plates should be sealed or kept in plastic bags to prevent desiccation during incubation.

- Colony morphology

The colony morphology of *X. fastidiosa* is variable (Chen et al., 2005; Davis et al., 1981). Colonies on the media recommended in this Protocol are as follows.

On all media, colonies are circular, smooth-edged and slightly convex.

On PD2 and BCYE they are opaque and whitish (Figures 24 and 25, respectively). On BCYE they contrast with the black (charcoal) medium (Figure 26).

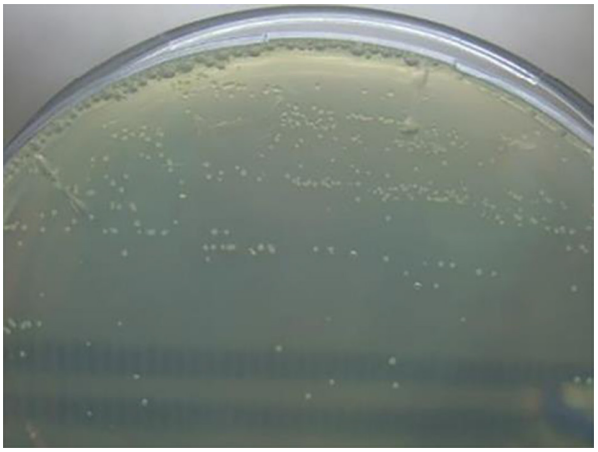

FIGURE 24 Colonies of *Xylella fastidiosa* subsp. *fastidiosa* on PD2 medium (size <2 mm after 3 weeks). Courtesy IVIA, ES.

FIGURE 25 Collection strain of *Xylella fastidiosa* subsp. *fastidiosa* ATCC 35879 on BCYE medium (size <2 mm after 3 weeks). Courtesy LSV, ANSES, FR.

On modified PWG colonies are shiny and translucent. They take the colour of the medium (light caramel) (Figures 27 and 28).

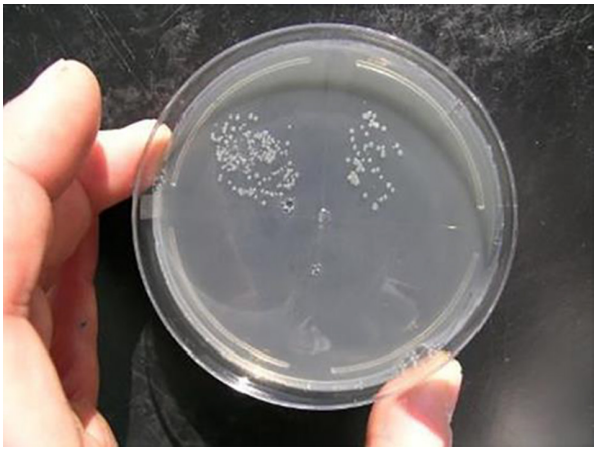

FIGURE 27 *Xylella fastidiosa*. subsp. *fastidiosa* isolated from *Coffea canephora* on modified PWG medium (size <2 mm after 3 weeks). Courtesy ANSES, FR.

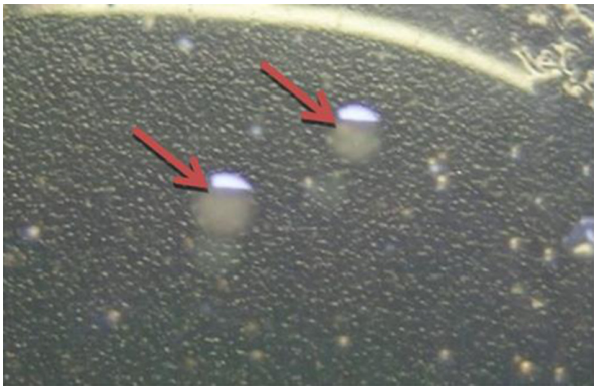

FIGURE 28 *Xylella fastidiosa* subsp. *pauca* isolated from *Coffea arabica* on modified PWG medium (size <2 mm after 3 weeks). Courtesy ANSES, FR (the background is a sheet of black paper below the plate).

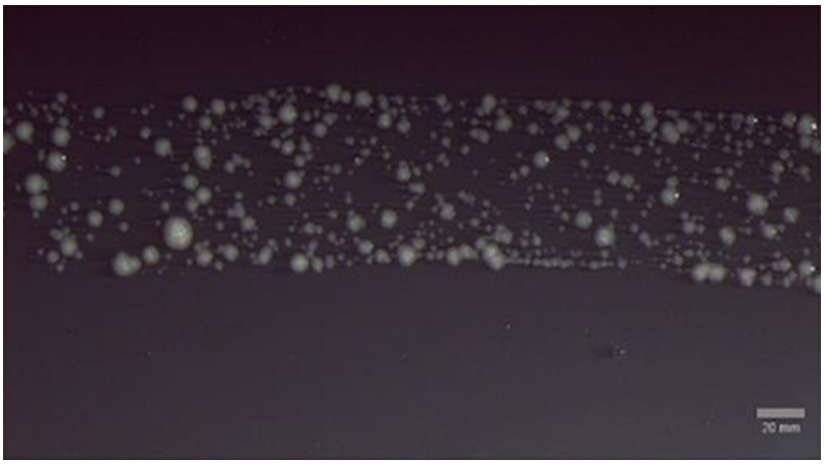

FIGURE 26 Colonies of *Xylella fastidiosa* subsp. *pauca* strain CoDiRO (ST53) on BCYE medium after 2 weeks. Courtesy M. Saponari, Institute for Sustainable Plant Protection (CNR, IT). (Other pictures of colonies are available in the EPPO Global database.)

- Cell morphology

Under dark field microscopy, the bacterium has a rod-shaped appearance with the following dimensions: 0.2–0.35 µm by 1–4 µm. Under the electron microscope, *X. fastidiosa* shows a characteristic rippled wall (Alves et al., 2009; Newman et al., 2003).

- Interpretation of isolation results

The isolation is negative if no bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed. Depending on the different subspecies, colonies can be visible after 2–3 weeks but the plates should be observed for up 6 weeks.

The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed within the above-mentioned period on at least one medium. The reference culture should also have been grown on the media used. The presumptive identification of *X. fastidiosa* colonies should be confirmed by serological or molecular tests (see Section 4.1).

4 | IDENTIFICATION AND SUBSPECIES DETERMINATION

For subspecies determination information is provided for the following subspecies *fastidiosa*, *morus*, *multiplex*, *pauca* and *sandyi*. For this fastidious pathogen, subspecies determination on plant extracts is performed after positive screening test(s) using PCR-based molecular tests described in Appendices 10, 11 and 16–19. Subspecies determination from insect(s) is also possible (Cunty et al., 2020), although it is more difficult due to difficulties of amplification (low concentration of bacteria and limited amount of DNA available from a single insect).

When a pure culture is obtained, the identification of *X. fastidiosa* should be performed using at least two tests, based on different biological principles or targeting two different parts of the genome for molecular tests. Relevant tests are described below.

4.1 | Identification of pure cultures as *X. fastidiosa*

4.1.1 | Serological tests

Serological tests can be used to identify a pure culture of *X. fastidiosa*; however, these tests do not allow the assignment of subspecies.

Instructions for performing ELISA are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010). Recommended antisera and validation data are given in Appendix 1.

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

4.1.2 | Molecular tests

The following molecular tests can be used for confirmation of a pure culture.

4.1.2.1 | *Conventional PCR*

The test based on Minsavage et al. (1994) is described in Appendix 4.

4.1.2.2 | *Real-time PCR*

The real-time PCR tests which are recommended, and which have been validated, are as follows:

- Harper et al. (2010), described in Appendix 5.
- Two tests based on Francis et al. (2006), described in Appendix 6.
- Ouyang et al. (2013), described in Appendix 7.
- Li et al. (2013), described in Appendix 8.
- Bonants et al. (2019), described in Appendix 9 (triplex real-time PCR).
- Dupas et al. (2019), described in Appendix 10 (tetraplex real-time PCR).
- Hodgetts et al. (2021), described in Appendix 11.

The latter two tests also allow subspecies assignment.

4.2 | Molecular tests for the identification of *X. fastidiosa* and assignment of *X. fastidiosa* subspecies

Although different tests are available for subspecies assignment (e.g. Dupas et al. (2019) and Hodgetts et al. (2021) on plant material see 4.1.2.2), MLST analysis is recommended for new findings (i.e. a new outbreak or new hosts). MLST is described in Appendix 16 (Yuan et al., 2010). A protocol for nested MLST is described in Appendix 17 (Cesbron et al., 2020) and is appropriate when the MLST analysis (Yuan et al., 2010) is not successful. In other cases, subspecies assignment may be performed by subspecies-specific molecular tests (Pooler & Hartung, 1995, see Appendix 18; Hernandez-Martinez et al., 2006, see Appendices 19 and 20) or Sanger sequencing. For Sanger sequencing, the PCR product of at least two housekeeping genes either *rpoD* (Minsavage et al., 1994, see Appendix 4) and *malF* (MLST analysis, see Appendix 16) or *cysG* and *malF* (MLST analysis, see Appendix 16) should be sequenced in both directions. These genes have been proven to be equivalent to MLST for the determination of subspecies (see Table

in Appendix 16). Sequencing at least a combination of two genes may allow possible recombinant strains to be detected.

Sequence data of *rpo*D and *mal*F can be analysed using the Basic Local Alignment Search Tool (BLASTN), available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov//), against the RefSeq Genome database, selecting *Xylella fastidiosa* (taxid: 2371) as the organism to search for. By selecting the 'distance tree of results' option the sequence will cluster with the *X. fastidiosa* isolates showing closerthe closest phylogenetic identity and the subspecies can be inferred.

Alternatively, a dataset containing the target sequences retrieved from reference strains of different subspecies is available (link to fasta file[4]) and can be used to align the sequences of the *rpoD* gene region amplified by the primers described by Minsavage.

CysG and *malF* sequences can be compared with data available in the pubMLST database for MLST genes (https://pubmlst.org/organisms/xylella-fastidiosa). Details on the analysis of *CysG* and *malF* sequences are available in Jacques et al. (2016).

In the case of inconsistent results for the two sequenced genes or atypical/new patterns, complete MLST analysis of the seven genes (see Appendix 16) should be performed and sequences compared with data available in the pubMLST database as indicated.

Additional information for subspecies assignment:

Another test for the assignment of subspecies *pauca* (CVC strains) is available (Li et al., 2013) but there is no experience with this test in the EPPO region.

The tests described above have primarily been developed on pure cultures but can be used on DNA from plants or insects, except for the multiplex PCR by Hernandez-Martinez et al. (2006) see Appendix 20. However, it is recognized that the quantity and quality of target DNA, or the occurrence of possible mixed infections, may prevent all amplicons from being obtained or the clear assignment of subspecies. The addition of bovine serum albumin (BSA) to the PCR mix improves the performance of the PCR (Yuan et al., 2010) for amplification of housekeeping genes. As stated before, amplification is even more difficult for insects than plant extracts due to the low concentration of bacteria and the limited amount of DNA from a single insect.

4.3 | Pathogenicity test

Verification of the pathogenicity of *X. fastidiosa* is sometimes difficult and can take several months. The pathogenicity test is described in Appendix 21.

[4]https://upload.eppo.int/download/479o8a5959753

4.4 | Bioassay

The bioassay test from Francis et al. (2008) and Pereira et al. (2017) on *Nicotiana tabacum* (tobacco) is described in Appendix 22. Pathogenicity of strains can be evaluated with *N. tabacum* ('Petite Havana SR1' or 'RP1'), but this has not been tested for all subspecies. Although comparisons of virulence among isolates from different subspecies can be difficult due to the lack of efficient protocols for inoculation and the limited host ranges of isolates, citrus variegated chlorosis strains of *X. fastidiosa* are capable of colonizing and causing leaf scorch symptoms in *N. tabacum* (Alves et al., 2003; Lopes et al., 2000), and *X. fastidiosa* isolates from almond and grape showed differences in tobacco colonization and symptomatology (Francis et al., 2008).

5 | REFERENCE MATERIAL

Reference strains are available at

CIRM-CFBP, Angers (FR)

BCCM/LMG Bacteria Collection, Ghent (BE)

NCPPB, Fera, York (GB)

EPPO-Q-bank (https://qbank.eppo.int/) includes sequences of *cysG*, *gltT*, *holC*, *leuA*, *malF*, *nuoL* and *petC* for properly documented species and strains present in collections.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CHARACTERISTICS

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

Reports of test performance studies and proficiency tests performed in the framework of XF-ACTORS, PONTE, PROMODE are available:

EU-XF-PT-2017-02 'Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*' (https://upload.eppo.int/download/217o22631f22a), molecular detection of *Xylella fastidiosa* by real-time tests (https://upload.eppo.int/download/298ocd8b7f525) and 17-XFAST-EU

'Interlaboratory test performance study (TPS) for the evaluation of molecular methods to detect *Xylella fastidiosa* in the vector *Philaenus spumarius*' (https://upload.eppo.int/download/268obc05d6355).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Anses-LSV, Unit of Bacteriology, Virology and GMO, 7 Rue Jean Dixméras, 49044 Angers Cedex 01 (FR). Contact: B. Legendre (bruno.legendre@anses.fr) or A. Cunty (amandine.cunty@anses.fr).

Institute for Sustainable Plant Protection, CNR, Via Amendola, 122/D 70126 Bari (IT). Contact: D. Boscia (donato.boscia@ipsp.cnr.it) or M. Saponari (maria.saponari@ipsp.cnr.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 regular 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 - ELISA

Instructions for performing ELISA are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010).

Tissue sources for ELISA tests can be leaves (including petioles) and twigs.

Samples can be prepared by macerating the leaves in extraction buffer (1:10, w:v) using a mortar and pestle or tissue homogenizer (e.g. Polytron, Homex). Samples can be frozen in liquid nitrogen for homogenization.

For twigs and canes, the bark is removed, and pieces of stem can be cut and minced with a razor blade and ground as described above.

Comment: It should be noted that for some host species (e.g. *Quercus*, *Platanus*) or some samples (due to the microbiota) high background signals resulting in false-positive reactions (not confirmed with molecular tests) can occur. In some cases, surface sterilization of the samples may help to overcome this problem.

The report EU-XF-PT-2017-02 'Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*' is available at https://upload.eppo.int/download/217o22631f22a.

1. *Double antibody sandwich (DAS)-ELISA test*

Kits for serological detection of *X. fastidiosa* can be supplied by different companies.

- The ELISA kits from Agritest and Loewe have been validated for olives, oleander, almond, citrus, oak, grape and other species (i.e. weeds; Loconsole et al., 2014 and data from the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests').

Analytical sensitivity

Test performance studies have been conducted with different hosts and preparation of spiked samples or artificially infected samples. Analytical sensitivity for both Agritest and Loewe varied between 10^4 and 10^5

For more details see the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests'.

Note*:* Loewe indicates an analytical sensitivity with pure type strain culture DSMZ10026 of 10^4 with inactivated cells and 10^3 with fresh cells from a plate.

Analytical specificity

Data from Loewe. No cross-reaction noted with:

Bacteria: 2 *Clavibacter michiganensis* subsp. *sepedonicus* and *Clavibacter michiganensis* subsp. *michiganensis*, 2 *Erwinia* spp., 2 *Pseudomonas* spp., *Ralstonia solanacearum*, 2 *Xanthomonas* spp., *Xylophilus ampelinus*.

Fungi: *Alternaria alternata*, *Botrytis cinerea*, *Pythium paroecandrum*, *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium albo-atrum*.

Agritest: data provided by Research Centre for Plant Protection and Certification (CREA-DC, Rome, IT)

Analytical specificity evaluated on 34 non-target bacterial strains.

No cross-reaction found with the following plant pathogens:

Bacteria: 2 *Agrobacterium tumefaciens* biovar 1, 1 *A. tumefaciens* biovar 2, 2 *Agrobacterium vitis*; 1 *Brenneria populi*, 1 *Brenneria quercina*, 1 *Brenneria rubrifaciens*; 1 *Burkholderia andropogonis*; 1 *Clavibacter michiganensis* subsp. *michiganensis*; 1 *Erwinia amylovora*; 1 *Pantoea agglomerans*, 2 *Pantoea stewartii* subsp. *stewartii*; 1 *Pseudomonas amygdali*, 2 *Pseudomonas marginalis* pv. *marginalis*, 1 *Pseudomonas savastanoi* pv. *savastanoi*, 2 *Pseudomonas syringae* pv. *syringae*, 1 *P. syringae* pv. *garcae*; 1 *Ralstonia solanacearum*; 1 *Xanthomonas arboricola* pv. *celebensis*, 1 *X. arboricola* pv. *corylina*, 2 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *pruni*; 1 *Xanthomonas campestris* pv. *citri*, 1 *X. campestris* pv. *populi*, 1 *X. campestris* pv. *vesicatoria*, 1 *X. campestris* pv. *viticola*, 2 *Xanthomonas hortorum* pv. *pelargonii*.

Diagnostic sensitivity: Institute for Sustainable Plant Protection (Bari, IT)

100% (with naturally infected samples).

Diagnostic specificity: Institute for Sustainable Plant Protection (Bari, IT)

100% (with naturally infected samples).

- ELISA kit from Agdia (data from Agdia)

Analytical sensitivity not yet available (evaluation in progress)

Analytical specificity

Cross-reaction noted with:

P. syringae pv. *syringae*, *X. arboricola* pv. *pruni*.

No cross-reaction noted with:

Bacteria: 2 *Acidovorax* spp., 2 *Agrobacterium* spp., *Burkholderia glumae*, 5 *Clavibacter michiganensis* pathovars, corn stunt spiroplasma, *Curtobacterium flaccumfaciens* subsp. *poinsettiae*, *Dickeya chrysanthemi*, 2 *Erwinia* spp., 2 *Pantoea* spp., 2 *Pectobacterium* spp., 4 *Pseudomonas* spp., *Ralstonia solanacearum*, *Rhizobium radiobacter*, *Rhizobium rhizogenes*, *Spiroplasma citri*, *Stenotrophomonas maltophilia*, *Xanthomonas albilineans*, 15 *Xanthomonas* spp.

Fungi: 1 *Phytophthora* sp., *Pythium ultimum*.

Data from ANSES (FR):
Analytical specificity
Inclusivity evaluated on 15 target strains: 100% (tested in duplicate)
Exclusivity evaluated on 26 non-target organisms: 100%
No cross-reaction found with the following plant pathogens:
Bacteria: 1 *Pseudomonas syringae* pv. *syringae*, 1 *P. syringae* pv. *morsprunorum*, *Pseudomonas syringae* pv. *persica*, 1 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 1 *Xanthomonas hortorum* pv. *pelargonii*, 1 *X. hortorum* pv. *hederae*, 1 *Xanthomonas citri*, 1 *Xanthomonas axonopodis* pv. *viticola*, *X. axonopodis* pv. *aurantifolia*, *Xanthomonas translucens* pv. *graminis*, *Xylophilus ampelinus*, 3 saprophytes of *Polygala myrtifolia*, 3 saprophytes of *Cistus monspeliensis*, 3 saprophytes de *Calicotome villosa*, 3 saprophytes of *Helichrysum italicum*, *Vitis* sp. sample infected with phytoplasma: 1 Bois noir, 1 Flavescence dorée

Test performance studies have been conducted with different hosts (the most common *X. fastidiosa*-infected hosts in France) and preparation of spiked samples.
Loewe analytical sensitivity varied between 10^4 (*Cistus monspeliensis*) and 10^5 (*Polygala myrtifolia*, *Helichrysum italicum*, *Calicotome villosa*) according to plant species. Agritest analytical sensitivity 10^5 (*C. monspeliensis*, *P. myrtifolia*, *H. italicum*, *C. villosa*)
To compare: analytical sensitivity of real-time PCR Harper et al., 2010 after DNA extraction QuickPick™ is 10^3 on same macerates.
Specificity: Loewe and Agritest 100%
Repeatability: Loewe 100%; Agritest 98.41%
Reproducibility: Loewe 100%; Agritest 98.15%

2. *Direct tissue blot immunoassay (DTBIA)*

DTBIA has been developed for the detection of *X. fastidiosa* in olive plant material for large-scale screening of symptomatic trees (Djelouah et al., 2014). Fresh cross-sections of young twigs are printed onto nitrocellulose membranes and the membranes incubated with the specific antiserum prior to development. This method has the advantages of being easy to perform and cost-effective in terms of reagents and labour and the membranes can be printed directly in the field preventing movement of infected plant materials to other areas.
The following performance characteristics are available:
In a test performance study performed at the Institute for Sustainable Plant Protection (Bari, IT), DTBIA was evaluated for the identification of *X. fastidiosa* strain CoDiRO in naturally infected olives (12 samples; 4 laboratories)
The DTBIA results were scored as the number of imprints showing specific purple coloration within the spotted sections.

Two different kits (Agritest and Enbiotech) were compared, which consisted of different detecting antisera. In the case of the protocol provided by Agritest, the imprints were made by squeezing the cuttings prior to spotting the fresh cut sections on the membrane.
Following both procedures, the olive samples were correctly categorized as positive and negative in the four laboratories. However, reactions seen with the Agritest kit were consistently stronger and easy to assess and interpret, even without observation of the imprinted membrane under the microscope.

APPENDIX 2 - IMMUNOFLUORESCENCE (IF) TEST

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009)
The IF test is usually performed on plant tissue that is mechanically homogenized in extraction buffer (e.g. 50 mM phosphate buffer) or demineralized water.
A commercial polyclonal antibody is available from Loewe.
Analytical specificity on pure cultures (data are provided by the supplier, Loewe), with concentrations of up to 10^4 cfu/mL tested on pure cell cultures.
Inclusivity: 100%
Number of *X. fastidiosa* strains tested: 5 (*X. fastidiosa*, *X. fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *fastidiosa*, CoDiRO; Lecce, IT).
Exclusivity: 100%
Number of non-target strains: 9 (*Agrobacterium vitis*, *Clavibacter michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, *Dickeya chrysanthemi*, *Pseudomonas syringae* pv. *syringae*, *Rhodococcus fascians*, *Xylophilus ampelinus*, *Xanthomonas vesicatoria*, *Xanthomonas campestris* pv. *campestris*).
No cross-reaction observed.
A preliminary test performance study on diagnostic sensitivity was performed during a workshop in Germany involving 13 laboratories using naturally infected coffee plant samples.
Diagnostic sensitivity: 100% of agreement at 10^4 cells/mL.
Repeatability: 100%

APPENDIX 3 - DNA EXTRACTION

Extraction of DNA for molecular analyses can be achieved using standard commercial kits and CTAB buffer. The following commercial kits are widely used and have been validated in several European Union (EU) laboratories to process samples from different plant species: DNeasy Plant Mini Kit-based extraction (Qiagen), modified DNeasy® Mericon™ Food Standard Protocol (Qiagen), QuickPick™ SML Plant DNA Kit-based extraction (Bio-Nobile). Validation data is available in the EPPO Database on Diagnostic Expertise.

Experiments conducted in the framework of the EU funded projects POnTE and XF-ACTORS and INRAe (FR) have shown that the analytical sensitivity of PCR tests was improved when an additional ultrasonication (1 min at 35–40 kHz) is performed on the plant extract prior to DNA extraction (CTAB-based extraction or QuickPick™ SML Plant DNA Kit-based extraction; Bio-Nobile). This has improved the release of bacteria from biofilms, in particular with difficult matrices such as *Olea* spp. and *Quercus* spp. Validation data from ANSES, FR is available on the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/validationlist).

For all PCR tests in addition to the undiluted DNA extract it is recommended to also use 10- and 100-fold dilutions to overcome possible inhibition.

1. Plant material

1.1. *DNA extraction from plant material other than large amounts (see 1.2)*

CTAB-based extraction

Weigh 0.5–1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized), put this into the extraction bags or into suitable tubes with 5 mL of CTAB buffer (see Appendix 14) and homogenize using a homogenizer (e.g. Homex, Polytron, etc.). For wood and xylem tissues crushing in liquid nitrogen may be needed depending on the type of homogenizer, or if no homogenizer is available.

Transfer 1 mL of extract into a 1.5-mL micro-centrifuge tube and heat the sample at 65°C for 30 min and centrifuge at 16 000 *g* for 5 min. Transfer the supernatant from centrifugation to a new 2-mL micro-centrifuge tube, with care being taken not to transfer any of the plant tissue debris. Add 1 mL of chloroform:isoamyl alcohol (24:1) and mix well by shaking. After centrifugation at 16 000 *g* for 10 min, transfer 700 µL of the supernatant to a 1.5-mL micro-centrifuge tube and add 490 µL (approximately 0.7 volumes) of cold 2-propanol. After mixing by inverting twice, incubate the tube at −20°C for 20 min. Centrifugation of the samples at 16 000 *g* for 20 min will allow recovery of a pellet which is washed with 1 mL of 70% ethanol. Perform an additional centrifugation at 16 000 *g* for 10 min and a decantation in 70% ethanol. Sample is air- or vacuum-dried. Resuspend the pellet in 100–150 µL of TE buffer or RNase- and DNase-free water.

Commercial kits

- DNeasy Plant Mini Kit-based extraction (Qiagen)

An aliquot of 200 mg of fresh small pieces of midribs and petioles is homogenized in 400 µL of lysis buffer using available equipment (Polytron, Homex, etc.). Lysis and purification are carried out following the manufacturer's instructions. The protocol can be performed manually or automated using a dedicated workstation.

- Modified DNeasy® Mericon™ Food Standard Protocol (Qiagen)

This kit, designed for the extraction of total DNA from a large-scale sample of raw or processed food material, has been successfully adapted to recover high-quality DNA from a wide range of plant species. For this purpose, plant samples of 0.5–1 g of fresh small pieces of midribs, petioles, basal leaf part or twigs (1/4 of the indicated amount, if lyophilized) are used. Transfer the recovered tissue into the extraction bags or suitable tubes, add 5 mL of Food Lysis Buffer and homogenize using a homogenizer (e.g. Homex, Polytron, etc.); transfer 1 mL of sap into a 1.5-mL micro-centrifuge tube and incubate for 30 min at 60°C. The sample is then processed following the manufacturer's instructions. The protocol can be performed manually or automated using a dedicated workstation.

The performance of the DNeasy Mericon Food kit combined with different real-time PCR tests (Francis et al., 2006; Harper et al., 2010) was evaluated for small (1 g) and large (10 g) amounts of plant tissue of different plants including (*Olea*, *Juniperus*, *Pelargonium*, *Prunus*, *Tagetes*, *Lavandula*, *Pistacia*, *Ficus* spp.). Results show that the real-time PCR protocol of Harper et al. (2010) performed well with DNA extracts from large amounts of tissue, which is important for pooled samples.

- QuickPick™ SML Plant DNA Kit-based extraction (Bio-Nobile)

Crush 0.5–1 g of fresh small pieces of midribs, petioles, basal leaf part or twigs in sterile water (5 mL/g) and perform a sonication step on the plant extract (1 min at 35–40 kHz). The plant extract is then left to soak for at least 15 min under gentle shaking. Then centrifuge 250 µL of the plant extract for 20 min at 20 000 *g*. The pellet (maximum 50 mg) is suspended in 75 µL of lysis buffer with 5 µL of proteinase K and the manufacturer's instructions followed. The extraction can be conducted manually or automated.

Before transferring the eluate into a new tube, the absence of magnetic particles should be verified by putting the last tube with the extract on a magnet for 10 s. When extraction is conducted manually, the durations indicated should be strictly followed.

- Maxwell® RSC PureFood GMO and Authentication Kit (Promega)

Weigh 0.5–1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized), put this into the extraction bags or into

suitable tubes with 5 mL of CTAB buffer (Promega) and homogenize using a homogenizer (e.g. Homex, Polytron, etc.). 20 µL of RNase A solution (to eliminate RNA) and 40 µL of Proteinase K (PK) solution are added to each microcentrifuge tube. The tubes are tapped, inverted and vigorously vortexed until the sample is resuspended. Tubes are placed in a heat block at 65°C for 30 min. During the incubation, cartridges are prepared according to the manufacturer's instructions. After incubation, tubes with the lysate are inverted or vortexed to mix thoroughly. Tubes are centrifuged at ≥16 000 *g* for 10 min to separate any oils and solids. 300 µL of clear lysate are transferred into well #1 of the reagent cartridge (avoid pipetting any solid material from the bottom of the tube or on the surface of the liquid and avoid putting oil on the surface; these materials may inhibit downstream tests). If necessary, transfer the cleared lysate to a new tube and centrifuge again to avoid oils and solids. Purify on the Mxwell® Instrument according to the manufacturer's instructions. For elution, 100 µL of Elution Buffer are used.

Validation of the Maxwell® RSC PureFood GMO and Authentication Kit extraction is included in the EPPO Database on Diagnostic Expertise.

1.2. *DNA extraction from large amounts of tissue*

Extraction efficiency depends on the matrix, and this is reported in the section on performance characteristics of the relevant tests.

1.2.1. Procedure validated for coffee plants (10 g)

The DNA extraction for composite samples has not been evaluated so far with other DNA extraction methods than the QuickPick™ SML Plant DNA Kit-based extraction.

The extraction methodology is based on initial maceration of approximately 10 g of petioles and midribs (latent material) and their subsequent homogenization and incubation under agitation for 30 min in phosphate-buffered saline (PBS; 0.01 M pH 7.2) (4 mL/g). The resulting extract is subjected to a concentration step, by centrifugation at 4°C at 10 000 *g* for 20 min. The pellet is resuspended in 1.5 mL of phosphate buffer (0.01 M pH 7.2). Total genomic DNA is isolated from 75 µL of the acquired plant extract with the QuickPick SML plant DNA kit (Bio-Nobile), using a King-Fisher isolation robot (Thermo Scientific). DNA is eluted in 50 µL of elution buffer and stored at –20°C.

1.2.2. Procedure validated for other plant species (Table 2).

- Homogenize the tissue in 1× PBS (see Appendix 14) using a volume of 1:1 or 1:1.5 (w:v) for herbaceous materials (the latter being used for more lignified tissues) and 1:3 (w:v) for non-herbaceous materials (e.g. leaves or portions of stems of perennial plants or woody trees or shrubs) as indicated in Table 2; make sure that the tissue is properly macerated.
- Incubate the macerate at room temperature for 30 min
- Recover at least 35 mL of plant sap and transfer to a clean tube
- Centrifuge the sap for 5 min at low speed (3000 *g*), recover the supernatant and discard the pellet containing the plant debris
- Centrifuge the supernatant at maximum speed (10 000 *g*) for 20 min
- Discard the supernatant and resuspend the pellet in 1.5 mL of 1× PBS and transfer to a micro-centrifuge tube
- Centrifuge the micro-centrifuge tube for 20 min at maximum speed (17 000 *g*)
- Discard the supernatant and resuspend the pellet in 1 mL of CTAB buffer or 1 mL of lysis buffer and proceed as indicated above for the extraction of the DNA using either the CTAB-based protocol or the commercial kit DNeasy Mericon Food Standard Protocol (Qiagen).

1.3. *Reduction of inhibition effects*

Evaluations performed in the framework of XF-ACTORS, aiming at reducing the inhibitory effects on target amplification to increase detectability of *Xylella fastidiosa* in plant DNA extracts by PCR-based methods have shown that:

- Elimination of inhibition effect with GeneRelaser® was only possible when used in a two-step reaction.
- A comparison of DNA extraction kits from Qiagen (DNeasy Plant Mini kit, DNeasy Plant Pro Kit, DNeasy Mericon Food kit) with and without modifications was made. Results have shown that DNeasy Mericon Food kit modified with the addition of 0.05% Tween 20 in the extraction buffer, and/or pretreatment of the plant material with hexane enhances the real time PCR performance in the case of *Olea europaea* samples and reduces the inhibitory effect of plant compounds (possibly aromatic secondary metabolites) in the case of spiked *Juniperus* sp. material.

2. DNA extraction from vectors

CTAB-based extraction

For small vectors (e.g. *Philaenus*) 1–5 heads can be pooled and for large vectors (e.g. *Cicadella viridis*, *Cicada orni* or *Aphrophora* spp.) a single insect head (from which the eyes have preferably been removed) should be used.

Homogenize a single insect head or a pool of 5 heads, in a 2-mL tube with one or two 5-mm tungsten carbide beads [for a maximum of 15–20 s at a frequency of

24 cycles/s, in Mill300 mixer/Tissue Lyser II (Qiagen) or similar equipment]. Add 500 µL of CTAB buffer and mix the tube well by shaking or vortexing. Heat the sample at 65°C for 30 min. Add 500 µL of chloroform:isoamyl alcohol 24:1 and mix the sample again by shaking or vortexing. After centrifugation at 16 000 *g* for 10 min, transfer 400 µL of the supernatant to a 1.5-mL micro-centrifuge tube and add 280 µL (approximately 0.7 volumes) of cold 2-propanol. After mixing by inverting twice, incubate the tube at −20°C for 20 min. The centrifugation of the samples at 16 000 *g* for 20 min will allow a pellet to be recovered; wash the pellet with 1 mL of 70% ethanol. Perform an additional centrifugation at 16 000 *g* for 10 min followed by decantation into 70% ethanol. The sample is air- or vacuum dried. Resuspend the pellet in 30–80 µL of TE buffer or RNase- and DNase-free water, depending on the amount of starting material (single or pooled insect heads).

Commercial kits

- QuickPick™ SML Plant DNA Kit-based extraction (Bio-Nobile)

Homogenize a single insect head (https://www.youtube.com/watch?v=q5DH1q66Llk) or a pool of up to 10 heads in a 2-mL tube with 10 stainless beads (diameter 3 mm) and 200 µL of demineralized sterile water for 2 min at 30 Hz in a mixer mill MM400 (Restch) or similar equipment. Then microtubes are placed on a magnetic rack and the whole macerate is transferred into a new 2-mL microtube. The microtubes are centrifuged for 20 min at 20 000 *g*. The pellet is suspended in 37.5 µL of lysis buffer with 2.5 µL of proteinase K and the manufacturer's instructions followed (buffer volumes: binding buffer 62.5 µL, beads 2.5 µL, washing buffer 125 µL, elution buffer 50 µL).

Validation of the QuickPick™ extraction for *P. spumarius* is included in the EPPO Database on Diagnostic Expertise.

- Maxwell® RSC PureFood GMO and Authentication Kit

Homogenize a single insect head in a 2 mL tube with one or two 5-mm tungsten carbide beads (for a maximum of 15–20 s at a frequency of 24 cycles/s, in Mill300 mixer/Tissue Lyser II (Qiagen) or similar equipment). 500 µL of CTAB buffer (Promega), 10 µL of RNase A solution (to eliminate RNA) and 20 µL of Proteinase K (PK) solution are added to each microcentrifuge tube. The tubes are tapped, inverted and vigorously vortexed until the sample is resuspended. Tubes are placed in a heat block at 65°C for 30 min. During the incubation, cartridges are prepared according to manufacturer's instructions. After incubation, tubes with the lysate are inverted or vortexed to mix thoroughly. Tubes are centrifuged at ≥16 000 *g* for 10 minutes to separate any oils and solids. 300 µL of clear lysate are transferred into well #1 of the reagent cartridge (avoid pipetting any solid material from the bottom of the tube or on the surface of the liquid and avoid putting oil on the surface; these materials may inhibit downstream tests). If necessary, transfer the cleared lysate to a new tube and centrifuge again to avoid oils and solids. Purify on the Maxwell® Instrument according to the manufacturer's instructions. For elution, 50 µL of Elution Buffer are used.

Validation of the Maxwell® RSC PureFood GMO and Authentication Kit extraction is included in the EPPO Database on Diagnostic Expertise.

Several commercial kits are available for insect DNA extraction (e.g. prepGEM™ Insect, ZyGEM, Solana Beach, CA, US; E.Z.N.A.® Insect DNA Kit, Omega Bio-Tek, Norcross, GA, US; EZgeneTM Insect gDNA Kit); however, there is no experience so far with these kits for detection of *X. fastidiosa* in the EPPO region.

One to 10 heads (Cunty et al., 2020) can be pooled, and for large vectors (e.g. *Cicadella viridis* or *Cicada orni*) a single insect head (from which the eyes have preferably been removed) should be used.

For the LAMP test (Appendix 12), DNA extracted from insects can be used or a single insect is used directly without DNA extraction.

3. DNA extraction from pure cultures

For pure cultures, a loop full of fresh pure culture is suspended in approximately 1 mL of molecular-grade water; lysis should be performed at 100°C for 5 min and the lysate put on ice.

APPENDIX 4 - CONVENTIONAL PCR (MINSAVAGE ET AL., 1994)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for the detection and identification of *X. fastidiosa*.

1.2. The test is based on Minsavage et al. (1994).

1.3. The target sequence is located in the 3′ end of the gene *rpoD*, coding for an RNA polymerase sigma-70 factor.

1.4. Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer RST31	5′-GCG TTA ATT TTC GAA GTG ATT CGA TTG C-3′	733 bp
Reverse primer RST33	5′-CAC CAT TCG TAT CCC GGT G-3′	

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Matrices: plants, insects, or pure culture suspension.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. Conventional PCR

2.2.1. Master mix

The conditions (below) are as implemented in ANSES (FR). Other master mixes and PCR conditions (not indicated in this Diagnostic Protocol) have given similar results.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	18.6	N.A.
Taq DNA polymerase buffer (Invitrogen)	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (RST31)	20 µM	0.375	0.3 µM
Reverse primer (RST33)	20 µM	0.375	0.3 µM
Platinum Taq DNA polymerase (Invitrogen)	10 U/µL	0.15	0.06 U/µL
Subtotal		23	
Genomic DNA from plant or from insect tissue extract or bacterial suspension		2	
Total		25	

2.2.2. PCR conditions

95°C for 1 min followed by 40 cycles of (95°C for 30 s, 55°C for 30 s, 72°C for 45 s) and a final step of 72°C for 5 min.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or by botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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).
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC: no band is visualized
- PIC and PAC a band of 733 bp is visualized. If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- A test will be considered positive if a band of the expected size (733 bp) is visualized.

- A test will be considered negative if it produces no band or band(s) of a different size is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

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

- **A From ANSES (FR) using the DNeasy® Plant Mini Kit (Qiagen):**

A4.1 Analytical sensitivity data

Vitis vinifera ≈ 10^2 bacteria/mL
Prunus persica ≈ 10^2 bacteria/mL
Citrus sinensis ≈ 10^3 bacteria/mL
Coffea arabica ≈ 10^4 bacteria/mL (diluted DNA 1/10)
Coffea canephora ≈ 10^3–10^4 bacteria/mL (non-specific bands are present near 750 bp; expected band is 733 bp)
The above concentrations gave a probability of detection of 100%.

A4.2 Analytical specificity data

Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' https://dc.eppo.int/validation_data/validationlist.
Inclusivity: 100% tested on 10 target strains (*X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *sandyi*, *X. fastidiosa* subsp. *multiplex*).
Exclusivity: 100% tested on 16 non-target stains (*Xylophilus ampelinus*, 15 *Xanthomonas* spp.).

A4.3 Data on repeatability

Vitis vinifera 80%
Prunus persica 92%
Citrus sinensis 98%
Coffea arabica 94%
Coffea canephora: 89%

A4.4 Data on reproducibility

Not available.

A4.5 Diagnostic sensitivity data

Vitis vinifera 81%
Prunus persica 81%
Citrus sinensis 82%
Coffea arabica 81%
Coffea canephora: 74%
Compared with spiked matrices with bacterial concentrations from 10 to 10^6 bacteria/mL: 21 samples per matrix, 63 DNA extractions per matrix, 126 amplifications per matrix. (On orange tree 18 samples per matrix, 54 DNA extractions per matrix, 108 amplifications per matrix.)

A4.6 Diagnostic specificity data

Citrus sinensis 100%
Coffea arabica 100%
Coffea canephora 100%

A4.7 Other information

In 2014, a test performance study was performed on a new set of spiked samples.
Performance characteristics
Analytical sensitivity (with a probability of detection of 100% on coffee and orange only):
Coffea spp. ≈ 10^4 bacteria/mL (100%, 4 labs/4)
Olea europaea ≈ 10^6 bacteria/mL (3 labs/4)
Vitis vinifera ≈ 10^6 bacteria/mL (2 labs/4)
Citrus sinensis ≈ 10^2 bacteria/mL (100%, 4 labs/4)
Prunus persica ≈ 10^4 bacteria/mL (3 labs/4)
Diagnostic sensitivity (based on results on spiked samples to the following concentrations):
Coffea spp. 70% (10^2–10^4 bacteria/mL)
Olea europaea 30% (10^4–10^6 bacteria/mL)
Vitis vinifera 40% (10^4–10^6 bacteria mL)
Citrus sinensis 80% (10–10^3 bacteria/mL)
Prunus persica 60% (10^2–10^4 bacteria mL)
Note: these results obtained by several laboratories are different from those obtained in the intra-laboratory evaluation, mainly on grapevine (variability linked to a matrix effect?)

Test performance study (TPS) performed with extraction kit from Qiagen (DNeasy Plant Mini Kit)
Diagnostic specificity: 100%
Reproducibility: 84%
Repeatability: 95% (from 88% to 100% in the 4 laboratories), four samples per matrix, two extractions per sample, two amplifications per DNA extract.

- **B Additional performance characteristics**

Additional performance characteristics obtained with slightly different master mixes are available and can be downloaded from the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validationlist.php).
A validation study with non-European *X. fastidiosa* strains (Harper et al., 2010) showed that the RST31/33 primer failed to detect the following American strains

from grapes and oaks: *X. fastidiosa*, *Vitis vitifolia*, US (PD0001); *X. fastidiosa*, *V. vitifolia*, US (R. Almeida); *X. fastidiosa*, *Vitis rotundifolia*, US (C. Chang); *X. fastidiosa*, *Quercus laevis*, US (OAK0023); *X. fastidiosa*, *Quercus rubra*, US (OAK0024); *X. fastidiosa*, *Quercus rubra*, US (C. Chang).

Accuracy: *Olea europaea* ≈60% [data from TPS involving CREA-DC (IT) and IPSP (IT)].

DNA (CTAB extraction) from olive extract samples spiked with *X. fastidiosa* CoDiRO strain suspensions at 10^3, 10^4, 10^6 cfu/mL in three repetitions, healthy olive extracts (three repetitions) for a total of 12 samples, tested by 15 laboratories.

The EU-XF-PT-2017-02 report 'Proficiency testing for the evaluation of molecular and serological diagnosis of *X. fastidiosa*' performed in the framework of the EU projects XF-ACTORS and PONTE and of the project Euphresco is available at https://upload.eppo.int/download/217o22631f22a.

APPENDIX 5 - REAL-TIME PCR (HARPER ET AL., 2010; ERRATUM 2013) SIMPLEX OR DUPLEX

The tests below are described as they weres carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that verification (see PM 7/98) is carried out.

1. General information

1.1. These PCR tests are suitable for the detection and identification of *X. fastidiosa*.

1.2. These tests are based on Harper et al. (2010; erratum 2013).

1.3. The target sequence is located in the gene coding for the 16S rRNA-processing RimM protein.

1.4. This PCR can be run in simplex for testing plants and pure cultures.

Primers	Sequence
Forward primer XF-F	5′-CAC GGC TGG TAA CGG AAG A-3′
Reverse primer XF-R	5′-GGG TTG CGT GGT GAA ATC AAG-3′
Probe XF-P	5′-6-FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1-3′

1.5. This PCR can be run in duplex for testing plants with an internal positive control based on the conserved plant cytochrome oxidase (COX) gene (Li et al., 2006).

Primers	Sequence
Forward primer COX-F	5′-GTATGCCACGTCGCATT CCAGA-3′
Reverse primer COX-R	5′-GCCAAAACTGCTAAGGG CATTC-3′
Probe COX-P	5′-Cy5-ATCCAGATGCTTAC GCTGG-BHQ-2-3′

1.6. This PCR can be used in duplex for testing insects with an internal PCR control based on the eukaryote 18S rDNA (Ioos et al., 2009).

Primers	Sequence
Forward primer 18S Uni-F	5′-GCA AGG CTG AAA CTT AAA GGA A-3′
Reverse primer 18S Uni-R	5′-CCA CCA CCC ATA GAA TCA AGA-3′
Probe 18S Uni-P	5′-Cy5-ACG GAA GGG CAC CAC CAG GAG T-BHQ-2-3′

1.7. The real-time PCR systems used to generate the validation data below are Applied Biosystems® 7500 Fast, ThermoFisher Scientific (ANSES) or CFX 96, Bio-Rad (ANSES, ISPP and CREA).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Matrices: plant, insects or pure cultures.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. Real-time PCR

2.2.1. Master mix for simplex PCR (plants and pure cultures).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	6.48	N.A.
TaqMan Fast Universal Master Mix, no AmpErase™ UNG (Applied Biosystems)	2×	10	1×
Forward primer (XF-F)	10 µM	0.6	0.3 µM
Reverse primer (XF-R)	10 µM	0.6	0.3 µM
Probe (XF-P)	10 µM	0.2	0.1 µM
Molecular-grade BSA (non-acetylated) (Invitrogen)	50 µg/µL	0.12	0.3 µg/µL
Subtotal		18	
Bacterial suspension or DNA extract		2	
Total		20	

2.2.2. Master mix for duplex PCR (for testing plants, CREA & CNR-IPSP, IT).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	5	N.A.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
TaqMan™ Fast Advanced PCR Master Mix, (Applied Biosystems)	2×	11	1×
Forward primer (XF-F)	10 μM	0.60	0.28 μM
Reverse primer (XF-R)	10 μM	0.60	0.28 μM
Probe (XF-P)	10 μM	0.40	0.18 μM
Forward primer (COX-F)	10 μM	0.80	0.36 μM
Reverse primer (COX-R)	10 μM	0.80	0.36 μM
Probe (COX-P)	10 μM	0.80	0.36 μM
Subtotal		20.0	
DNA		2.0	
Total		22	

2.2.3. Master mix for duplex PCR (for testing insects).

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	5.78	N.A.
TaqMan™ Fast Universal PCR Master Mix, no AmpErase™ UNG (Applied Biosystems)	2×	10.0	1×
Forward primer (XF-F)	10 μM	0.60	0.30 μM
Reverse primer (XF-R)	10 μM	0.60	0.30 μM
Probe (XF-P)	10 μM	0.20	0.10 μM
Forward primer (18S Uni-F)	10 μM	0.30	0.15 μM
Forward primer (18S Uni-R)	10 μM	0.30	0.15 μM
Probe (18S Uni-P)	10 μM	0.10	0.05 μM
Molecular-grade BSA (non-acetylated) (Invitrogen)	50 μg/μL	0.12	0.30 μg/μL
Subtotal		18.0	
DNA		2.0	
Total		20	

2.2.4. PCR conditions

95°C for 10 min, followed by 40 cycles of (94°C for 10 s and 62°C for 40 s). Heating ramp speed 5°C/s.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: the molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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).
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table described in 2.2.1. Consequently, if a laboratory plans to use an IPC in multiplex reactions with this test, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) 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.

Comment: some laboratories have noted the occurrence of late Ct *values (above 38) with this test. Such cases should be considered as inconclusive. Retesting and/or resampling are recommended.*

4. Performance characteristics available

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

A ANSES (Angers, FR)

DNA extraction using the QuickPick™ SML Plant DNA kit (BioNobile) performed automated using KingFisher™ mL (Thermo Scientific) or KingFisher™.

Test with the Master mix 2.2.1

A4.1 Analytical sensitivity data

Vitis vinifera: 10^3 cells/mL
Citrus sinensis: 10^2 cells/mL

A4.2 Analytical specificity data

Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist). Inclusivity 100% evaluated on 55 target strains belonging to *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *sandyi*, *X. fastidiosa* subsp. *multiplex*.

Exclusivity 100% evaluated on 18 non-target strains: *Xylophilus ampelinus* CFBP 2098, *Xanthomonas arboricola* pv. *pruni* CFBP 3901, *Xanthomonas arboricola* pv. *juglandis* NCPPB 362, *Xanthomonas axonopodis* pv. *citri* CFBP 2904, *Xanthomonas axonopodis* pv. *aurantifolia* CFBP 3529, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas arboricola* pv. *fragariae*, *Xanthomonas fragariae* CFBP 2157, *Xanthomonas campestris* pv. *carotae*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas hortorum* pv. *hederae*, *Xanthomonas translucens* pv. *graminis* CFBP 2058, *Xanthomonas translucens* pv. *translucens* CFBP 2544, *Xanthomonas arboricola* pv. *pruni* CFBP 3900, *Xanthomonas oryzae* pv. *oryzae* LMG 806, 'Ca. Liberibacter asiaticus', 'Ca. Liberibacter africanus'.

A4.3 Data on repeatability

Vitis vinifera: 100%
Citrus sinensis: 100%

A4.6 Data on accuracy

Vitis vinifera: 100%
Citrus sinensis: 100%

B ANSES (Angers, FR)

DNA extraction using CTAB-based extraction with a prior sonication step (1 min at 35 kHz).

Test with the Master mix 2.2.1

B4.1 Analytical sensitivity data

Olea europaea: 10^4 cells/mL
Quercus ilex: 10^3 cells/mL

B4.2 Analytical specificity data

Same as A4.2

B4.3 Data on repeatability

Olea europaea: 100%
Quercus ilex: 100%

B4.4 Data on reproducibility

Olea europaea: 100%
Quercus ilex: 100%

B4.6 Data on accuracy

Olea europaea: 100%
Quercus ilex: 100%

C CREA (IT) in collaboration with CNR-IPSP (IT)

DNA extraction using the DNeasy Mericon Food Kit (Qiagen) and CTAB DNA extraction method.

Samples consisted of olive extracts spiked with X. fastidiosa subsp. *pauca strain De Donno suspensions at 10^6, 10^4, 10^3 cfu/mL and healthy olive extracts, all in three repetitions for a total of 12 samples, tested by 15 Italian laboratories.*

Test with the Master mix 2.2.2

C4.1 Data on diagnostic sensitivity

Olea europaea: 100%

C4.2 Data on diagnostic specificity

Olea europaea: 100%

C4.3 Data on accuracy

Olea europaea: 100%

C4.4 Data on repeatability

Olea europaea: 100%

C4.5 Data on reproducibility

Olea europaea: 100%

D Additional validation data

Additional performance characteristics obtained with slightly different master mixes are available and can be downloaded from the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/validationlist)

The report EU-XF-PT-2017-02 'Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*' is available at https://upload.eppo.int/download/217o22631f22aa (participants used the Master mix in use in their laboratories).

The report of the TPS on 'Molecular detection of *Xylella fastidiosa* by real-time tests' is available at https://upload.eppo.int/download/298ocd8b7f525.

The 17-XFAST-EU interlaboratory TPS for the evaluation of molecular methods to detect *Xylella fastidiosa* in the vector *Philaenus spumarius* is available at https://upload.eppo.int/download/268obc05d6355 (test performed according to the EPPO Diagnostic protocol). The performance characteristics obtained during this TPS are presented in the table below.

	Real-time PCR			
	Harper et al. (2010), cut-off = 38		Duplex (Harper et al., 2010, Ioos et al., 2009)[a], cut-off = 38	
17-XFAST-EU TPS performance criteria	**QuickPick™**	**CTAB**	**QuickPick™**	**CTAB**
Diagnostic sensitivity				
%	88.46	99.11	84.81	100.00
% (restricted series)[b]	95.83	99.11	94.37	100.00
No. of labs with false negatives	2	1	3	1
Diagnostic specificity				
%	96.15	92.86	90.00	91.67
% (restricted series)[c]	97.92	100.00	100.00	100.00
No. of labs with false positives	2	1	1	1
Accuracy				
%	91.03	97.02	87.27	96.53
% (restricted series)[d]	96.21	99.36	95.83	99.24
Repeatability				
%	99.65	98.72	99.31	96.97
Reproducibility				
%	82.49	95.51	76.37	94.76
% (restricted series)[e]	92.94	98.98	91.90	98.78
No. of labs	13	14	10	12

[a] Corresponding to Master mix 2.2.3

[b] Results of one laboratory excluded (systematic false negatives).

[c] Results of one laboratory excluded (high number of false positives, about 40% on healthy insects).

[d] Results of one laboratory excluded (high number of false positives, about 40% on healthy insects) and of another laboratory excluded (systematic false negatives).

[e] Results of three laboratories excluded: one for a high number of false positives (about 40% on healthy insects), another carried out one repetition only for amplification and a third laboratory gave a systematic false negative.

APPENDIX 6 - REAL-TIME PCR TESTS (FRANCIS ET AL., 2006)

The tests below are described as they were carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that verification (see PM 7/98) is carried out.

APPENDIX 6A SYBR Green version

1. General information

1.1. This PCR is suitable for the detection and identification of *X. fastidiosa*.

1.2. The test is based on Francis et al. (2006).

1.3. The target sequence is a conserved hypothetical protein HL gene.

1.4. Oligonucleotides:

Primers	Sequence
Forward primer HL5	5′-AAG GCA ATA AAC GCG CAC TA-3′
Reverse primer HL6	5′-GGT TTT GCT GAC TGG CAA CA-3′

1.5. The real-time PCR systems used to generate the validation data presented below were: Applied Biosystems® 7500 Fast (ThermoFisher Scientific) or CFX 9600 (Bio-Rad).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Matrices: plants, insects or pure cultures.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	3.88	N.A.
SYBR Select Master Mix (Applied Biosystems)	2×	5.5	1×
Forward primer (HL5)	10 µM	0.31	0.28 µM
Reverse primer (HL6)	10 µM	0.31	0.28 µM
Subtotal		10	
Bacterial suspension or DNA extract		1	
Total		11	

2.2.2. PCR conditions

Pre-incubation at 50°C for 2 min, followed by 95°C for 5 min, followed by 40 cycles of (95°C for 20 s and 60°C for 40 s); melt-curve analysis is performed immediately after the amplification protocol by collecting data over a temperature range of 65–95°C in 0.5°C increments.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if a laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) 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 and the specific melting peak is in the range of 83–85
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. It should be noted that frequently curves for which the values of Ct (cycle threshold) are between 35 and 40 do not exhibit a characteristic curve. In this case, the result is interpreted as being undetermined.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

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

A Data provided by Research Centre for Plant Protection and Certification (CREA-DC, Rome, IT) in collaboration with Institute for Sustainable Plant Protection (CNR-IPSP, Bari, IT).

4.1. Analytical sensitivity data

Olea europaea plant extracts spiked with 10-fold dilution of *X. fastidiosa* subsp. *pauca* CoDiRO strain suspensions ≈ 10 cfu/mL.

4.2. Analytical specificity data (CREA-DC, Rome, Italy)

Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

Exclusivity: 100%. Evaluated on 34 non-target bacterial strains: 3 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *fragariae*, 1 *X. arboricola* pv. *corylina*, 1 *X. arboricola* pv. *celebensis*, 1 *Xanthomonas axonopodis* pv. *citri*, 1 *Xanthomonas campestris* pv. *campestris*, 1 *X. campestris* pv. *populi*, 2 *Xanthomonas hortorum* pv. *pelargonii*, 3 *Pseudomonas savastanoi* pv. *savastanoi*, 1 *Pseudomonas marginalis*, 4 *Pseudomonas syringae* pv. *syringae*, 4 *Brenneria* (spp. *rubrifaciens*, *quercina*, *salicis*, *populi*), 2 *Pantoea stewartii*, 1 *Pantoea agglomerans*, 1 *Erwinia amylovora*, 3 *Agrobacterium tumefaciens* and 2 *Rhizobium vitis*.

DNA (CTAB extraction) from olive extract samples spiked with *X. fastidiosa* CoDiRO strain suspensions at 10^3, 10^4, 10^6 cfu/mL in three repetitions, healthy olive extracts (three repetitions) for a total of 12 samples, tested by 15 laboratories.

X. arboricola pv. *celebensis* (NCPPB 1832) and *Brenneria populi* (NCPPB 4299^T) gave an amplification curve corresponding to melting peak values of respectively 87°C and 87.5°C (different from *Xylella fastidiosa* that has a melting peak at 84–85°C). *Pantoea agglomerans* (ISF 438), *B. quercina* (NCPPB 1852^T), *Pseudomonas marginalis* (CREA-PAV 1229), *X. hortorum* pv. *pelargonii* gave amplification curves with inconsistent melting peaks.

4.3. Data on repeatability

Olea europaea: 91%.

4.4. Data on reproducibility

Olea europaea: extraction 85%.

4.5. Data on diagnostic sensitivity

Olea europaea: 90%.

4.6. Data on accuracy

Olea europaea: 92%.

4.7. Data on diagnostic specificity

Olea europaea: 100%.

B Additional validation data

The report EU-XF-PT-2017-02 'Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*' is available at https://upload.eppo.int/download/217o22631f22a (participants used the Master mix in use in their laboratories).

The report of the TPS on 'Molecular detection of *Xylella fastidiosa* by real-time tests' is available at https://upload.eppo.int/download/298ocd8b7f525.

The 17-XFAST-EU interlaboratory TPS for the evaluation of molecular methods to detect *Xylella fastidiosa* in the vector *Philaenus spumarius* is available at https://upload.eppo.int/download/268obc05d6355 (test performed according to the EPPO Diagnostic protocol). The performance characteristics obtained during this TPS are presented in the table below.

17-XFAST-EU TPS performance criteria	TaqMan PCR		SYBR green	
	Francis et al. (2006) cut-off = 38		Francis et al. (2006) cut-off = 35/melting peak 83–85°C	
	QuickPick™	CTAB	QuickPick™	CTAB
Diagnostic sensitivity				
%	60.42	95.54	56.25	79.17
% (restricted series)[a]	65.91	95.54	56.25	79.17
No. of labs with false negatives	8	3	2	1
Diagnostic specificity				
%	100.00	100.00	87.50	100.00
% (restricted series)[b]	100.00	100.00	87.50	100.00
No. of labs with false positives	0	0	1	0
Accuracy				
%	73.61	97.62	66.67	86.11
% (restricted series)[c]	78.33	97.44	66.67	86.11
Repeatability				
%	97.73	99.36	100.00	100.00
Reproducibility				
%	64.77	96.45	72.66	81.94
% (restricted series)[d]	69.14	96.18	72.66	81.94
Number of laboratories	12	14	2	3

[a] Results of one laboratory excluded (systematic false negatives).

[b] Results of one laboratory excluded (high number of false positives, about 40% on healthy insects).

[c] Results of two laboratories excluded: one laboratory with a high number of false positives (about 40% on healthy insects) and another with systematic false negatives.

[d] Results of three laboratories excluded: one with a high number of false positives (about 40% on healthy insects), one with repetition for only one amplification and a third with systematic false negatives.

APPENDIX 6B Taqman version

1. General information

1.1. This PCR is suitable for the detection and identification of *X. fastidiosa*.

1.2. The test is based on Francis et al. (2006). Modified protocol developed at the National Institute of Biology (NIB), SI (2007, unpublished).

1.3. The target sequence is a conserved hypothetical protein HL gene.

1.4. Oligonucleotides:

Primers	Sequence
Forward primer HL5	5′-AAG GCA ATA AAC GCG CAC TA-3′
Reverse primer HL6	5′-GGT TTT GCT GAC TGG CAA CA-3′
Probe	5′-FAM-TGG CAG GCA GCA ACG ATA CGG CT-BHQ1-3′

1.5. The validation data below has been generated using the real-time PCR system ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Matrices: plants, insects or pure bacterial suspensions

2.1.2. For extraction procedures see Appendix 3 (this test was not evaluated by NIB on insects).

2.1.3. Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for later use.

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	1	N.A.
Real-time PCR buffer (TaqMan® Universal PCR Master Mix, ThermoFisher Scientific, 2×)	2×	5	1×
Forward primer (HL5)	10 µM	0.9	0.9 µM
Reverse primer (HL6)	10 µM	0.9	0.9 µM
Probe 1 (probe)	10 µM	0.2	0.2 µM
Subtotal		8	
Bacterial suspension or DNA extract		2	
Total		10	

2.2.2. PCR conditions

Pre-incubation (UNG step) at 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s.

Heating and cooling ramp speed: standard temperature ramping mode, corresponding to ± 1.6°C on 7900HT Fast Real-Time PCR System (Applied Biosystems) and ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific).

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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).
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential
- NIC and NAC should give no amplification.

When these conditions are met:

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

4. Performance characteristics available

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

A Data provided by NIB (SI), DNA extraction QuickPick Plant Mini Kit.

4.1. Analytical sensitivity data

Determined on *X. fastidiosa* DNA dilutions. The lowest concentrations tested in which all replicates were positive was found to be 2.6, 3.2 and 3.5 [log (target copies of DNA/mL), determined with digital PCR using the same primers and probe as in real-time PCR, and corresponding to concentration of cells/mL], *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* and *X. fastidiosa* subsp. *pauca* CoDiRO strain, respectively.

Plant material (spiked): 94% determined in plant material prepared as for symptomatic testing (31/33) spiked

with *X. fastidiosa* at 10^5 cells/mL and 100% determined in plant material prepared as for latent testing (3/3) spiked with *X. fastidiosa* at 10^5 cells/mL.

Details on the preparation of the spiked samples are provided in the validation report available through the EPPO Database on Diagnostic Expertise in the section 'Validation data for diagnostic tests *Xylella fastidiosa*' [LabID NIB-FITO, complementary files Validation data on the modified real-time PCR for detection of *Xylella fastidiosa* adapted from Francis et al., 2006 (no. D0002/16)].

4.2. Analytical specificity data

Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

Inclusivity: 100%.

Strains evaluated: *X. fastidiosa* subsp. *fastidiosa* (2), *X. fastidiosa* subsp. *multiplex*, and *X. fastidiosa* subsp. *pauca* CoDiRO strain.

Exclusivity 100%: 90 bacterial strains evaluated 5 endophytic bacteria isolated from *Olea europaea*, 9 isolates isolated from *Nerium oleander*, 10 endophytic bacteria isolated from *Rosmarinus*, 10 endophytic bacteria isolated from *Lavandula* and 56 other pathogenic bacteria.

4.3. Data on repeatability

No data available.

4.4. Data on reproducibility

97% at an approximate concentration of 10^5 cells/mL of plant extract.

B Additional validation data

Validation studies with non-European *X. fastidiosa* strains showed that a real-time PCR adapted from Francis et al. (2006) with a modified protocol failed to detect some strains from the Americas: from *Morus alba* (mulberry, DC (Mull)), *Acer negundo* (box elder, MD BE1), three Brazilian strains from *Citrus sinensis* (sweet orange, CVC 3BA, CVC 5AB, CVC 6-B; Li et al., 2013), as well as *Quercus rubra* (red oak, US OAK0024) and *Liquidambar styraciflua* (sweetgum, US) (Harper et al., 2010). All these strains were detected with real-time PCR based on Harper et al., (Harper et al., 2010; Li et al., 2013), however, most of these strains are no longer available and the analytical specificity of other tests for these strains cannot be verified.

The report EU-XF-PT-2017-02 'Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*' is available at https://upload.eppo.int/download/217o22631f22a and 'Molecular detection of *Xylella fastidiosa* by real-time tests' at https://upload.eppo.int/download/298ocd8b7f525.

APPENDIX 7 - REAL-TIME PCR (OUYANG 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 verification (see PM 7/98) is carried out.

1. General information

1.1. This PCR is suitable for the detection and identification of *X. fastidiosa*.

1.2. The test is adapted from Ouyang et al. (2013).

1.3. The target sequence is the cobalamin synthesis protein-coding gene.

1.4. Oligonucleotides:

Primers	Sequence
Forward primer Xf.Csp6F	5′-CCC ATT ACG CTT CAA CCA TT-3′
Reverse primer Xf.Csp6R	5′-CCC AAT CCA TAC GAC TTG CT-3′
Probe Xf.Csp6P[a]	5′-6-FAM-GGT GTG ATT [ZEN] CGC AGC AAG GGC-IBFQ-3′

[a] This probe with an internal ZEN and an Iowa Black FQ quencher can be ordered at Integrated DNA Technologies, Inc.

1.5. The real-time PCR system used to generate the validation data below was an Eppendorf Realplex 4 Mastercycler S.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Matrices: plants, insects or pure cultures.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	3.3	N.A.
PerfeCTa qPCR ToughMix (without ROX or UNG), QuantaBio (Quanta Biosciences)	2×	5	1×
Forward primer (Xf.Csp6F)	10 µM	0.3	0.3 µM

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Reverse primer (Xf.Csp6R)	10 µM	0.3	0.3 µM
Probe (Xf.Csp6P)	10 µM	0.1	0.1 µM
Subtotal		9	
Bacterial suspension or DNA extract		1	
Total		10	

2.2.2. PCR conditions

Initial denaturation for 10 min at 95°C, followed by 40 cycles of (95°C for 15 s and 60°C for 60 s). Heating ramp speed: 6°C/s.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: the molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential
- NIC and NAC should give no amplification.

When these conditions are met:

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

4. Performance characteristics available

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

AGES (Vienna, AT) DNA extraction using the DNeasy Plant Mini Kit (Qiagen).

4.1. Analytical sensitivity data

Data obtained from the original publication: 8.41 copies per reaction (bacterial suspension); the number of copies was calculated according to the 2.679-Mb genome size of *X. fastidiosa* subsp. *pauca* (9a5c; GenBank accession number AE003849) using an online calculator (http://cels.uri.edu/gsc/resources/cndna.html) (Ouyang et al., 2013). Additional data from AGES is not yet available.

4.2. Analytical specificity data

According to the original publication: for inclusivity 27 different *X. fastidiosa* strains from 5 different host plants tested (including defined subsp. *pauca*, *multiplex* and *fastidiosa*), 4 *X. fastidiosa* strains from leafhopper and for exclusivity 15 closely related or host related non-targets (13 bacteria strains, 2 fungi) tested.

Inclusivity 100% evaluated using strains from ST76 (*sandyi*), a cysG variant of 'ST76', ST53 (*pauca*), ST2 (*fastidiosa*) and ST26 (*multiplex*) (AGES).

In an additional analysis along with the TPS (EU-XF-PT-2017-02, Bari) *X. fastidiosa* subsp. *pauca* (CODIRO) could be detected by this test (AGES).

4.3. Data on repeatability

Evaluated by AGES alongside the TPS (EU-XF-PT-2017-02, Bari) with two different extractions methods [CTAB and Mericon Food Kit (Qiagen)] on spiked olive plant sap, 100%.

4.4. Data on reproducibility

100% with two different real-time PCR machines, two operators and two different master mixes.

4.5. Data on diagnostic sensitivity

Evaluated by AGES: manual extraction 100% on more than 500 natural samples (infected: not infected; 1:10) compared with the real-time data of Harper et al. (2010).

4.6. Data on diagnostic specificity

More than 500 natural samples (infected: not infected, 1:10) compared with the real-time PCR test of Harper et al. (2010): 100%.

4.7. Data on accuracy

Data evaluated by AGES alongside the TPS (EU-XF-PT-2017-02, Bari) compared with Harper and Li with two different extraction methods [CTAB and Mericon Food Kit (Qiagen)] on spiked olive plant sap: 100%. Accuracy was also evaluated on more than 500 natural samples (infected: not infected, 1:10) compared with the real-time of Harper et al. (2010): 100%

APPENDIX 8 - REAL-TIME PCR (LI 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 verification (see PM 7/98) is carried out.

1. General information

1.1. This PCR is suitable for the detection and identification of *X. fastidiosa*.
1.2. The test is based on Li et al. (2013).
1.3. The target sequence is 16S rRNA (XF_r01).
1.4. Oligonucleotides:

The amplification conditions have been modified from the original paper and adapted to the real-time PCR conditions validated for other real-time PCR tests used to detect *X. fastidiosa*. Two different modifications of the TaqMan probes have been tested: (A) TaqMan probe labelled with the MGB technology (Applied Biosystem); (B) TaqMan probe labelled with standard fluorophore and quencher.

(A) Test with the MGB-TaqMan probe (labelled with FAM)

Primers	Sequence
Forward primer XF16Sf	5′-CGG CAG CAC GTT GGT AGT AA-3′
Reverse primer XF16Sr	5′-CCG ATG TAT TCC TCA CCC GTC-3′
Probe XF16Sp	5′-FAM-CA TGG GTG GCG AGT GGC-MGBNFQ-3′

(B) Real-time PCR (Li et al., 2013) with standard TaqMan probe

Primers	Sequence
Forward primer XF16Sf	5′-CGG CAG CAC GTT GGT AGT AA-3′
Reverse primer XF16Sr	5′-CCG ATG TAT TCC TCA CCC GTC-3′
Probe XF16Sp	5′-FAM-CA TGG GTG GCG AGT GGC-BHQ1-3′

2. Methods

2.1. Nucleic acid extraction and purification
2.1.1. Matrices: plants, insects or pure cultures
2.1.2. See Appendix 3 for extraction procedures

It should be noted that the diagnostic sensitivity of the test was lower for olive samples with the DNA extraction based on the CTAB protocol than with DNA

extracted using the DNeasy Mericon Food Kit (Qiagen). A link to the report is available in Section 4.

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	6.63	N.A.
TaqMan Fast Universal Master Mix (Applied Biosystems)	2×	10	1×
Forward primer (XF16Sf)	10 µM	0.5	0.25 µM
Reverse primer (XF16Sr)	10 µM	0.5	0.25 µM
Probe MGB (XF16Sp)[a]	10 µM	0.25	0.125 µM
BSA	50 µg/µL	0.12	0.3 µg/µL
Subtotal		18	
Bacterial suspension or DNA extract		2	
Total		20	

[a] For test A the MGB-TaqMan probe labelled with FAM is used, for test B the standard TaqMan probe (FAM-BHQ1) is used

2.2.2. PCR conditions

95°C for 10 min, followed by 40 cycles of (94°C for 10 s and 60°C for 40 s).

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: the molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential
- NIC and NAC should give no amplification

When these conditions are met:

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

4. Performance characteristics available

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

Fourteen laboratories participated in a comparison of different real-time PCR tests on extracts from olive tissues spiked with bacterial suspension of *X. fastidiosa* subsp. *pauca* strain De Donno, at known concentrations (cfu/mL). This comparison was organized in the framework of the EU projects XF-ACTORS and PONTE and a Euphresco project. See 'Molecular detection of *Xylella fastidiosa* by real-time tests' at https://upload.eppo.int/download/298ocd8b7f525. Values of performance characteristics obtained for the real-time PCR Li et al., 2013 with standard TaqMan probe and MGB-TaqMan probe using DNA extracts prepared using for CTAB-based and DNeasy® mericon™ Food kit (Qiagen) protocols are presented:

TPS performance criteria (by CNR)	Test with standard TaqMan probe		Test with the MGB-TaqMan probe	
	CTAB	Mericon	CTAB	Mericon
Diagnostic sensitivity				
%	100	100	100	100
N. of laboratories with false negative	0	0	0	0
Diagnostic specificity				
%	92	97	67	97
N. of laboratories with false positive	3	1	6	1
Accuracy				
%	96	99	92	99
Repeatability				
%	97	99	97	99
Reproducibility				
%	96	99	92	99
Number of laboratories	13	11	13	11

APPENDIX 9 - TRIPLEX REAL-TIME PCR (BONANTS ET AL., 2019)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This triplex PCR is suitable for the detection and identification of *X. fastidiosa*.
1.2. The test is based on Bonants et al. (2019).
1.3. The target sequences are located in the cobalamin synthesis protein coding gene and the gene coding for the 16S rRNA processing RimM protein.
1.4. An internal control is included. *Acidovorax cattleyae* is a bacterium added in a known quantity to check extraction and PCR efficiency. The primer set was originally developed by Syngenta and modified by Naktuinbouw.
1.5. Oligonucleotides:

Primers	Sequence
Forward primer XF-F	5′-CAC GGC TGG TAA CGG AAG A-3′
Reverse primer XF-R	5′-GGG TTG CGT GGT GAA ATC AAG-3′
Probe XF-P	5′-FAM-TCG CAT CCC GTG GCT CAG TCC-31ABkFQ-3′
Forward primer Xf.Csp6F	5′-CCC ATT ACG CTT CAA CCA TT-3′
Reverse primer Xf.Csp6R	5′-CCC AAT CCA TAC GAC TTG CT-3′
Probe Xf.Csp6P	5′-HEX-GGT GTG ATT CGC AGC AAG GGC-31ABkFQ-3′
Internal control forward primer Acat-2-F	5′-TGT AGC GAT CCT TCA CAA G-3′
Internal control reverse primer Acat 2-R	5′-TGT CGA TAG ATG CTC ACA AT-3′
Probe Acat 2-P	5′-Texas Red-CTT GCT CTG CTT CTC TAT CAC G-31AbRQsp-3′

1.6. The real-time PCR system used to generate the validation data below was a Bio-Rad CFX384 thermocycler.

2. Methods

2.1. Nucleic acid extraction and purification
2.1.1. Matrices: plant or pure cultures.
2.1.2. See Appendix 3 for extraction procedures.
2.1.3. The internal control bacterium *Acidovorax cattleyae* (Acat) is grown in Tryptic Soy Broth medium to a density of OD_{600}=0.8. The cell suspension is diluted 100× with 0.01 M PBS and 5 µL is added to the plant extract before DNA extraction, giving a C_t value of about 30 for the Acat TaqMan PCR. Alternatively, a gBlock, based on sequences of Acat, can be used as internal control, as described by Bonants et al. (2019).

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	3.8	N.A.
Premix ExTaq (TaKaRa RR390A)	2×	10	1×
Forward primer XF-F	10 µM	0.6	0.3 µM
Forward primer Xf.Csp6F	10 µM	0.6	0.3 µM
Forward primer Acat-2-F	10 µM	0.6	0.3 µM
Reverse primer XF-R	10 µM	0.6	0.3 µM
Reverse primer Xf.Csp6R	10 µM	0.6	0.3 µM
Reverse primer Acat 2-R	10 µM	0.6	0.3 µM
Probe XF-P	10 µM	0.2	0.1 µM
Probe Xf.Csp6P	10 µM	0.2	0.1 µM
Probe Acat 2-P	10 µM	0.2	0.1 µM
Subtotal		18	
DNA extract		2	
Total		20	

2.2.2. PCR conditions: 2 min at 95°C, 40 cycles of (15 s at 95°C, 40 s at 60°C).

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: the molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL.

The gBlock containing the Harper et al. amplicon sequence and the Ouyang et al. amplicon sequence as described by Bonants et al. (2019) can be used for detection of *X. fastidiosa*.

The gBlock containing the *Acidovorax cattleyae* (Acat) internal control amplicon sequence as described by Bonants et al. (2019) can be used to detect the Acat internal control.

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (and IPC) amplification curves should be exponential
- NIC and NAC should give no amplification

When these conditions are met:

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

4. Performance characteristics available

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

Data are from Bonants et al. (2019).

4.1. Analytical sensitivity: 10 copies of target DNA.

4.2. Analytical specificity (inclusivity) 3 strains of *X. fastidiosa* subsp. *pauca* (CoDiRo, CFBP 8074, CFBP 8072), 3 strains of *X. fastidiosa* subsp. *multiplex*

(LMG 09063, CFBP 8430, CFBP 8434) and 2 strains of *X. fastidiosa* subsp. *fastidiosa* (LMG 17159, Temecula) were tested positively with the triplex TaqMan PCR.

Exclusivity: 20 strains of *Xanthomonas* species (including *X. fragariae*, *X. populi*, *X. arboricola* and *X. vesicatoria*) and 14 strains of other bacteria (including *Pantoea*, *Erwinia*, *Agrobacterium*, *Pseudomonas*, *Clavibacter*, *Rhodococcus*, *Bacillus* and *Dickeya*) did not react positively with the triplex TaqMan PCR.

4.3. Repeatability: dilution series of the *Xylella* gBlock and DNA of *X. fastidiosa* subsp. *pauca* (CoDiRo) and *X. fastidiosa* subsp. *multiplex* (LMG 09063) were tested many times (>10) with 100% repeatability. DNA extracts from infected plants were tested in duplicate with triplex TaqMan PCR with the same C_t values.

4.4. Reproducibility: samples for repeatability were tested by three different people with equal results on different days. Three real-time PCR thermocyclers (ABI7500, Bio-Rad CFX384, ThermoFisher Quantstudio) were used.

4.5. Other information

Bonants et al. (2019) state that: '[the diagnostic] sensitivity of the triplex assay was compared with a duplex assay based on a combination of the Harper TaqMan and the TaqMan for the internal control, using symptomatic tobacco plant material that had been infected artificially with the CoDiRO strain. In this comparison, the Ct-values of the Harper TaqMan assay in the duplex and triplex format were largely similar. Thirdly, the [diagnostic] sensitivity of the triplex assay with a Harper simplex assay was compared using 24 DNA extracts from spiked, artificially inoculated and naturally infected or *X. fastidiosa* -free plant extracts from different origins within Europe. Also in this experiment, the Ct-values of the triplex and simplex assay were largely similar indicating the same [analytical] sensitivity.'

APPENDIX 10 - TETRAPLEX REAL-TIME PCR (DUPAS ET AL., 2019)

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 verification (see PM 7/98) is carried out.

1. General Information

1.1. This tetraplex real-time PCR is suitable for the detection and the identification of *X. fastidiosa* and assignment of subspecies on plant samples and pure cultures.

1.2. The test is based on Dupas et al. (2019)

1.3. The target sequences are: (a) the gene coding for the Ketol-acid reductoisomerase (WP_004084873) (M23) (b) the restriction modification system (ACB93575) (M23); (c) a genome region with unknown function (M23); (d) the gene coding for the DNA adenine methylase (WP_004083560) (M12); (e) the gene coding for the Peptidase S24 (AIC14009) (MUL0034); (f) the gene coding for the Histidine kinase, ABC transporter substrate-binding (ARO67912, ARO69620) (De Donno);

1.4. Oligonucleotides:

Primers[a]	Sequence	Species/ subspecies
Forward primer XF-F	5′-AAC CTG CGT GAC TCT GGT TT-3′	*X. fastidiosa*
Reverse primer XF-R	5′-CAT GTT TCG CTG CTT GGT CC-3′	
Probe XF-P	5′-FAM-GCT CAG GCT GAC GGT TTC ACA GTG CA-BHQ1-3′	
Forward primer XFF-F	5′-TTA CAT CGT TTT CGC GCA CG-3′	*X. fastidiosa* subsp. *fastidiosa*
Reverse primer XFF-R[a]	5′-TCG GTT GAT CGC AAT ACC CA-3′	
Probe XFF-P[a]	5′-HEX-CCC GAC TCG GCG CGG TTC CA-BHQ1-3′	
Forward primer XFFSL-F	5′-TAG TAT GCG TGC GAG CGA C-3′	*X. fastidiosa* subsp. *morus*/ *fastidiosa*/ *sandyi*
Reverse primer XFFSL-R	5′-CGC AAT GCA CAC CTA AGC AA-3′	
Probe XFFSL-P	5′-HEX-CGC GTA CCC ACT CAC GCC GC-BHQ1-3′	
Forward primer XFM-F	5′-ACG ATG TTT GAG CCG TTT GC-3′	*X. fastidiosa* subsp. *multiplex*
Reverse primer XFM-R	5′-TGT CAC CCA CTA CGA AAC GG-3′	
Probe XFM-P	5′-ROX- ACG CAG CCC ACC ACG ATT TAG CCG-BHQ2-3′	
Forward primer XFMO-F	5′-TAA CGC TAT CGG CAG GTA GC-3′	*X. fastidiosa* subsp. *morus*
Reverse primer XFMO-R	5′-GCA TCA GCT TCA CGT CTC CT-3′	
Probe XFMO-P	5′-CY5- GGT TCC GCA CCT CAC ATA TCC GCC C-BHQ2-3′	
Forward primer XFP-F	5′-TGC GTT TTC CTA GGT GGC AT-3′	*X. fastidiosa* subsp. *pauca*
Reverse primer XFP-R	5′-GTT GGA ACC TTG AAT GCG CA-3′	
Probe XFP-P	5′-CY5- CCA AAG GGC GGC CAC CTC GC-BHQ2-3′	

[a] Primers and probes from Harper et al. (2010) and Ioos et al. (2009) can also be used in this tetraplex real-time PCR. These are described in Appendix 5 Real-time PCR (Harper et al., 2010; erratum 2013).

1.5. The real-time PCR system used to generate the validation data presented below is CFX 384, Bio-Rad thermocycler.
1.6. The software used for data analysis was Bio-Rad CFX Manager 3.1 and the regression mode was applied.

2. Methods

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

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	2.8	N.A.
Master mix SsoAdvanced™ Universal Probes Supermix (Bio-Rad)	2×	5	1×
Forward Primer 1[a]	50 µM	0.115	0.575 µM
Reverse Primer 1	50 µM	0.115	0.575 µM
Probe 1	50 µM	0.04	0.2 µM
Forward Primer 2	50 µM	0.115	0.575 µM
Reverse Primer 2	50 µM	0.115	0.575 µM
Probe 2	50 µM	0.04	0.2 µM
Forward Primer 3	50 µM	0.115	0.575 µM
Reverse Primer 3	50 µM	0.115	0.575 µM
Probe 3	50 µM	0.04	0.2 µM
Forward Primer 4	50 µM	0.115	0.575 µM
Reverse Primer 4	50 µM	0.115	0.575 µM
Probe 4	50 µM	0.04	0.2 µM
BSA (non-acetylated) (Invitrogen)	50 µg/µL	0.12	600 ng/µL
Subtotal		9	
DNA dilution		1	
Total		10	

[a] Different sets of primers and probes are described in Dupas et al., 2019. Due to limitations of the real-time PCR system only four primers and probes can be mixed together with different fluorophores. It is up to the user to decide which ones they wish to use.

2.2.2. PCR conditions: 95°C for 3 min, followed by 40 cycles of (95°C for 15 s and 60°C for 30 s).

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: extracted DNA or boiled cells from a suspension of *X. fastidiosa* at approximately 10^8 cfu/mL ($OD_{600\,nm}$ = 0.1).

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results:

Verification of the controls

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

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

4. Performance characteristics available

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

A Validation data from Dupas et al. (2019)

4.1. Analytical sensitivity data

Sample	Primers	Detection threshold (100%)
Cistus monspeliensis	XF, XFF, XFFSL, XFM primers	10^4 bacteria/mL
Cistus monspeliensis	Harper's primers	10^2 bacteria/mL
Citrus clementina	XF primers	10^4 bacteria/mL
Citrus clementina	XFM, XFP primers	10^3 bacteria/mL
Citrus clementina	Harper's primers	10^2 bacteria/mL
Helichrysum italicum	XF, XFM, Harper's primers	10^3 bacteria/mL
Lavandula angustifolia	XF, XFM, XFP primers	10^4 bacteria/mL
Lavandula angustifolia	Harper's primers	10^3 bacteria/mL
Nerium oleander	XF, XFM, XFP primers	10^4 bacteria/mL
Nerium oleander	Harper's primers	10^3 bacteria/mL
Olea europaea	XF, XFM primers	10^5 bacteria/mL
Olea europaea	XFP primers	10^4 bacteria/mL
Olea europaea	XFMO, Harper's primers	10^3 bacteria/mL
Polygala myrtifolia	XFF, XFM, XFP primers	10^4 bacteria/mL
Polygala myrtifolia	XF, XFFSL, XFMO, Harper's primers	10^3 bacteria/mL
Prunus cerasus	XFF, XFM primers	10^5 bacteria/mL
Prunus cerasus	XF, XFFSL primers	10^4 bacteria/mL
Prunus cerasus	Harper's primers	10^3 bacteria/mL
Prunus dulcis	XF, XFF, XFFSL, XFM, XFP primers	10^4 bacteria/mL
Prunus dulcis	Harper's primers	10^3 bacteria/mL
Quercus ilex	XF, XFM primers	10^4 bacteria/mL
Quercus ilex	XFF primers	10^3 bacteria/mL
Quercus ilex	Harper's primers	10^2 bacteria/mL
Quercus robur	XF primers	10^5 bacteria/mL
Quercus robur	XFM primers	10^4 bacteria/mL
Quercus robur	Harper's primers	10^3 bacteria/mL
Rosmarinus officinalis	XF, XFM primers	10^4 bacteria/mL
Rosmarinus officinalis	Harper's primers	10^3 bacteria/mL

4.2. Analytical specificity data

Inclusivity: 100% evaluated on 39 target strains of *X. fastidiosa* (10 strains of *X. fastidiosa* subsp. *fastidiosa*, 16 strains of *X. fastidiosa* subsp. *multiplex*, 1 strain of *X. fastidiosa* subsp. *morus*, 7 strains of *X. fastidiosa* subsp. *pauca*, 5 strains of *X. fastidiosa* subsp. *sandyi*). Each primer amplified the strains of the subspecies they were designed for.

Exclusivity: 100% evaluated on 30 non-target strains: 3 *Agrobacterium* spp., 1 *Clavibacter*, 1 *Dickeya*, 1 *Ensifer*, 1 *Erwinia*, 2 *Pantoea* spp., 4 *Pseudomonas* spp., 1 *Rhizobium* spp., 1 *Stenotrophomonas* spp., 14 *Xanthomonas* spp., 1 *Xylophilus* spp. In addition, the subspecies specific primers were evaluated on the *X. fastidiosa* belonging to the other subspecies. No cross-reactions were observed.

B Other validation data

Performance characteristics of the tetraplex set #5 (XF Harper, XFM, XFP, XFFSL), generated by ANSES, for a total volume of Master Mix of 20 µL including a DNA template of 2 µL can be downloaded from the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/validationlist).

APPENDIX 11 - REAL-TIME PCR (HODGETTS ET AL., 2021)

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 verification (see PM 7/98) is carried out.

1. General Information

1.1. These real-time PCR tests can be used for the detection and identification of *Xylella fastidiosa* subspecies.

1.2. These tests are based on Hodgetts et al., 2021
1.3. The target sequences are located in the following genes: the gene coding for pilA (*X. fastidiosa* subsp. *fastidiosa*), the gene coding for the histone-like protein (*X. fastidiosa* subsp. *multiplex*), a gene coding for a hypothetical protein (*X. fastidiosa* subsp. *pauca*), the gene coding for a putative hemagglutinin-related protein (*X. fastidiosa* subsp. *morus*), a gene coding for a hypothetical protein (*X. fastidiosa* subsp. *sandyi*)
1.4. Oligonucleotides for subsp. *fastidiosa*:

Primers	Sequence
Forward primer	5′-TCG TTG TGG GAT TAC CGT TAA A-3′
Reverse primer	5′-ACC TGA GAA TTG CCC TTA ATC G-3′
Probe	5′-FAM-TCG AAA ACA CCG GAC TTG CCA ACA-BHQ1-3′

1.5. Oligonucleotides for subsp. *multiplex*:

Primers	Sequence
Forward primer	5′-CAA TCG CTT TTG AGG TCA TCC-3′
Reverse primer	5′-GCG ATT GTT TCT TCT CTA CAC CAA G-3′
Probe	5′-FAM-TCT GCA AAC GCT TTA AAA ACT GCT CGC C-BHQ1-3′

1.6. Oligonucleotides for subsp. *pauca*:

Primers	Sequence
Forward primer	5′-GCA TCC TCA CCA CCG AAG G-3′
Reverse primer	5′-TCC ACA TCC AGC AAG GTG AC-3′
Probe	5′-FAM-CCT TGG ACG CGG ATA CCC GCA-BHQ1-3′

1.7. Oligonucleotides for subsp. *morus*:

Primers	Sequence
Forward primer	5′-CCA CCT CGC TTT AGT TAC GTG ATT-3′
Reverse primer	5′-GGA GTT TAT TTG GCT GAA CTG AGT G-3′
Probe	5′-FAM- AAG CGT GAT ACT ACT CC- MGB-NFQ-3′

1.8. Oligonucleotides for subsp. *sandyi*:

Primers	Sequence
Forward primer	5′-CCC CGC TGT GGC AGA A-3′
Reverse primer	5′-GGT CCG AGC CAT ACG GC-3′
Probe	5′-FAM- CAG CGC CTT CAA TC- MGB-NFQ-3′

1.9. The real-time PCR systems used to generate the validation data below is 7900HT Real-time PCR system (Applied Biosystems®)

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. Matrices: plants or pure cultures
2.1.2. See Appendix 3 for extraction procedures
2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time Polymerase Chain Reaction – real-time PCR
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	5.38	N.A.
TaqMan Fast Universal PCR Master Mix no AmpErase® UNG (*Applied Biosystems*®)	2×	10	1×
Bovine Serum Albumin (UltraPure™ BSA, non-acetylated, Invitrogen™)	50 µg/µL	0.12	0.3 µg/µL
Forward Primer	7.5 µM	1	375 nM
Reverse Primer	7.5 µM	1	375 nM
Probe	5 µM	0.5	125 nM
Subtotal		18	
Nucleic acid extract		2	
Total		20	

2.2.2. PCR conditions: 95°C for 10 min followed by 40 cycles of (95°C for 10 s, 62°C for 40 s).

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: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results:

Verification of the controls

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

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

4. Performance characteristics available

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

The test was validated by Fera (GB) in accordance with PM 7/98.

4.1. Analytical sensitivity data

124 fg of DNA of *X. fastidiosa* subsp. *fastidiosa* are detected.

182 fg of DNA of *X. fastidiosa* subsp. *multiplex* are detected.

84.2 fg of DNA of *X. fastidiosa* subsp. *pauca* are detected.

59.2 fg of DNA of *X. fastidiosa* subsp. *morus* are detected.

90.8 fg of DNA of *X. fastidiosa* subsp. *sandyi* are detected.

4.2. Analytical specificity data

Inclusivity evaluated on the following targets: *X. fastidiosa* subsp. *fastidiosa* (LMG 17159; LMG 15099; NCPPB 4605), *X. fastidiosa* subsp. *multiplex* (LMG 9063; NCPPB 4604), *X. fastidiosa* subsp. *pauca* (NCPPB 4595), *X. fastidiosa* subsp. *morus* (NCPPB 4589), *X. fastidiosa* subsp. *sandyi* (NCPPB 4606), 6 naturally infected plant samples and 5 artificially infected samples: 100%

Exclusivity evaluated on the other *X. fastidiosa* subspecies, 38 non-target species (*Burkholderia* spp. NCPPB 2889, 'Ca. Liberibacter africanus', 'Ca. Liberibacter solanacearum', 'Ca. Phytoplasma asteris', 'Ca. Phytoplasma ulmi', *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 3896, *Cupriavidus necator* NCPPB 4048, *Enterobacter* spp. NCPPB 4168, *Ochrobactrum anthropi* NCPPB 4170, *Paenibacillus polymyxa* NCPPB 4162, *Pantoea agglomerans* NCPPB 1931, *Pseudomonas marginalis* pv. *marginalis* NCPPB 4163, *Pseudomonas syringae* pv. *persicae* NCPPB 3686, *Pseudomonas syringae* pv. *persicae* NCPPB 3687, *Ralstonia pickettii* NCPPB 3899, *Ralstonia solanacearum* NCPPB 787, *Ralstonia solanacearum* NCPPB 3205, *Ralstonia syzygii* NCPPB 3792, *Ralstonia syzygii* subsp. *celebesensis* NCPPB 3726, *Agrobacterium radiobacter* NCPPB 2404, *Rhizobium vitis* NCPPB 2562, *Rhizobium vitis* NCPPB 3269, *Rhizobium vitis* NCPPB 3554, *Spiroplasma citri* NCPPB 2565, *Xanthomonas albilineans* NCPPB 887, *Xanthomonas arboricola* pv. *celebensis* NCPPB 1630, *Xanthomonas arboricola* pv. *fragariae* NCPPB 4183, *Xanthomonas arboricola* pv. *juglandis* NCPPB 411, *Xanthomonas axonopodis* pv. *dieffenbachiae* NCPPB 1833, *Xanthomonas axonopodis* pv. *dieffenbachiae* NCPPB 3380, *Xanthomonas axonopodis* pv. *vasculorum* NCPPB 186, *Xanthomonas axonopodis*

pv. *vasculorum* NCPPB 899, *Xanthomonas campestris* pv. *campestris* NCPPB 45, *Xanthomonas campestris* pv. *campestris* NCPPB 529, *Xanthomonas campestris* pv. *musacearum* NCPPB 4389, *Xanthomonas campestris* pv. *musacearum* NCPPB 4387, *Xanthomonas citri* NCPPB 409, *Xanthomonas citri* subsp. *citri* NCPPB 3799, *Xanthomonas fuscans* subsp. *aurantifolii* NCPPB 3233, *Xanthomonas hortorum* pv. *pelargonii* NCPPB 2985, *Xanthomonas hortorum* pv. *pelargonii* NCPPB 4031, *Xanthomonas oryzae* pv. *oryzae* NCPPB 3002, *Xanthomonas perforans* NCPPB 4321, *Xanthomonas vasicola* pv. *holcicola* NCPPB 989, *Xanthomonas vasicola* pv. *holcicola* NCPPB 1060, *Xanthomonas vasicola* pv. *vasculorum* NCPPB 206, *Xanthomonas vasicola* pv. *vasculorum* NCPPB 702, *Xanthomonas vesicatoria* NCPPB 422, *Xanthomonas vesicatoria* NCPPB 701, *Xylella taiwanensis* NCPPB 4612), 1 insect species (*Philaenus spumarius*): 100%

4.3. Data on Repeatability

High bacterial DNA concentration: 100%; Low bacterial DNA concentration: 100%; Very low bacterial DNA concentration: test dependent (0%–62.5%)

4.4. Data on Reproducibility

High bacterial DNA concentration: 100%; Low bacterial DNA concentration: 92%–100%; Very low DNA concentration: test dependent (8%–63%)

4.5. Diagnostic sensitivity

100%

4.6. Diagnostic specificity

100%

APPENDIX 12 - REAL-TIME LAMP TEST

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This test is suitable for the detection of *X. fastidiosa* in plants or insects.
1.2. The test is based on primers developed by Harper et al. (2010; erratum 2013) and was modified by Yaseen et al. (2015).
1.3. The target sequence is located at the 16S rRNA processing gene *rimM* of *X. fastidiosa*.
1.4. Oligonucleotides:

Primers	Sequence
External XF-F3 primer	5′-CCG TTG GAA AAC AGA TGG GA-3′
External XF-B3 primer	5′-GAG ACT GGC AAG CGT TTG A-3′
Internal XF-FIP primer	5′-ACC CCG ACG AGT ATT ACT GGG TTT TTC GCT ACC GAG AAC CAC AC-3′
Internal XF-BIP primer	5′-GCG CTG CGT GGC ACA TAG ATT TTT GCA ACC TTT CCT GGC ATC AA-3′
Loop XF-LF primer	5′-TGC AAG TAC ACA CCC TTG AAG-3′
Loop XF-LB primer	5′-TTC CGT ACC ACA GAT CGC T-3′

2. Methods

2.1. Nucleic acid extraction and purification
2.1.1. Matrices: plants or insects (Yaseen et al., 2015).
2.1.2. Plant tissues (thin slices of 1-year-old twigs, 1–2 mm thick) or single captured insects (whole specimen) are immersed in 1 mL of extraction buffer (1% Triton X-100, 20 mM Tris-HCl, 20 mM EDTA) and denatured at 95°C for 10 min.
2.1.3. Alternatively, DNA extracts prepared using different extraction procedures (see Appendix 3) can be used for plants or for insects.
2.1.4. DNA should preferably be stored at approximately −20°C.
2.2. LAMP
2.2.1. Ready to use kits are commercially available to perform the test on a specific device or using a standard real-time thermal cycler (e.g. Enbiotech, Qualiplante).
2.2.2. PCR conditions: follow the manufacturer's instructions.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures or *X. fastidiosa* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^4 cfu/mL

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC should produce no fluorescence
- PIC and PAC: for real-time measurement, a positive reaction is defined by time of positivity (25 minutes, ICGENE) and/or melting temperature (TM; 88–88.5°C) as given by the manufacturer Genie®II (OptiGene Limited, Horsham, GB).

When these conditions are met:

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

4. Performance characteristics available

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

Data was generated by the Research Centre for Plant Protection and Certification (CREA-DC, Rome, IT) and the Institute for Sustainable Plant Protection, CNR (Bari, IT).

4.1. Analytical sensitivity data

Olive crude extract spiked with 10-fold dilution of *X. fastidiosa* CoDiRO strain suspensions (from 10^8 to 10 cfu/mL) (Enbiotech s.r.l. kit: 10^3–10^4 cfu/mL).

DNA (CTAB DNA extraction) from olive extracts spiked with 10-fold dilution of *X. fastidiosa* CoDiRO strain suspensions (from 10^8 to 10 cfu/mL) (Qualiplante SAS kit, 10^2–10^3 cfu/mL; Enbiotech s.r.l. kit, 10–10^2 cfu/mL).

4.2. Analytical specificity data

Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (http://dc.eppo.int/validationlist.php).

Exclusivity evaluated by LAMP-PCR (Enbiotech s.r.l. kit) 100% No cross-reactions were observed with the following bacterial strains: 3 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *fragariae*, 1 *X. arboricola* pv. *corylina*, 1 *X. arboricola* pv. *celebensis*, 1 *Xanthomonas campestris* pv. *campestris*, 1 *X. campestris* pv. *populi*, 2 *Xanthomonas hortorum* pv. *pelargonii*, 3 *Pseudomonas savastanoi* pv. *savastanoi*, 1 *Pseudomonas marginalis*, 4 *Pseudomonas syringae* pv. *syringae*, 4 *Brenneria* spp. (*rubrifaciens*, *quercina*, *salicis*, *populi*), 2 *Pantoea stewartii*, 1 *Pantoea agglomerans*, 1 *Erwinia amylovora*, 3 *Agrobacterium tumefaciens*, *2 Rhizobium vitis*.

4.3. Data on repeatability

Olive crude extracts spiked with 10^3, 10^4, 10^6 cfu/mL of *X. fastidiosa* in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 68%.

DNA (CTAB DNA extraction) from olive extracts spiked with 10^3, 10^4, 10^6 cfu/mL in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 91%.

4.4. Data on reproducibility

Olive crude extracts spiked with 10^3, 10^4, 10^6 cfu/mL of *X. fastidiosa* in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 63%.

DNA (CTAB DNA extraction) from olive extracts spiked with 10^3, 10^4, 10^6 cfu/mL of *X. fastidiosa* in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 85%.

4.5. Diagnostic sensitivity

Olive crude extract spiked with 10^3, 10^4, 10^6 cfu/mL of *X. fastidiosa* in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 70%.

DNA (CTAB DNA extraction) from olive extracts spiked with 10^3, 10^4, 10^6 cfu/mL of *X. fastidiosa* in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 90%.

4.6. Diagnostic specificity (Enbiotech s.r.l. kit)

Olive crude extract spiked with 10^3, 10^4, 10^6 cfu/mL in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 97%.

DNA (CTAB DNA extraction) from olive extracts spiked with 10^3, 10^4, 10^6 cfu/mL in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 100%.

4.7. Accuracy

Olive crude extract spiked with 10^3, 10^4, 10^6 cfu/mL in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 77%.

DNA (CTAB DNA extraction) from olive extracts spiked with 10^3, 10^4, 10^6 cfu/mL in three repetitions (15 laboratories) and healthy olive extracts, in three repetitions for a total of 12 samples: (Enbiotech s.r.l. kit) 92%.

4.8. Additional validation data

17-XFAST-EU interlaboratory TPS for the evaluation of molecular methods to detect *Xylella fastidiosa* in the vector *Philaenus spumarius*, available at: https://upload.eppo.int/download/268obc05d6355.

APPENDIX 13 - THE *XF* AMPLIFYRP XRT TEST (LI ET AL., 2016)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This test is suitable for the detection of *X. fastidiosa* in symptomatic plants. The test has not been evaluated on insects.

1.2. This test is based on Li et al. (2016).

1.3. The target sequence is located in the disulphide isomerase gene. The probe and primer sequences are protected by IP.

2. Methods

2.1. Sample preparation

2.1.1. Place 300–500 mg of petioles in AMP1 buffer from the kit at a 1:10 weight to volume ratio. Grind the petioles in an extraction bag using a marker pen and let AMP1 buffer lyse the bacterial cells for 10 min.

	LAMP			
	Harper et al. (2010) Modified by Yaseen et al. (2015), no cut-off			
	Thermocycler		Portable device	
TPS performance criteria	QuickPick™	CTAB	QuickPick™	CTAB
Diagnostic sensitivity				
%	82.14	89.58	100.00	97.50
% (restricted series)[a]	95.83	89.58	100.00	97.50
No. of labs with false negatives	3	3	0	1
Diagnostic specificity				
%	100.00	95.83	100.00	100.00
% (restricted series)[b]	100.00	100.00	100.00	100.00
No. of labs with false positive	0	0	1	0
Accuracy				
%	88.10	91.67	100.00	98.33
% (restricted series)[c]	98.33	93.33	100.00	98.33
Repeatability				
%	98.61	95.83	100.00	100.00
Reproducibility				
%	78.13	87.24	100.00	95.50
% (restricted series)[d]	97.63	92.00	100.00	95.50
No. of labs	7	6	2	5

[a] Results of one laboratory excluded (systematic false negatives).

[b] Results of one laboratory excluded (high number of false positives, about 40% on healthy insects).

[c] Results of two laboratories excluded: one had a high number of false positives (about 40% on healthy insects) and another gave systematic false negatives.

[d] Results of three laboratories excluded: one had a high number of false positives (about 40% on healthy insects), one had only one repetition for amplification and a third laboratory gave systematic false negatives.

2.1.2. Prior to testing, dilute the extract 1:200 in PD1.
2.1.3. Rehydrate the pellet with 25 µL of the diluted PD1 extract.

2.2. Test procedure
2.2.1. *Xf* AmplifyRP XRT+ kits are commercially available to perform real-time tests (20 min) on a portable fluorometer (AmpliFire, Douglas Scientific).
2.2.2. *Xf* AmplifyRP XRT+ kits are commercially available to perform end-point tests (20 min) on a portable heat block (Agdia) followed by placing the completed reaction inside an amplicon detection chamber (20 min) that houses a lateral flow strip.

3. Essential procedural information
3.1. Controls
3.1.1. Negative control 1 (AMP1 buffer): to monitor contamination.
3.1.2. Negative control 2 (healthy host tissue): to rule out possible background reaction caused by plant tissue.
3.1.3. Positive control (inactivated *X. fastidiosa* bacterial culture or infected leaf petioles).

3.2. Interpretation of results
3.2.1. Real-time test result is available next to the well designation on the screen:

(+) = positive for *X. fastidiosa*
(–) = *X. fastidiosa* not detected
(!) = invalid.

3.2.2. End-test result shown on lateral flow strip:

Positive = control and test lines are both visible
Negative = control line is visible and test line is not visible
Invalid = control line not visible.

4. Performance criteria available

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

(A) Data from INRAE (Angers, FR)

Additional data is available in the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (http://dc.eppo.int/validationlist.php).

A4.1 Analytical sensitivity

Plant crude extracts:

Plant crude extracts spiked with 10-fold dilutions of *X. fastidiosa* suspensions (from 10^6 to 10^2 cells/mL) from different subspecies (3 independent series/*Xf* strain). Recombinase polymerase amplification (RPA) was conducted with the AmplifyRP® XRT+ for Xf (*Xylella fastidiosa*) kit (Agdia) (real-time tests with fluorometer); prior to testing, the extract was diluted 1:100 in PD1 pellet diluent which was the protocol at the time of the evaluation presented below:

10^6 cells/mL for *Quercus* sp. and *Vitis vinifera*;

10^5 cells/mL for Prunus dulcis, Prunus cerasifera, Polygala myrtifolia, Citrus sp., Helichrysum italicum, Nerium oleander, Lavandula sp.;

10^4 cells/mL for Olea europaea

Inhibitions of RPA observed with *Holm oak* and *Cistus monspeliensis* (60% and 11% detection at 10^6 cells/mL respectively).

Bacterial DNA (CFBP 8416 strain):

Extraction with PROMEGA Wizard® Genomic DNA Purification Kit; series of 10-fold dilution in PD1 pellet diluent (from 25.2 ng/µL to 0.252 fg/µL) from the AmplifyRP® XRT+ for Xf kit:

2.52 fg/µL: which corresponds to 1 copy/µL or 25 copies/reaction; number of copies were calculated using an estimated genome size for CFBP 8416, of 2506765 bp, knowing that 1 pg = 9.78×10^8 bp (Doležel et al., 2003)

A4.2 Analytical specificity

Inclusivity:

22 target strains: *X. fastidiosa* subsp. *fastidiosa* (4 strains), *X. fastidiosa* subsp. *multiplex* (12 strains), *X. fastidiosa* subsp. *pauca* (3 strains), *X. fastidiosa* subsp. *morus* (1 strain), *X. fastidiosa* subsp. *sandyi* (2 strains): 100% positive reactions

Exclusivity:

30 non-target species were tested, (*Xanthomonas arboricola* pv. *juglandis* CFBP 2528, *Xanthomonas arboricola* pv. *pruni* CFBP 2535, *Xanthomonas axonopodis* pv. *axonopodis 9.3* CFBP 4924, *Xanthomonas campestris* pv. *campestris* CFBP 5241, *Xanthomonas citri* pv. *aurantifolii 9.6* CFBP 2901, *Xanthomonas citri* pv. *citri 9.5* CFBP 2525, *Xanthomonas citri* pv. *viticola 9.5* CFBP 7660, *Xanthomonas gardneri* CFBP 2625, *Xanthomonas hortorum* pv. *pelargonii* CFBP 2533, *Xanthomonas hyacinthi* CFBP 1156, *Xanthomonas oryzae* pv. *oryzae* CFBP 2532, *Xanthomonas translucens* pv. *translucens* CFBP 2054, *Xanthomonas vasicola* pv. *holcicola* CFBP 2543, *Xylophilus ampelinus* CFBP 1192, *Xanthomonas maltophilia* 13100, *Pseudomonas amygdali* CFBP 3205, *Agrobacterium rubi* CFBP 6448, *Agrobacterium tumefaciens* CFBP 2413, *Agrobacterium vitis* CFBP 5523, *Clavibacter michiganensis* subsp. *insidiosus* CFBP 2404, *Dickeya dianthicola* CFBP 1200, *Ensifer meliloti* CFBP 5561, *Erwinia amylovora* CFBP 1232, *Pantoea agglomerans* CFBP 3845, *Pantoea stewartii* subsp. *stewartii* CFBP 3167, *Pseudomonas cerasi*

CFBP 8305, *Pseudomonas syringae* pv. *persicae* CFBP 1573, *Pseudomonas syringae* pv. *syringae* CFBP 1392, *Rhizobium nepotum* CFBP 7436, *Xanthomonas alfalfae* subsp. *citrumelonis 9.2* CFBP 3371) and no cross reactions were observed.

A 4.3 Data on Repeatability

	10^6 cells/mL	10^5 cells/mL	10^4 cells/mL	10^3 cells/mL	10^2 cells/mL
Holm oak	60	20	20	0	0
Cistus monspeliensis	11	0	0	0	0
Prunus dulcis	100	100	87	0	0
Prunus cerasifera	89	100	89	11	0
Polygala myrtifolia	100	100	22	0	0
Vitis vinifera	100	83	67	17	0
Quercus sp.	100	80	60	60	0
Citrus sp.	93	100	47	20	0
Helichrysum italicum	100	100	67	33	0
Nerium oleander	100	100	83	17	0
Lavandula sp.	100	100	67	17	0
Olea europaea	100	100	100	20	0

When an amplicon detection chamber (Agdia) is used after RPA with samples showing a small amplification by RPA under the threshold of the AmpliFire fluorometer, detection can be improved to 10^4 and 10^3 cells/mL.

A4.4 Data on Reproducibility

Not evaluated

A4.5 Diagnostic sensitivity

Not evaluated

A4.6 Diagnostic specificity

100%

(B) Data provided by AGDIA

B4.1 Analytical sensitivity data

The limit of detection (LoD): 22 copies of *X. fastidiosa* genomic DNA spiked in crude petiole extract

Plant species	Extraction buffer	Host tissue	Positive samples/total samples
Citrus	AMP1	Petiole/midrib	52/52
Vitis vinifera	AMP1	Petiole	12/12
Prunus dulcis	AMP1	Petiole/midrib	12/12
Olea europaea	AMP1	Petiole/midrib	12/12

B4.2 Analytical specificity data

4.2.1. The test reacts to all *X. fastidiosa* isolates collected by Agdia Inc.
4.2.2. The test does not react to closely related bacterial species.
4.2.3. The test does not react to host leaf tissue such as *Citrus*, *Vitis vinifera*, *Prunus dulcis*, *Olea europaea*, *Coffea* spp. and *Vaccinium* spp.

B4.3 Data on repeatability

100% detection was obtained for *X. fastidiosa*-infected grapevine, al3mond and blueberry in the laboratory across 14 replicates for each sample preparation of each species.

B4.4 Data on reproducibility

100% detection was obtained for *X. fastidiosa*-infected grapevine, almond and blueberry in the laboratory across three sample preparations for each species.

B4.5 Diagnostic specificity

100% on olive tree samples (Dr Giuliana Loconsole evaluated six infected olive tree samples and 2 healthy olive tree samples).

APPENDIX 14 - BUFFERS AND MEDIA

All buffers and media should be sterilized by autoclaving at 121°C for 15 min unless stated otherwise.

(A) Buffers

Sterile succinate–citrate–phosphate buffer	
Disodium succinate ($Na_2C_4H_4O_4$)	1.0 g
Trisodium citrate ($C_6H_5Na_3O_7$)	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Distilled water to make up to	1 L
Adjust pH to 7.0 before autoclaving	

Phosphate Buffer (PB 10 mM (=0.01 M))	
$Na_2HPO_4.12H_2O$	2.7 g
$NaH_2PO_4.2H_2O$	0.4 g
Distilled water to make up to	1 L
Adjust pH to 7.2 before autoclaving	

Phosphate Buffer Saline (PBS 10 mM (=0.01 M)),	
$Na_2HPO_4.12H_2O$	2.7 g
$NaH_2PO_4.2H_2O$	0.4 g
NaCl	8 g
Distilled water to make up to	1 L
Adjust pH to 7.2 before autoclaving	

CTAB buffer[a]

CTAB	2.0 g
TRIS (1 M autoclaved solution pH 8.0)	10 mL
EDTA (0.5 M autoclaved solution pH 8.0)	4.0 mL
NaCl (5 M autoclaved solution)	28 mL
PVP-40	1.0 g
Distilled sterile water to make up to	100 mL

[a]Do not autoclave. It is recommended to keep the buffer for no longer than 1 week.

TE buffer (100 mL)

TRIS (1 M solution pH 8.0)	1.0 mL
EDTA (0.5 M solution pH 8.0)	0.2 mL
Distilled water to make up to	100 mL

(B) Media

Ingredients should be dissolved in the order given.

- PD2 medium (Davis et al., 1980) (this medium can be used for the isolation of *X. fastidiosa* from several host plants including grapevine)

Soy peptone (BD Difco™, 0436-01)	2.0 g
Bacto tryptone (Oxoid, LP0042)	4.0 g
Disodium succinate (Sigma, S-2378)	1.0 g
Trisodium citrate (Sigma, S-4641)	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH) (Sigma, H-5533)	10.0 mL
Microbiological grade agar (Oxoid, LP0028 or Bacto agar BD Difco™)	15.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Sterile distilled or deionized water to make up to	1.0 L
Adjust pH to 6.9	
BSA fraction V (20% w/v)[a] (Sigma Aldrich, A7030)	10.0 mL

[a] Bovine serum albumin is filter-sterilized and added to the rest of the medium at 50°C after autoclaving.

Modified BCYE medium

Demineralized water	940 mL
Aces buffer (Sigma/A-3594)	10 g
KOH solution 1 M	40 mL[a]
Yeast extract (BD Difco™/212750)	10 g
Activated charcoal (Sigma/C-9157)	2 g
Agar no. 1 (Oxoid/LP011 or Bacto agar BD Difco™/214010)	17 g
Agitate for at least 1 min. The final pH is approximately 6.9	
Cysteine hydrochloride stock solution, see below (Sigma/C-7880)	5 mL
Ferric pyrophosphate stock solution, see below (Sigma/P-6526)	15 mL

[a] Adjust the pH to 6.9 before adding the agar. This is done by adding approximately 40 mL KOH 1 M until the appropriate pH value is reached. Adjust the total volume to 980 mL with the demineralized water.

Stock solutions (filter sterile)

Component (supplier/ order no.)	Final per L	Concentration	Dissolve in
Cysteine hydrochloride (Sigma/C-7880)	400 mg	400 mg per 5 mL	Distilled water
Ferric pyrophosphate (Sigma/P-6526)	250 mg	250 mg per 15 mL	Distilled water

The compound ferric pyrophosphate needs to be heated, under agitation, at 75°C for approximately 15–20 min.

- Modified PWG medium [ANSES, FR, based on Hill & Purcell, 1995 and information provided at the COST Workshop – Bari 2010 (R. Almeida, pers. comm.)]

Gelrite gellan gum (Gelzam™ CM; Sigma G 1910)	9.0 g
Phytone peptone (BD/211906)	4.0 g
Bacto tryptone (Fisher Scientific 11778143 = BD Difco™ 211705)	1.0 g
$MgSO_4 \cdot 7H_2O$	0.4 g
K_2HPO_4	1.2 g
KH_2PO_4	1.0 g
Stock solution of red phenol (0.2% aqueous solution), see below	10 mL
Stock solution of hemin chloride (0.1% solution NaOH 0.05 N) see below	10 mL
Sterile distilled or deionized water	830 mL
BSA (Sigma Aldrich, A7030)	3 g
l-glutamine (Sigma Aldrich, G3126)	4 g

Use a 2-L bottle and autoclave at approximately 121°C for 20 min. Ingredients except BSA and l-glutamine are added mixed and dissolved in the order given.

After autoclaving, allow to cool down to approximately 50°C. Under a horizontal air flow add filtered sterile BSA dissolved in 50 mL of deionized water and l-glutamine dissolved in 100 mL of water at about 50°C.

Stock solution of red phenol (0.2% aqueous solution)

Red phenol	50 mg
Sterile distilled or deionized water	25 mL
Store for a maximum of 1 month at 5 ± 4°C	

In case of solubility problems in water, dissolving in 70% ethanol is possible.

Stock solution of hemin chloride (0.1% solution NaOH 0.05 N)

Hemin chloride	50 mg
Solution NaOH 0.05 N	50 mL
Store for a maximum of 1 month at 5 ± 4°C	

APPENDIX 15 - ISOLATION PROCEDURES

Isolation procedures as currently implemented in different laboratories are presented below. No comparison of these procedures has been performed. Consequently, no recommendation can be made so far regarding the advantages and disadvantages of different procedures.

The conditions for surface disinfection can vary according to the plant tissue, the most commonly used procedures are reported below.

The implementation of an additional ultrasonication step before plating has been shown to increase the success of isolation. Ultrasonication is performed on crushed plant material at a frequency of 40 kHz for 30 s. Durations of 45 s and 60 s have been tested but duration does not influence the number of isolates obtained (Bergsma-Vlami et al., 2017).

1. Isolation from several leaves, option 1: 0.5–1 g of tissue

For each sample, at least 0.5–1 g of tissue (petioles and midribs or basal leaf portions) is used.

Soak sequentially the leaf midribs, petioles or basal leaf portions in a bleach solution (e.g. 2% for 2 min or 0.5% for 5 min) followed by immersion in 70% ethanol for 2 min then three rinses in sterile distilled water.

After surface sterilization, tissues are cut into pieces, placed in a mortar or in a container/test tube with sterile succinate–citrate–phosphate buffer or PBS (see Appendix 14) at a ratio of 1:10 (w:v). Tissues are then ground with a homogenizer (Polytron, Homex, etc.). An aliquot of 100 µL of sap is added to 900 µL of sterile succinate–citrate–phosphate buffer or PBS and used to prepare a serial 10-fold dilution (up to 10^{-5}). Aliquots of 100 µL of 10^{-2}, 10^{-3}, 10^{-4} dilutions are then plated on the specific media, incubated at approximately 28°C and monitored for colony development over 6 weeks. Plates should be sealed or kept in plastic bags to prevent desiccation.

An alternative procedure can also be followed for isolation from twigs and branches. A branch (4–5 cm long) is surface sterilized and cut in the middle, the internal cut ends are squeezed with a pair of pliers and the sap blotted onto agar plates. BCYE medium is the most commonly used medium with this procedure (Coletta-Filho & Machado, 2003). Plates are then incubated as described above.

2. Isolation from several leaves, option 2: up to 10 g of tissue collected from 100 to 200 leaves

For each sample, up to 10 g of tissue collected from 100 to 200 leaves (petioles and midribs or basal leaf portions) is used.

Plant material is disinfected by soaking in a bleach solution (0.5% for 5 min or 2% for 2 min), then rinsed three times with sterile water. The plant material is then dried in tissue paper and briefly disinfected with 70% alcohol. Then the material is dried in a flow cabinet. After disinfection, the plant material is crushed in a stomacher bag. Forty millilitres of buffer (PBS 0.01 M, see Appendix 14) is added and agitated for approximately 30 min at room temperature. The required volume of the extract for screening and for isolation is directly used from the extract obtained after the agitation step. The remaining extract volume is subsequently concentrated (centrifugation for 20 min at 10 000 *g* and 4°C) and resuspended in 1.5 mL PB 0.01 M. This concentrated extract is also used for screening and for isolation. In both cases, i.e. non-concentrated and concentrated extract, isolation is performed by preparation of serial dilutions (non-diluted; 1:10; 1:100) and plating on the specific medium. Incubation should be done at approximately 28°C, and plates monitored for colony development for up to 6 weeks. Plates should be sealed or kept in plastic bags to prevent desiccation.

3. Isolation from individual leaves

After disinfection of the leaf with 70% (v/v) ethanol, a petiole or midrib approximately 1 cm long is collected with a sterile scalpel. Symptomatic leaves should be used for preference, if available. The midrib or petiole is briefly soaked in ethanol at 96% (v/v) and flamed very quickly to achieve surface disinfection without causing a significant temperature rise in the tissues which could kill the bacteria. The sample is immediately placed in a sterile Petri dish with 1–2 mL of sterile saline solution or sterile demineralized water, comminuted and left to soak for at least 15 min, under gentle shaking. One hundred microlitres of the macerate is plated without dilutions. Plates should be sealed or kept in plastic bags to prevent desiccation.

APPENDIX 16 - MULTILOCUS SEQUENCE TYPING (MLST) (YUAN ET AL., 2010)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This test is suitable for the assignment of an isolate to one of the known subspecies using DNA from a pure bacterial culture. It has been used with DNA from plant extracts (Bergsma-Vlami et al., 2017; Denancé et al., 2017; Loconsole et al., 2016) or insects

(Cunty et al., 2020). However, it is recognized that the quantity and quality of target DNA, or the occurrence of possible mixed infections, may prevent all amplicons from being obtained or a clear assignment of subspecies. This is also more difficult for insects than for plant extracts (low concentration of bacteria and limited amount of DNA from a single insect).

1.2. The test on pure culture is based on Yuan et al. (2010). Modifications may be needed for DNA from plant extracts (see Section 2.3 of this appendix).

1.3. The target sequences are those of seven housekeeping genes amplified individually: 2-isopropylmalate synthase (*leuA*); ubiquinol cytochrome *c* oxidoreductase C1 subunit (*petC*); ABC transporter sugar permease (*malF*); sirohaem synthase (*cysG*); DNA polymerase III holoenzyme chi subunit (*holC*); NADH-ubiquinone oxidoreductase NQO12 subunit (*nuoL*); and glutamate symport protein (*gltT*).

1.4. Amplicon size (without primers): 708 bp for l*euA*, 533 bp for *petC*, 730 bp for *malF*, 600 bp for *cysG*, 379 bp for *holC*, 557 bp for *nuoL*, 654 bp for *gltT*.

1.5. Oligonucleotides:

Forward primers	Reverse primer
leuA-for 5′-GGT GCA CGC CAA ATC GAA TG-3′	leuA-rev 5′-GTA TCG TTG TGG CGT ACA CTG-3′
petC-for 5′-GCT GCC ATT CGT TGA AGT ACC T-3′	petC-rev 5′-GCA CGT CCT CCC AAT AAG CCT-3′
malF-for 5′-TTG CTG GTC CTG CGG TGT TG-3′	malF-rev 5′-GAC AGC AGA AGC ACG TCC CAG AT-3′
cysG-for 5′-GCC GAA GCA GTG CTG GAA G-3′	cysG-rev 5′-GCC ATT TTC GAT CAG TGC AAA AG-3′
holC-for 5′-ATG GCA CGC GCC GAC TTC T-3′	holC-rev 5′-ATG TCG TGT TTG TTC ATG TGC AGG-3′
nuoL-for 5′-TAG CGA CTT ACG GTT ACT GGG C-3′	nuoL-rev 5′-ACC ACC GAT CCA CAA CGC AT-3′
gltT-for 5′-TCA TGA TCC AAA TCA CTC GCT T-3′	gltT-rev 5′-ACT GGA CGC TGC CTC GTA AAC C-3′

1.6. The workflow is described in the PubMLST *X. fastidiosa* database (http://pubmlst.org/xfastidiosa).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Nucleic acid source: pure culture, plant extract or insects.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. PCR for MLST (for pure cultures)

2.2.1. Master mix (per reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	35.9	N.A.
PCR buffer (Invitrogen)	10×	5	1×
$MgCl_2$	50 mM	1.5	1.5 mM
BSA (non-acetylated)	50 µg/µL	0.3	0.3 µg/µL
DNTPs	20 mM	0.5	0.2 mM
Forward primer (leuA-for/petC-for/malF-for, cysG-for/holC-for/nuoL-for/gltT-for)	20 µM	0.75	0.3 µM
Reverse primer (leuA-rev/petC-rev/malF-rev/, cysG-rev/holC-rev/nuoL-rev/gltT-rev)	20 µM	0.75	0.3 µM
DNA Polymerase Platinum (Invitrogen)	5 U/µL	0.3	0.03 U/µL
Subtotal		45	
Genomic DNA		5	
Total		50	

2.2.2. PCR conditions (for pure cultures)

95°C for 3 min, 35 cycles of (95°C for 30 s, 65°C for 30 s and 72°C for 60 s) and a final step of 72°C for 10 min. If the amplicons are of good quality and at the expected size, a template should be sent for sequencing with reverse and forward primers. The results of sequencing should be compared with available sequences on https://pubmlst.org/organisms/xylella-fastidiosa/ (Scally et al., 2005).

2.3. DNA from plant extracts or insects

The test should be performed as described in Section 2.2 above. If erratic amplification occurs, the following PCR parameters can be adjusted: dilution of the DNA extract (to limit inhibition) or increase of DNA input, use of a different Taq polymerase/master mix, decrease of annealing temperature from 65 to 60°C or 58°C or increase of primer concentration from 0.3 to 0.5 µM.

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 cross-reaction with the host tissue and/or 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

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

Verification of the controls for each PCR:

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of the relevant size (*leuA* = 749, *petC* = 576, *malF* = 773, *cysG* = 642, *holC* = 422, *nuoL* = 599, gltT = 698). If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- Sequencing is performed when the expected amplicons are produced (as written under the PIC and PAC just above).
- Subspecies assignment is not possible if no band, a different number of bands or band(s) of a different size are produced.
- The test should be repeated or adjusted (see Section 2.3) if any contradictory or unclear results are obtained.

3.3. Interpretation of sequencing results

A table of correspondence between sequence types (ST) and subspecies based on the allele numbers for the different genes is presented below.

ST	leuA	petC	malF	cysG	holC	nuoL	gltT	Clonal complex	Reference
1	1	1	1	1	1	1	1	*fastidiosa*	Yuan et al. (2010)
2	1	1	4	1	1	1	1	*fastidiosa*	Yuan et al. (2010)
3	1	1	1	20	1	1	1	*fastidiosa*	Yuan et al. (2010)
4	1	1	1	4	1	1	1	*fastidiosa*	Yuan et al. (2010)
5	2	2	2	2	2	2	2	*sandyi*	Yuan et al. (2010)
6	3	3	3	3	3	3	3	*multiplex*	Yuan et al. (2010)
7	3	3	3	7	3	3	3	*multiplex*	Yuan et al. (2010)
8	3	3	5	5	4	3	7	*multiplex*	Nunney et al. (2013)
9	3	3	5	5	4	3	4	*multiplex*	Yuan et al. (2010)
10	5	4	3	3	6	3	5	*multiplex*	Yuan et al. (2010)

ST	leuA	petC	malF	cysG	holC	nuoL	gltT	Clonal complex	Reference
11	7	7	7	9	10	8	8	*pauca*	Nunney et al. (2010)
12	7	7	7	9	13	8	8	*pauca*	Nunney et al. (2012)
13	7	6	7	9	10	7	8	*pauca*	Yuan et al. (2010)
14	8	8	8	11	12	9	9	*pauca*	Yuan et al. (2010)
15	5	3	3	3	4	3	5	*multiplex*	Nunney et al. (2013)
16	7	6	8	10	11	8	8	*pauca*	Nunney et al. (2010)
17	1	1	10	12	18	10	1	*fastidiosa*	Nunney et al. (2010)
18	9	1	9	13	14	5	10	*fastidiosa*	Nunney et al. (2010)
19	10	1	10	14	15	11	1	*fastidiosa*	Nunney et al. (2010)
20	1	1	10	12	17	11	11	*fastidiosa*	Nunney et al. (2010)
21	10	1	10	14	15	11	12	*fastidiosa*	Nunney et al. (2010)
22	3	3	5	12	4	3	3	*multiplex*	Nunney et al. (2013)
23	3	3	5	3	6	3	3	*multiplex*	Nunney et al. (2013)
24	3	3	5	3	4	3	7	*multiplex*	Nunney et al. (2013)
25	3	3	3	17	3	3	3	*multiplex*	Nunney et al. (2013)
26	5	3	3	3	6	3	5	*multiplex*	Yuan et al. (2010)
27	6	3	5	6	7	3	7	*multiplex*	Nunney et al. (2013)
28	6	3	5	18	7	4	7	*multiplex*	Nunney et al. (2013)
29	4	3	6	18	5	4	3	*morus*	Nunney, Schuenzel, et al. (2014)
30	4	5	6	8	5	4	3	*morus*	Nunney, Schuenzel, et al. (2014)
31	4	3	6	18	8	6	3	*morus*	Nunney, Schuenzel, et al. (2014)
32	4	3	5	12	4	4	3	*multiplex*	Nunney et al. (2013)
33	11	9	14	15	19	13	10	*fastidiosa/sandyi*	Yuan et al. (2010)
34	3	3	3	3	3	3	6	*multiplex*	Nunney et al. (2013)
35	3	10	3	3	3	3	3	*multiplex*	Nunney et al. (2013)
36	5	3	5	19	6	3	5	*multiplex*	Nunney et al. (2013)
37	3	3	5	21	4	3	3	*multiplex*	Nunney et al. (2013).
38	3	3	5	16	4	3	7	*multiplex*	Nunney et al. (2013)
39	3	3	5	19	4	3	7	*multiplex*	Nunney et al. (2010)
40	6	3	5	18	7	3	7	*multiplex*	Nunney et al. (2013)
41	3	3	5	18	9	3	3	*multiplex*	Nunney et al. (2013)
42	6	3	5	12	4	3	3	*multiplex*	Nunney et al. (2013)
43	3	3	5	18	4	3	7	*multiplex*	Nunney et al. (2013)
44	3	3	5	5	6	3	4	*multiplex*	Nunney et al. (2013)
45	3	3	5	3	4	3	3	*multiplex*	Nunney et al. (2013)
46	5	3	3	3	6	3	3	*multiplex*	Nunney et al. (2013)
47	13	1	10	23	20	5	1	*fastidiosa*	Nunney et al. (2010)
48	3	3	12	3	6	3	3	*multiplex*	Nunney et al. (2013)
49	3	3	5	3	6	3	7	*multiplex*	Nunney et al. (2013)
50	3	11	13	22	21	14	13	*multiplex*	Nunney et al. (2013)
51	3	3	5	3	4	15	3	*multiplex*	Nunney et al. (2013)
52	10	1	10	14	18	10	1	*fastidiosa*	Nunney et al. (2010)
53	7	6	16	24	10	16	14	*pauca*	Nunney, Schuenzel, et al. (2014)
54	11	9	11	25	19	12	1	*fastidiosa/sandyi*	Nunney et al. (2010)
55	1	1	10	12	18	10	10	*fastidiosa*	Nunney et al. (2010)

ST	leuA	petC	malF	cysG	holC	nuoL	gltT	Clonal complex	Reference
56	11	9	11	15	17	12	10	*fastidiosa/sandyi*	Nunney et al. (2010)
57	1	1	10	12	18	11	11	*fastidiosa*	Nunney et al. (2010)
58	6	3	5	12	4	3	7	*multiplex*	Nunney et al. (2013)
59	9	1	9	13	14	5	1	*fastidiosa*	L. Nunney (pers. comm.)
60	9	1	1	13	14	5	1	*fastidiosa*	L. Nunney (pers. comm.)
61	11	9	11	15	16	12	10	*fastidiosa/sandyi*	Nunney, Ortiz, et al. (2014)
62	4	3	6	18	5	6	3	*morus*	Nunney, Schuenzel, et al. (2014)
63	5	6	3	3	6	3	5	*multiplex*	Coletta-Filho et al. (2017)
64	7	7	7	9	10	7	8	*pauca*	Coletta-Filho et al. (2017)
65	7	6	7	9	10	8	8	*pauca*	Coletta-Filho et al. (2017)
66	7	8	8	10	11	8	8	*pauca*	Coletta-Filho et al. (2017)
67	5	3	8	3	12	3	5	*multiplex*	Coletta-Filho et al. (2017)
68	14	8	8	11	12	9	8	*pauca*	Coletta-Filho et al. (2017)
69	7	6	7	9	23	17	8	*pauca*	Coletta-Filho et al. (2017)
70	14	7	8	11	22	9	8	*pauca*	Coletta-Filho et al. (2017)
71	5	8	8	11	12	9	9	*pauca*	Coletta-Filho et al. (2017)
72	12	12	15	26	24	18	1	*sandyi*	Loconsole et al. (2016)
73	7	6	8	27	10	16	8	*pauca*	Loconsole et al. (2016)
74	7	6	8	28	25	16	8	*pauca*	Jacques et al. (2016)
75	9	1	10	29	1	19	1	*fastidiosa*	Jacques et al. (2016)
76	12	13	15	26	24	18	1	*sandyi*	Loconsole et al. (2016)
77	1	1	6	30	26	5	1	*fastidiosa*	Bergsma-Vlami et al. (2017)
78	7	6	7	9	23	8	8	*pauca*	Tolocka et al. (2017)
79	3	3	3	26	3	3	3	*multiplex*	Denancé et al. (2017)
80	7	6	17	31	10	16	15	*pauca*	B. Landa (pers. comm.)
81	3	3	3	32	3	3	3	*multiplex*	B. Landa (pers. comm.)
82	3	3	5	12	4	3	16	*multiplex*	Ferguson (2016)
83	6	3	5	33	7	4	7	*multiplex*	Ferguson (2016)
84	7	6	7	34	10	20	8	*pauca*	Safady et al. (2019)
85	7	6	8	10	10	8	8	*pauca*	Safady et al. (2019)
86	7	6	8	10	11	20	8	*pauca*	Safady et al. (2019)
87	5	3	5	3	3	21	3	*multiplex*	Marchi et al. (2019), Saponari et al. (2019)
88	3	3	19	7	3	3	3	*multiplex*	Cunty et al. (2022)
89	6	2	5	18	4	23	7	*multiplex*	Cunty et al. (2022)
90	16	15	20	36	27	24	18	*pauca*	MLST database (2022, Jolley et al., 2018)

4. Performance characteristics available

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

Data generated during an intra-laboratory study performed at ANSES, FR. Additional data available in the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity

Pure cultures (DNA after thermal lysis): 100% detection at 10^5–10^6 bacteria/mL

Plant extracts:

Two intra-laboratory test performance studies have been conducted with a preparation of spiked samples (range 10^3–10^6 bacteria/mL):

- One (TPS-A) with *Polygala myrtifolia*, *Cistus monspeliensis*, *Helichrysum italicum* (the most common *X. fastidiosa*-infected hosts in France): effect of DNA dilution on Yuan et al. (2010) PCR sensitivity.
- One (TPS-B) with *Lavandula* × *intermedia*, *Rosmarinus officinalis*, *Helichrysum italicum* [hosts in France with the highest concentration of inhibitors affecting the Yuan et al., 2010 PCR]: effect of BSA addition on PCR Yuan sensitivity.

Effect of DNA dilution on the analytical sensitivity of the Yuan et al. (2010) PCR (detection threshold) without the addition of BSA (TPS-A)

PCR Yuan/7 genes	DNA dilution	Detection threshold (100%)
Helichrysum italicum	Undiluted DNA	No detection (10^6)
	Diluted 1/10	10^5 bacteria/mL
Cistus monspeliensis	Undiluted DNA	10^4 bacteria/mL
	Diluted 1/10	10^5 bacteria/mL
Polygala myrtifolia	Undiluted DNA	10^4 bacteria/mL
	Diluted 1/10	10^5 bacteria/mL

Effect of the addition of BSA on the analytical sensitivity of the Yuan et al. (2010) PCR (detection threshold) (TPS-B)

PCR Yuan/7 genes	BSA 0.3 µg/µL	Detection threshold (100%)
Helichrysum italicum	Without	Not reached (68% for 10^6 bacteria/mL)
Undiluted DNA	With	10^4 bacteria/mL (89% for 10^3 bacteria/mL)
Rosmarinus officinalis	Without	Not detected (86% for 10^6 bacteria/mL)
Undiluted DNA	With	10^5 bacteria/mL (98% for 10^4 bacteria/mL)
Lavandula × *intermedia*	Without	10^6 bacteria/mL (89% for 10^5 bacteria/mL)
Undiluted DNA	With	10^4 bacteria/mL (94% for 10^3 bacteria/mL)

It is important to note that the addition of BSA contributes to a better quality of the amplicon and, consequently, to sequencing success. A figure with pictures of gels is shown in Figure A1.

Further information is available in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

4.2. Analytical specificity (same results with/without BSA)

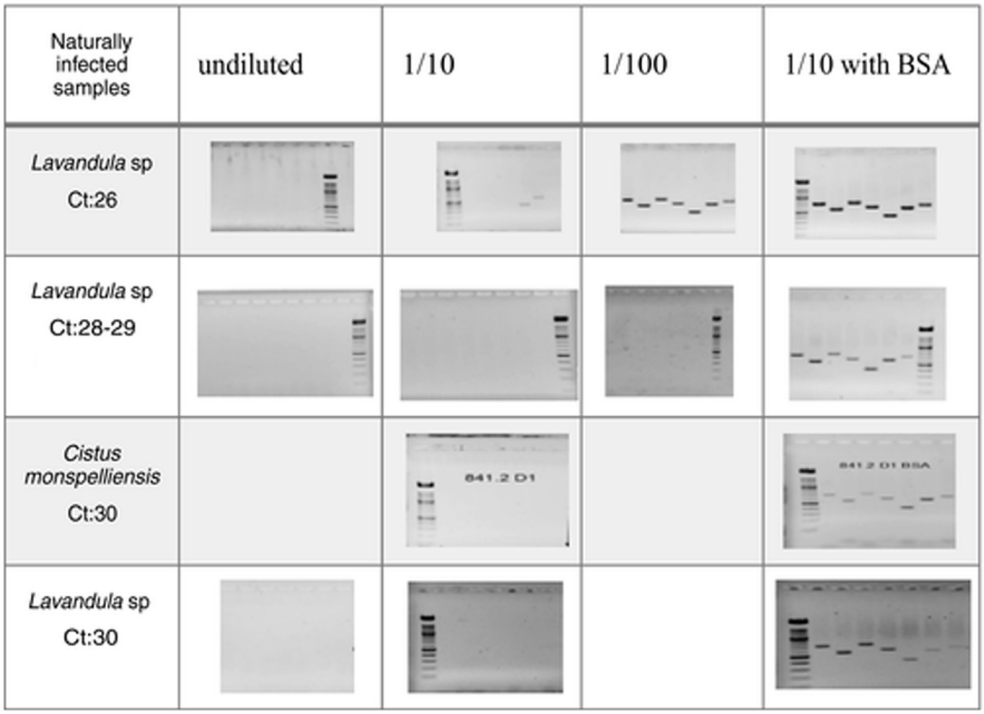

FIGURE A1 Pictures of gels for different plant species with and without BSA/tenfold dilutions.

Inclusivity evaluated with 14 target strains (all subspecies): 100%.

Exclusivity evaluated with 39 non-target strains (24 plant pathogenic bacteria and 15 saprophytes): 100%.

For details see the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

4.3. Repeatability (criteria calculated for a target concentration higher than or equal to the detection threshold)

TPS-A:

- undiluted DNA: 100% (*Polygala*, *Cistus*, *Helichrysum*)
- 1/10 diluted DNA: 100% (*Polygala*, *Cistus*, *Helichrysum*)

TPS-B:

- without BSA: between 70.4% (*Helichrysum*/malF), 85.2% (*Helichrysum*/petC/holC/nuoL; *Rosmarinus* /leuA/petC/malF/cysG/gltT) and 100% (other genes on *Helichrysum* and *Rosmarinus*, all genes on *Lavandula*)
- with 0.3 µg/µL BSA: 100% (*Helichrysum*, *Rosmarinus*, *Lavandula*).

4.4. Reproducibility (criteria calculated for a target concentration higher than or equal to the detection threshold)

TPS-A:

- undiluted DNA: 100% (*Polygala*, *Cistus*, *Helichrysum*)
- 1/10 diluted DNA: 100% (*Polygala*, *Cistus*, *Helichrysum*)

TPS-B

- without BSA: between 56% (*Helichrysum*/leuA/malF/cysG/gltT) and 100% (other genes *Helichrysum*; all genes on *Rosmarinus*, *Lavandula*)
- with 0.3 µg/µL BSA: 100% (*Helichrysum*, *Rosmarinus*, *Lavandula*).

4.5. Other information

Information on diagnostic sensitivity and specificity is available from the validation sheet in the EPPO Database on Diagnostic Expertise section 'Validation data for diagnostic tests' (https://dc.eppo.int/validation_data/validationlist).

APPENDIX 17 - NESTED-MULTILOCUS SEQUENCE TYPING (CESBRON ET AL., 2020)

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 verification (see PM 7/98) is carried out.

1. General Information

1.1. This test is suitable for the identification of *Xylella fastidiosa* subspecies from plant samples and insects.

1.2. The test is based on Cesbron et al., 2020.

1.3. The target sequences are those of seven housekeeping genes amplified individually: 2-isopropylmalate synthase (*leuA*); ubiquinol cytochrome *c* oxidoreductase C1 subunit (*petC*); ABC transporter sugar permease (*malF*); sirohaem synthase (*cysG*); DNA polymerase III holoenzyme chi subunit (*holC*); NADH-ubiquinone oxidoreductase NQO12 subunit (*nuoL*); and glutamate symport protein (*gltT*).

1.4. Oligonucleotides:

Primers	Sequence	Amplicon size (bp)
Forward primer *cysG* round 1	5′-CCA AAC ATA GAA GCA CGC CG-3′	776
Reverse primer *cysG* round 1	5′-GCG AGT GTT TTC AGC GTT CC-3′	
Forward primer *cysG* round 2[a]	5′-GCC GAA GCA GTG CTG GAA G-3′	642
Reverse primer *cysG* round 2[a]	5′-GCC ATT TTC GAT CAG TGC AAA AG-3′	
Forward primer *gltT* round 1	5′-GGT GCC ATC CAA TCC GTT TT-3′	916
Reverse primer *gltT* round 1	5′-TCA GGA TGT CCC AAT TCC AAC G-3′	
Forward primer *gltT* round 2[a]	5′-TCA TGA TCC AAA TCA CTC GCT T-3′	700
Reverse primer *gltT* round 2	5′-TTA CTG GAC GCT GCC TCG-3′	
Forward primer *holC* round 1	5′-CCG ATG GTG AAG AAC AGT AGA CA-3′	549
Reverse primer *holC* round 1	5′-GCT CGA GAA ACT SGA TTA ATG G-3′	
Forward primer *holC* round 2	5′-GGT CAC ATG TCG TGT TTG TTC-3′	424
Reverse primer *holC* round 2	5′-CAC GCG CCG ACT TCT ATT T-3′	
Forward primer *leuA* round 1	5′-CGA AGG TGC AAA CAA AGT GA-3′	886
Reverse primer *leuA* round 1	5′-CGC ACT GGC TTC GAT AAT GTC T-3′	

Primers	Sequence	Amplicon size (bp)
Forward primer *leuA* round 2[a]	5′-GGT GCA CGC CAA ATC GAA TG-3′	774
Reverse primer *leuA* round 2	5′-ACT GGT CCC TGT ACC TTC GT-3′	
Forward primer *malF* round 1	5′-AAC GTC GTC ACC CCA AGA A-3′	845
Reverse primer *malF* round 1	5′-ATG AGG CGG GCT TCT TTG G-3′	
Forward primer *malF* round 2	5′-AGC AGA AGC ACG TCC CAG AT-3′	767
Reverse primer *malF* round 2	5′-CTG GTC CTG CGG TGT TGG-3′	
Forward primer *nuoL* round 1	5′-TTG GTA CGT TGG CTT TGG TG-3′	845
Reverse primer *nuoL* round 1	5′-GAC AAA ACC AGA TTG CGT GC-3′	
Forward primer *nuoL* round 2	5′-GCG ACT TAC GGT TAC TGG GC-3′	597
Reverse primer *nuoL* round 2[a]	5′-ACC ACC GAT CCA CAA CGC AT-3′	
Forward primer *petC* round 1	5′-TCA ATG CAC GTC CTC CCA AT-3′	582
Reverse primer *petC* round 1	5′-GGC TGC CAT TCG TTG AAG TA-3′	
Forward primer *petC* round 2	5′-ACG TCC TCC CAA TAA GCC T-3′	551
Reverse primer *petC* round 2	5′-CGT TAT TCA CGT ATC GCT GC-3′	

[a] Oligonucleotides from Yuan et al. (2010).

1.5. Thermocycler Applied Biosystems.
1.6. Forward and reverse nucleotide sequences were assembled, and aligned using Geneious 9.1.8 software (French samples) or Bionumerics V7.6.3 software (Spanish samples) to obtain high quality sequences. ST or loci assignation was performed according to https://pubmlst.org/organisms/xylella-fastidiosa/

2. Methods

2.1. Nucleic Acid Extraction and Purification.
2.1.1. Matrices: plants, insects.
2.1.2. See Appendix 3 for extraction procedures from plants and insects.
2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Conventional PCR round 1
2.2.1. Master Mix Table

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	13.63	N.A.
PCR buffer GoTaq G2 (*Promega*)	5×	5	1×
$MgCl_2$ (*Invitrogen*)	50 mM	0.75	1.5 mM
dNTPs (*Promega*)	10 mM	0.5	200 µM
Forward primer round 1 (for the 7 housekeeping genes)	5µM	1.5	300nM (each)
Reverse primer round 1 (for the 7 housekeeping genes)	5µM	1.5	300 nM (each)
GoTaq G2 (*Promega*)	5 U/µL	0.12	0.6 U
Subtotal		23	
Nucleic acid extract		2	
Total		25	

2.3. Conventional PCR round 2
2.3.1. Master Mix Table

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	27.25	N.A.
PCR buffer GoTaq G2 (*Promega*)	5×	10	1×
$MgCl_2$ (*Invitrogen*)	50 mM	1.5	1.5 mM
dNTPs (*Promega*)	10 mM	1	200 µM
Forward primer round 2 (for the 7 housekeeping genes)	5 µM	3	300 nM (each)
Reverse primer round 2 (for the 7 housekeeping genes)	5 µM	3	300 nM (each)
GoTaq G2 (*Promega*)	5 U/µL	0.25	1.25 U
Subtotal		46	
First-round PCR product		4	
Total		50	

2.3.2. PCR conditions first round: 3 min at 95°C followed by 35 cycles of (30 s denaturation at 95°C, 30 s at the relevant temperature according to each gene (see below) and 60 s at 72°C) followed by a final extension step of 10 min at 72°C

2.3.3. PCR conditions second round: 30 cycles of (30 s denaturation at 95°C, 30 s at the relevant temperature according to each gene (see below) and 60 s at 72°C) followed by a final extension step of 10 min at 72°C

2.3.4. Annealing temperatures:

Primers	Annealing temperature (°C)
Forward primer *cysG* round 1	64
Reverse primer *cysG* round 1	
Forward primer *cysG* round 2	56
Reverse primer *cysG* round 2	
Forward primer *gltT* round 1	60
Reverse primer *gltT* round 1	
Forward primer *gltT* round 2	56
Reverse primer *gltT* round 2	
Forward primer *holC* round 1	62
Reverse primer *holC* round 1	
Forward primer *holC* round 2	59
Reverse primer *holC* round 2	
Forward primer *leuA* round 1	58
Reverse primer *leuA* round 1	
Forward primer *leuA* round 2	60
Reverse primer *leuA* round 2	
Forward primer *malF* round 1	56
Reverse primer *malF* round 1	
Forward primer *malF* round 2	60
Reverse primer *malF* round 2	
Forward primer *nuoL* round 1	60
Reverse primer *nuoL* round 1	
Forward primer *nuoL* round 2	54
Reverse primer *nuoL* round 2	
Forward primer *petC* round 1	60
Reverse primer *petC* round 1	
Forward primer *petC* round 2	56
Reverse primer *petC* round 2	

3. Essential Procedural Information

3.1. Controls

For positive controls inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 cross-reaction with the host tissue and/or 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2 and 2.3). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

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

Verification of the controls for each PCR:

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of the relevant size (*leuA* = 774 bp, *petC* = 551 bp, *malF* = 767 bp, *cysG* = 642 bp, *holC* = 424 bp, *nuoL* = 597 bp, gltT = 700 bp). If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- Sequencing is performed when the expected amplicons are produced
- Subspecies assignment is not possible if no band, a different number of bands or band(s) of a different size are produced
- The test should be repeated or adjusted if any contradictory or unclear results are obtained.

3.3. Interpretation of sequencing results

A table of correspondence between sequence types (ST) and subspecies is presented in Appendix 16.

4. Performance characteristics available

4.1. Analytical sensitivity data

On *Xylella fastidiosa* DNA dilution series: the first round PCR tests gave a signal of varying intensity for concentrations up to 2.2 ng/mL (0.8×10^6 copies/mL) for all house-keeping genes except *malF* and *cysG* (220 pg/mL). The second round of PCR tests allowed a sufficiently strong signal for sequencing for concentrations up to 22 pg/mL (0.8×10^4 copies/mL) for *gltT*, *holC*, *petC*, *leuA*, *cysG*, and up to 2.2 pg/mL (0.8×10^3 copies/mL) for *nuoL* and *malF*.

4.2. Analytical specificity data

Inclusivity: 100% evaluated on the following targets: *Xylella fastidiosa* subsp. *fastidiosa* (CFBP 7970), *Xylella fastidiosa* subsp. *multiplex* (CFBP 8416; CFBP 8070), *Xylella fastidiosa* subsp. *morus* (CFBP 8084), *Xylella fastidiosa* subsp. *pauca* (CFBP 8402) in vitro, and on 58 *Xylella fastidiosa* genome sequences in silico (including all subspecies).

Exclusivity: 100% evaluated on 34 non-target species (*Agrobacterium rubi* CFBP 6448, *Agrobacterium tumefaciens* CFBP 2413, *Agrobacterium vitis* CFBP 5523, *Clavibacter insidiosus* CFBP 2404, *Clavibacter michiganensis* CFBP 4999, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* CFBP 3414, *Dickeya dianthicola* CFBP 1200, *Ensifer meliloti* CFBP 5561, *Erwinia amylovora* CFBP 1232, *Pantoea agglomerans* CFBP 3845, *Pantoea stewartii* pv. *stewartii* CFBP 3167, *Pseudomonas amygdali* CFBP 3205, *Pseudomonas cerasi* CFBP 7436, *Pseudomonas congelans* CFBP 7019, *Pseudomonas syringae* pv. *persicae* CFBP 1573, *Pseudomonas syringae* pv. *syringae* CFBP 1392, *Rhizobium nepotum* CFBP 7436, *Stenotrophomonas maltophilia* CFBP 13100, *Xanthomonas euvesicatoria* pv. *citrumelonis* CFBP 3371, *Xanthomonas arboricola* pv. *juglandis* CFBP 2528, *Xanthomonas arboricola* pv. *pruni* CFBP 2535, *Xanthomonas axonopodis* pv. *axonopodis* CFBP 4924, *Xanthomonas campestris* pv. *campestris* CFBP 5241, *Xanthomonas citri* pv. *aurantifolii* CFBP 2901, *Xanthomonas citri* pv. *citri* CFBP 2525, *Xanthomonas citri* pv. *viticola* CFBP 7660, *Xanthomonas gardneri* CFBP 2625, *Xanthomonas hortorum* pv. *hederae* CFBP 4925, *Xanthomonas hortorum* pv. *pelargonii* CFBP 2533, *Xanthomonas hyacinthi* CFBP 1156, *Xanthomonas oryzae* pv. *oryzae* CFBP 2532, *Xanthomonas translucens* CFBP 2054, *Xanthomonas vasicola* pv. *holcicola* CFBP 2543) in vitro, and on 194 380 bacterial whole genome shotgun sequences in silico.

No amplification was detected on the non-target strains except for strain CFBP 2532 (*Xanthomonas oryzae* pv. *oryzae*) and CFBP 2533 (*Xanthomonas hortorum* pv. *pelargonii*) in the first round of the nested PCR for the petC outer primers, providing a product of the expected size. However, these products were not amplified in the second round of the nested PCR and therefore a false positive signal was not obtained.

4.3. Diagnostic sensitivity

On samples with low and high concentrations of the bacterium: 206 plant samples and 26 insect samples, also analysed with Harper's real-time PCR without a cut-off.

cysG: from 11% to 90%
gltT: from 9% to 90%
holC: 27%–93%
leuA: 27%–90%
malF: 16%–90%
nuoL: 26%–90%
petC: 25%–90%

4.4. Diagnostic specificity

100%

APPENDIX 18 - CONVENTIONAL PCR (POOLER & HARTUNG, 1995)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for the detection and identification of *X. fastidiosa* subsp. *pauca in planta* or for an isolate. There is little experience for insects (low concentration of bacteria and limited amount of DNA from a single insect).
1.2. The test is based on Pooler and Hartung (1995).
1.3. The primers target a gene coding for a hypothetical protein (BLASTing; CVC strain *X. fastidiosa* 9a5c).
1.4. Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer CVC-1	5′-AGA TGA AAA CAA TCA TGC AAA-3′	500 bp
Reverse primer 272-2-Int	5′-GCC GCT TCG GAG AGC ATT CCT-3′	

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Tissue source: plant, pure culture suspension or insects.

2.1.2. See Appendix 3 for extraction procedures.

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	N.A.	18.3	N.A.
Taq DNA polymerase buffer (Invitrogen)	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (CVC-1)	20 µM	0.5	0.4 µM
Reverse primer (272-2-Int)	20 µM	0.5	0.4 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U/µL	0.2	0.04 U/µL
Subtotal		23	
Genomic DNA (final concentration and its 10- and 100-fold dilutions) or bacterial suspension		2	
Total		25	

2.2.2. PCR conditions

94°C for 4 min followed by 30 cycles of (94°C for 1 min, 62°C for 1 min, 72°C for 1 min) and a final step of 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* subsp. *pauca* can be used instead of living cultures. 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC: no band is visualized
- PIC and PAC a band of the expected size (500 bp) is

visualized. If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- A test will be considered positive if a band of the expected size (500 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

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

In France two different strains, isolated from coffee and identified as subsp. *pauca* by MLST (Yuan et al., 2010), have tested positive with the Pooler and Hartung (1995) method. It has not been possible to test other strains, in particular from Brazil, as these are not available in reference collections.

APPENDIX 19 - CONVENTIONAL SIMPLEX PCR (HERNANDEZ-MARTINEZ ET AL., 2006)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for assignment of subspecies *in planta* and assignment of an isolate to *X. fastidiosa* subsp. *fastidiosa*, *multiplex* and *sandyi* isolates. A multiplex PCR for isolates is described in Appendix 20. There is little experience for insects (low concentration of bacteria and limited amount of DNA from a single insect).

1.2. The test is based on Hernandez-Martinez et al. (2006).

1.3. The target sequences are a gene that encodes a putative methyltransferase of the restriction/methylation system for the XF1968 primers, a gene that encodes a putative fimbrial protein for the XF2542 primers (these were assigned to the CVC *X. fastidiosa* 9a5c strain) and a gene that encodes an intergenic region between the genes coding for a conserved hypothetical protein and a glycine cleavage H protein for the ALM primers (this target area was assigned to the genome of the ALS strain M12).

1.4. Oligonucleotides for subsp. *sandyi*, *multiplex*:

Primers	Sequence	Amplicon size
Forward primer XF1968-L	5′-GGA GGT TTA CCG AAG ACA GAT-3′	638 bp
Reverse primer XF1968-R	5′-ATC CAC AGT AAA ACC ACA TGC-3′	

1.5. Oligonucleotides for subsp. *multiplex*:

Primers	Sequence	Amplicon size
Forward primer ALM1	5′-CTG CAG AAA TTG GAA ACT TCA G-3′	521 bp
Reverse primer ALM2	5′-GCC ACA CGT GAT CTA TGA A-3′	

1.6. Oligonucleotides for subsp. *fastidiosa* and *multiplex*:

Primers	Sequence	Amplicon size
Forward primer XF2542-L	5′-TTG ATC GAG CTG ATG ATC G-3′	412 bp
Reverse primer XF2542-R	5′-CAG TAC AGC CTG CTG GAG TTA-3	

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. The test can be performed on DNA extracts (plants or insects) or on pure culture suspension.

2.1.2. See Appendix 3 for extraction procedures.

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

2.2. Conventional simplex PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	6	N.A.
FIREPol® master mix ready to load with 7.5 mM $MgCl_2$ (Solis Biodyne)	5×	2	1×
Forward primer (XF1968-L or ALM1 or XF2542-L)	10 µM	0.5	0.5 µM
Reverse primer (XF1968-R or ALM2 or XF2542-R)	10 µM	0.5	0.5 µM
Subtotal		9	
Genomic DNA extract		1	
Total		10	

2.2.2. PCR conditions

95°C for 3 min, 40 cycles of (95°C for 30 s, 55°C for 30 s and 72°C for 30 s) and a final step at 72°C for 5 min before cooling at 15°C.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 cross-reactions with the host tissue and/or 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

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

Verification of the controls:

- NIC and NAC: no band is visualized
- PIC and PAC bands of the expected size (638 bp with subsp. *sandyi* and *multiplex*; 521 bp with subsp. *multiplex*; 412 bp with subsp. *fastidiosa* and *multiplex*) are visualized. If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- A test will be considered positive if bands of the expected size 638 bp (subsp. *sandyi* and *multiplex*), 521 bp (subsp. *multiplex*) or 412 bp (subsp. *fastidiosa* and *multiplex*) are visualized
- Subspecies assignment is not possible if no band, a different number of bands or band(s) of a different size are visualized
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available from the Austrian Agency for Health and Food Safety (AGES, AT)

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

This test was established at the AGES lab and has been in use there since 2014. It was tested on symptomatic and asymptomatic samples. Bacterial suspensions of different *X. fastidiosa* subspecies, e.g. DSM 10026 (*fastidiosa*) and LMG 9063 (*multiplex*) can be used as controls. More than 100 routine samples including olives, coffee, deciduous trees, oleander, *Carex* spp. and *Polygala* spp. have been tested. All samples were run in duplicates (undiluted and 1:20). *Xylella fastidiosa* subsp. *sandyi* was detected in 10 coffee samples.

4.1. Analytical sensitivity data

Sensitivity data were not provided in the original publication because the test was developed for subspecies determination and applied on pure cultures.

At the AGES lab this test had the same diagnostic sensitivity as the test described by Minsavage et al. (1994) when used for detection and subspecies determination.

4.2. Analytical specificity data

In the original publication the PCR was successfully tested on 53 *X. fastidiosa* strains isolated from *Cercis* spp., *Citrus* spp., *Gingko* spp., *Hemerocallis* spp., *Jacaranda* spp., *Lagerstroemia* spp., *Liquidambar* spp., *Magnolia* spp., *Morus* spp., *Nandina* spp., *Nerium* spp., *Olea* spp., *Prunus* spp., *Pyrus* spp., *Quercus* spp., *Spartium* spp. And *Vitis* spp. from the USA, Brazil and Taiwan attributed to the *X. fastidiosa* subsp. *fastidiosa*, *sandyi* and *multiplex* (for details see Hernandez-Martinez et al., 2006).

4.3. Data on repeatability

100% when using PACs of *X. fastidiosa* subsp. *fastidiosa* and *multiplex*.

4.4. Data on reproducibility

100% when using PACs of *X. fastidiosa* subsp. *fastidiosa* and *multiplex*.

APPENDIX 20 - CONVENTIONAL MULTIPLEX PCR (HERNANDEZ-MARTINEZ ET AL., 2006)

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 verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR technique is mainly used for assignment of an isolate to *X. fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*. There is little experience for insects (low concentration of bacteria and limited amount of DNA from a single insect).

1.2. The test is based on Hernandez-Martinez et al. (2006).

1.3. The target sequences are a gene that encodes a putative methyltransferase of the restriction/methylation system for the XF1968 primers, a gene that encodes a putative fimbrial protein for the XF2542 primers (these were assigned to the CVC *X. fastidiosa* 9a5c strain) and a gene that encodes an intergenic region between the genes coding for a conserved hypothetical protein and a glycine cleavage H protein for the ALM primers (this target area was assigned to the genome of the ALS strain M12).

1.4. Oligonucleotides for subsp. *multiplex* and *sandyi*:

Primers	Sequence	Amplicon size
Forward primer XF1968-L	5′-GGA GGT TTA CCG AAG ACA GAT-3′	638 bp
Reverse primer XF1968-R	5′-ATC CAC AGT AAA ACC ACA TGC-3′	

1.5. Oligonucleotides for subsp. *multiplex*:

Primers	Sequence	Amplicon size
Forward primer ALM1	5′-CTG CAG AAA TTG GAA ACT TCA G-3′	521 bp
Reverse primer ALM2	5′-GCC ACA CGT GAT CTA TGA A-3′	

1.6. Oligonucleotides for subsp. *fastidiosa* and *multiplex*:

Primers	Sequence	Amplicon size
Forward primer XF2542-L	5′-TTG ATC GAG CTG ATG ATC G-3′	412 bp
Reverse primer XF2542-R	5′-CAG TAC AGC CTG CTG GAG TTA-3′	

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. See Appendix 3 for extraction procedures.

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

2.2. Multiplex PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	10.25	N.A.
PCR buffer (Promega)	10×	2.5	1×
$MgCl_2$	50 mM	1.25	2.5 mM
dNTPs	20 mM	1	0.8 mM
Forward primers (XF1968-L, ALM1, XF2542-L) for each	20 µM	1.25 for each (total 3.75)	1 µM
Reverse primers (XF1968-R, ALM2, XF2542-R) for each	20 µM	1.25 for each (total 3.75)	1 µM
Promega Taq DNA polymerase	5 U/µL	0.5	0.1 U/µL
Subtotal		23	
Genomic DNA extract		2	
Total		25	

2.2.2. PCR conditions

94°C for 5 min, 40 cycles of (94°C for 1 min, 55°C for 1 min and 72°C for 1 min) and a final step at 72°C for 10 min before cooling at 4°C.

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures of *X. fastidiosa* can be used instead of living cultures. 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 cross-reactions with the host tissue and/or 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 plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or per botanical genus
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*, isolated from a suspension with approximately 10^5 cfu/mL.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC: no band is visualized
- PIC and PAC bands of the expected size (638 bp with subsp. *sandyi* and *multiplex*, 521 bp with subsp. *multiplex* and 412 bp with subsp. *fastidiosa* and *multiplex*) are visualized. If relevant, a band of the expected size is visualized for the IC and IPC

When these conditions are met:

- A test will be considered positive if amplicons of 638 bp (subsp. *sandyi* and *multiplex*), 521 bp (subsp. *multiplex*) or 412 bp (subsp. *fastidiosa* and *multiplex*) are visualized. Some strains of subsp. *multiplex* have two bands (638 bp and 521 bp, Type ST6) and others three bands (638, 521 and 412 bp, Type ST7) (see Figure A2)
- Subspecies assignment is not possible if no band, a different number of bands, or band(s) of a different size is/are visualized
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

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

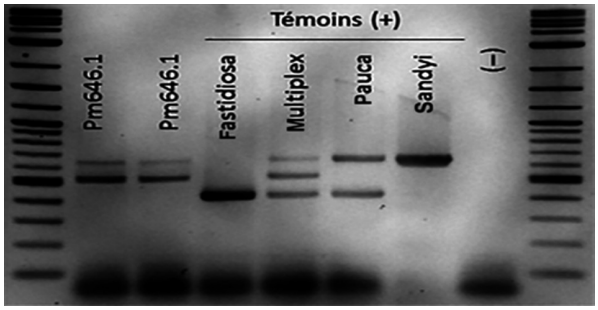

FIGURE A2 Electrophoretic analysis of PCR amplicons obtained from samples of *Polygala myrtifolia* (Pm646.1) by Hernandez-Martinez et al. (2006). It should be noted that although the test is not recommended for the detection of *Xylella fastidiosa* subsp. *pauca* some sequence types of subsp. *pauca* can produce bands with this PCR

From the DNA extraction method available with the Qiagen DNA Tissue Kit (Qiagen):

4.1. Analytical sensitivity data

Not available, but this is not critical as the test is mainly performed on cultures.

4.2. Analytical specificity data

Inclusivity 100% evaluated on 15 strains of *X. fastidiosa* subsp. *fastidiosa*, 12 strains of *X. fastidiosa* subsp. *sandyi* and 25 strains of *X. fastidiosa* subsp. *multiplex*.

4.3. Data on repeatability

No data available.

4.4. Data on reproducibility

No data available.

4.5. Data on diagnostic sensitivity

Oleander: 100%
Grape: 100%

APPENDIX 21 - PATHOGENICITY TEST

General guidance on a pathogenicity test for *X. fastidiosa* is provided. Plant growing conditions are specific to the host used and only examples are provided.

1. Test plants

Pathogenicity tests use host plants grown in pots. The plants should not be herbaceous and xylem tissue should be well differentiated. Optimal stages for inoculation are illustrated in Figure A3. When known, the most susceptible cultivars should be selected. Examples for some hosts are given below.

Olea europaea: successful inoculation has been obtained with Cellina di Nardò, Frantoio, Leccino, Coratina (Saponari et al., 2016, 2017).

Vitis vinifera: Chardonnay, Cabernet Sauvignon, Chenin Blanc and Pinot Noir are recommended. All these varieties develop symptoms of Pierce's disease within a short period (1–3 months) after inoculation with *X. fastidiosa* subsp. *fastidiosa*.

Citrus sinensis: Pera, Hamlin, Natal and Valencia.

Coffea: Coffea arabica (the cultivar Nana is used in the NRC, NPPO-NL), *Coffea canephora* (no data is available to allow recommendation of specific cultivars).

Actively growing susceptible plants should be maintained in an insect-proof greenhouse or growth chamber at 25–28°C. For inoculations, the soil in the pots should be dry and experimental conditions should favour plant transpiration (i.e. the inoculation should be done on a sunny day).

Ideally each experiment should include 10–15 inoculated plants and at least 3–5 controls, but this can vary depending on the test plants. Conditions after inoculation are as before inoculation. Water stress may favour the appearance of symptoms.

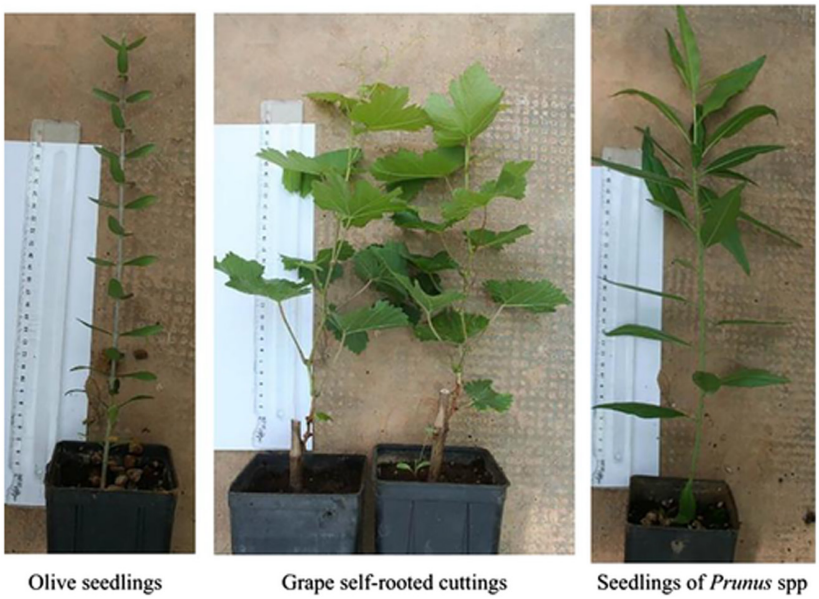

FIGURE A3 Illustration of the size of plants used for the inoculations. Courtesy M. Saponari, CNR – Institute for Sustainable Plant Protection (IT). The ruler in the figures is 30 cm long.

2. Inoculation

Inoculation techniques should ensure infiltration directly into the xylem vessels in order for symptoms to develop. The most widely used method for plant inoculation is by needle puncture in the stem at the base of the petiole (Figure A4).

A general inoculation procedure consisting of the pinprick inoculation method (Almeida et al., 2001; Hill & Purcell, 1995) is described below.

Low-passage (2–3) cultures of the bacterium grown for 8–10 days on the most suitable medium (see Appendix 14) at 28–30°C should be used for inoculation. Bacteria are removed from solid media and resuspended in PBS or succinate–citrate-phosphate buffer (see Appendix 14). The bacterial suspension should contain a high bacterial concentration (approximately 10^9 cfu/mL).

Plant parts to be inoculated should be placed in a horizontal position. A drop (10–50 µL) of inoculum is placed on a leaf axil and punctured through several times with a fine needle. After inoculation, plants should be maintained in a horizontal position for 5–10 min to allow absorption of the inoculum. Inoculation should be made in different parts of the test plants. Control plants are treated in the same way except that buffer is used instead of bacterial suspension.

Another conventional procedure consists of the inoculation of plants using a syringe (i.e. a 1-mL tuberculin syringe) to infiltrate droplets of inoculum containing *X. fastidiosa* at approximately 10^8 cfu/mL.

Alternative methods for inoculating citrus are:

(A) raise a flap of bark tissue by cutting tangentially upward with a razor blade, and apply 10–30 µL of suspension (10^8 cfu/mL) under the flap;
(B) excise a piece of bark tissue and place it in an Eppendorf tube containing 500 µL of bacterial suspension for 2 h then replace the tissue piece and wrap with grafting tape.

If plants have multiple stems (e.g. *Polygala myrtifolia* or blueberry) inoculations should be performed on at least two stems. To increase the effectiveness of the inoculations, the plants can be subjected to a second round of inoculation 3–8 weeks after the first inoculations.

FIGURE A4 Needle inoculation. Courtesy M. Saponari, CNR – Institute for Sustainable Plant Protection (IT).

3. Symptom monitoring

Symptoms usually appear (see Section 4) 60–80 days after inoculation in grapes and 8–10 months in citrus. In Brazil it took 6 years to complete the pathogenicity tests (and fulfil Koch's postulates) for the *X. fastidiosa* strains infecting coffee. In Italy, symptoms on the most susceptible cultivars of olive started to appear after 13 months.

The test plants showing symptoms should be tested as recommended in Section 3.4 and isolation should be attempted, although it may not be successful. As it is not easy to obtain symptoms, the testing of asymptomatic test plants is also recommended.

APPENDIX 22 - BIOASSAY ON TOBACCO PLANTS (FRANCIS ET AL., 2008; PEREIRA ET AL., 2017)

Tobacco plants are propagated in a greenhouse and inoculated with *X. fastidiosa* as described below.

Nicotiana tabacum 'Petite Havana SR1' or 'RP1' seeds are germinated at temperatures of approximately 20–25°C and under a day length of 14 h or under natural daylight if greater.

After approximately 1 month, 50 seedlings are transplanted into small individual pots (10 cm^2). From this point onward plants are fertilized occasionally when yellowing of leaves (deficiency) is observed. These conditions, which can be considered as stressful for tobacco plants, result in the rapid development of symptoms.

Around 1 month after transplant, tobacco plants are prepared for inoculation by cutting the top of the stem and removing the lower juvenile leaves so that only three

healthy adult leaves in the lower portion of the plant remain (numbered 1–3).

Bacterial inoculum is prepared from *X. fastidiosa* cultured on solid media at 28°C for about 1 week. Bacteria from two plates are scraped off and resuspended in 1.5 mL of succinate–citrate–phosphate buffer (Appendix 14).

A 1-mL tuberculin syringe with a 23-gauge needle is used to inject half of the plants with approximately 20 μL of inoculum in each remaining tobacco petiole, near the axils. The other half of the tobacco plants (control plants) are injected in the same manner with buffer only.

Plants continue growing from the site where the stem was cut. Leaves are classified according to their appearance as control (healthy) or senescent (showing browning symptoms) from buffer-inoculated control plants and asymptomatic (healthy) or symptomatic (marginal leaf scorch) from *X. fastidiosa*-inoculated plants.

Symptoms start to develop 10–14 days after inoculation (leaf scorch symptoms). Francis et al. (2008) reports that tobacco inoculated with stains associated with almond leaf scorch and Pierce's disease showed typical symptoms resembling those of grapes and almond infected with *X. fastidiosa* (Figure A5).

A reduction of plant size after inoculation with three strains of *X. fastidiosa* subsp. *pauca*, namely CoDiRO (ST53), 9a5c (ST13) and MF01 (ST16), is reported on both cultivars by Pereira et al. (2017). Symptoms of leaf yellow blotch were mainly found in RP1. The strain *X. fastidiosa* subsp. *pauca* CoDiRO induced the lowest percentage of infections with respect to the other *X. fastidiosa* strains, with a higher percentage in RP1 (78%) than in Petite Havana (53%; Pereira et al., 2017).

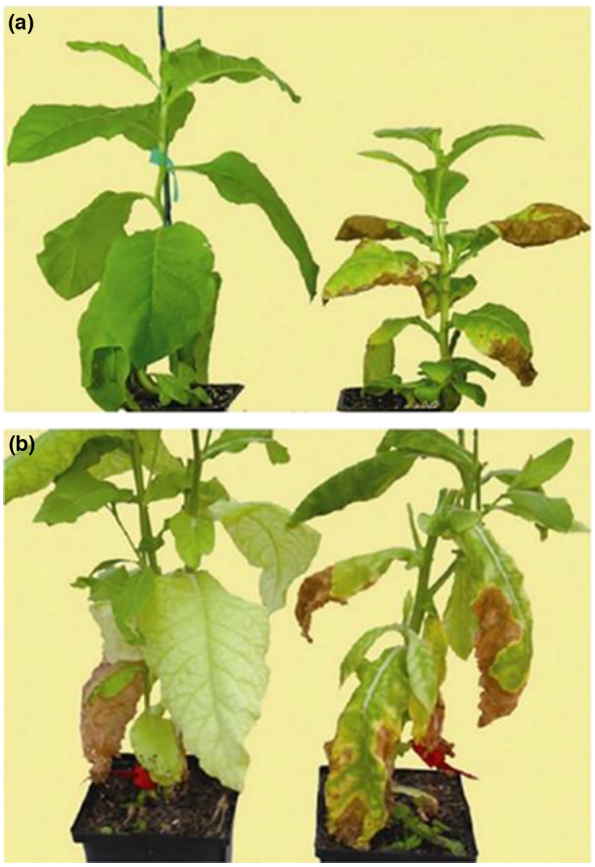

FIGURE A5 Symptoms on *Nicotiana tabacum* cv. SR1 after inoculation with *Xylella fastidiosa* Pierce's disease Temecula-1 strain. Symptoms were fully developed 6 weeks after inoculation. (a) The control plant mock inoculated with water (left) and plant inoculated with *X. fastidiosa* Temecula-1 (right). (b) Advanced symptoms at flowering time (2–3 months after inoculation). The water mock-inoculated control plant is showing normal leaf senescence (left) and the *X. fastidiosa*-inoculated plant is showing marginal leaf scorching and a chlorotic halo around the edge of the scorch symptoms (right). Reproduced from Francis et al. (2008).

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CORRIGENDUM

Corrigendum PM 7/024 (5) *Xylella fastidiosa*

Background information
In the EPPO diagnostic protocol PM 7/024 (5) *Xylella fastidiosa* (EPPO, 2023), the test of Hodgetts et al. (2021) is recommended under Section 3.4 on Screening test in Section 3.4.2.2 and as an identification and subspecies determination test in Section 4. It is described in full in Appendix 11.

It should be noted that during a Proficiency Test (PT) organized by the European Union Reference Laboratory for bacteriology (EURL-BAC) on *Xylella fastidiosa* some issues concerning analytical sensitivity and analytical specificity have been encountered with this test (Vreeburg et al., 2024a). A lower analytical sensitivity for subsp. *pauca* ST74 has been observed compared to the other subspecies. Therefore, using Hodgetts et al. (2021) as a single test for screening is not recommended.

In terms of assignment of subspecies in Section 4.2, the results of the PT showed that for Hodgetts et al. (2021) some of the subsp. *pauca* strains from sequence type 74 (ST74) can cross react with the test for subsp. *fastidiosa* (Vreeburg et al., 2024a). It is therefore recommended to laboratories using this test to include ST74 strains in the set of strains selected for verification of this test.

In Section 4.2 Molecular tests for the identification of *X. fastidiosa* and assignment of *X. fastidiosa* subspecies it is stated: 'In other cases, subspecies assignment may be performed by subspecies-specific molecular tests (Pooler & Hartung, 1995, see Appendix 18; Hernandez-Martinez et al., 2006, see Appendices 19 and 20) or Sanger sequencing.'

It should be noted that during the PT organized by the EURL-BAC on *Xylella fastidiosa* subspecies determination, several participants used the conventional PCR tests of Hernandez-Martinez et al. (2006). These participants found that these conventional PCR tests could not distinguish between subsp. *sandyi* and subsp. *morus*, since both subspecies produced a PCR product of 638 bp (Vreeburg et al., 2024b).

It is therefore recommended to laboratories performing Hernandez-Martinez et al. (2006), that if a band of 638 bp is obtained, then additional tests (Appendices 10, 11, 16 or 17) should be performed to distinguish between subsp. *sandyi* and subsp. *morus*.

List of changes:
In Figure 1 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* on plant material, note (1) should be modified as follows (new text in bold):

(1) It is highly recommended to perform a real-time PCR test for the detection on asymptomatic material.

Using Hodgetts et al. (2021) as a single test for screening on plant samples is not recommended.

The same information should also be added to the last bullet point in Section 3.4.2.2. Real-time PCR.

- real-time PCR test of Hodgetts et al. (2021) is described in Appendix 11. **Using Hodgetts et al. (2021) as a single test for screening is not recommended**.

Section 4 of Appendix 11 for exclusivity is modified as follows (new text in bold):
[ ]: 100%.

With Hodgetts et al. (2021) some of the subsp. *pauca* strains from sequence type 74 (ST74) can cross react with the test for subsp. *fastidiosa* (Vreeburg et al., 2024a). It is therefore recommended to laboratories using this test to include ST74 strains in the set of strains selected for this test.

Section 4 of Appendix 19 and 20 for Analytical specificity is modified as follows (new text in bold):
[ ]

Hernandez-Martinez et al. (2006) cannot distinguish between subsp. *sandyi* and subsp. *morus*, since both subspecies produce a PCR product of 638 bp (Vreeburg et al., 2024b).

It is therefore recommended to laboratories performing Hernandez-Martinez et al. (2006), that if a band of 638 bp is obtained, then additional tests (Appendices 10, 11, 16 or 17) should be performed to distinguish between subsp. *sandyi* and subsp. *morus*.

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