

This diagnostic protocol was first adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in August 2018.

The first revision of this diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in August 2024.

The annex is a prescriptive part of ISPM 27.

ISPM 27

Diagnostic protocols for regulated pests

DP 25: *Xylella fastidiosa*

Adopted 2024; published 2025

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1. Pest information

Xylella fastidiosa Wells *et al.*, 1987 is a xylem-limited bacterium that causes many economically important plant diseases of agronomic, horticultural or forestry crops such as *Vitis vinifera*, *Prunus domestica*, *Prunus dulcis*, *Citrus sinensis*, *Olea europaea*, *Ulmus* spp. and *Quercus* spp. *X. fastidiosa* has a wide, expanding host range and comprehensive lists of susceptible hosts are available (EFSA, 2023; EFSA, n.d). *X. fastidiosa* is also expanding its geographical range. It was mainly distributed throughout the Americas (Almeida and Nunney, 2015) but has been reported in Asia and Europe (EPPO, n.d.(a)).

X. fastidiosa is genetically diverse and consists of several subspecies. Three subspecies are widely accepted: *X. fastidiosa* subsp. *fastidiosa* causes Pierce's disease in *V. vinifera* and infects a large host range, including *Acer* spp., *Medicago sativa* and *P. dulcis* (EPPO, 2023a; Schuenzel *et al.*, 2005); *X. fastidiosa* subsp. *multiplex* is associated with scorch diseases of a range of trees that include *Platanus occidentalis*, *P. dulcis*, *Prunus persica*, *Quercus* spp. and *Vaccinium* spp. as well ornamentals (e.g. *Helichrysum italicum*, *Polygala myrtifolia*); and *X. fastidiosa* subsp. *pauca* (Schaad *et al.*, 2004) infects most *Citrus* species (mainly *C. sinensis*), *Coffea* species and *O. europaea*. Three other subspecies are currently described: *X. fastidiosa* subsp. *morus* (Nunney *et al.*, 2014) infects *Morus* spp.; *X. fastidiosa* subsp. *sandyi* causes oleander leaf scorch in *Nerium oleander* (Schuenzel *et al.*, 2005); and *X. fastidiosa* subsp. *tashke* causes leaf scorch in *Chitalpa tashkentensis* (Nunney *et al.*, 2014). Recent work has shown that *X. fastidiosa* taxonomy needs re-evaluation (Kahn and Almeida, 2022).

X. fastidiosa is a Gram-negative bacterium with fastidious growth requirements. The bacterial cells are non-flagellate and rod-shaped, with rounded or tapered ends and numerous irregular ridges or folds on the cell-wall surface (Wells *et al.*, 1987). The bacterium is inoculated into the water-transporting xylem elements of its host plants by xylem sap-feeding insects. The colonization of the xylem blocks the transport of mineral nutrients and water in the infected plants. Many diseases caused by *X. fastidiosa* are characterized by leaf scorch, defoliation, foliage wilt and a general decline in vigour, but expression of symptoms is heterogeneous, depending on the host plant species, *X. fastidiosa* genotype and the climatic conditions. Many host plants infected with *X. fastidiosa* show no symptoms (Almeida and Purcell, 2003). The bacterium proliferates in the xylem of an infected host and invades the plant's shoot and root system systemically (Aldrich, Gould and Martin, 1992; He *et al.*, 2000; Li *et al.*, 2003). The pathogen overwinters in the xylem of its host.

Insect transmission is responsible for the local spread of *X. fastidiosa*. The vectors belong to the order Hemiptera, suborder Auchenorrhyncha, families Cicadellidae (sharpshooter leafhopper), Cercopidae and Aphrophoridae (spittlebugs) (Redak *et al.*, 2004; Chatterjee, Almeida and Lindow, 2008), and Cicadidae. The transmission of *X. fastidiosa* by insects is persistent. Nymphs and adults are able to acquire the bacteria by feeding on the xylem fluid of an infected plant, and they then transmit the pathogen to susceptible plant hosts. While nymphs are able to acquire (and inoculate) the bacterium, they lose the infection at each moult; to become infected again, the insects must reacquire the bacterium by feeding on infected plants after moulting (Almeida *et al.*, 2014). Once infected, adults can transmit throughout their whole lifetime (as they do not moult), with the bacterium multiplying and persisting in the foregut of the vector (cibarium and precibarium) (Brlansky *et al.*, 1983; Almeida *et al.*, 2005). There is no evidence of transovarial transmission (transmission from a female to her eggs; Redak *et al.*, 2004). The movement of infected plants and propagative planting material (e.g. budwood, seedlings) is assumed to be responsible for the long-distance spread of the disease and its introduction into new areas.

2. Taxonomic information

Name: *Xylella fastidiosa* Wells *et al.*, 1987

Synonyms: none

Taxonomic position: Bacteria, Pseudomonadota (ex-Proteobacteria), Gammaproteobacteria, Lysobacterales (ex-Xanthomonadales), Lysobacteraceae (ex-Xanthomonadaceae) (Tindall, 2014; Whitman, Lawson and Losey, 2015)

Common names: alfalfa dwarf, bacterial leaf scorch disease, dwarf lucerne, citrus variegated chlorosis, olive quick decline syndrome, periwinkle wilt, phony peach disease, Pierce's disease of grapevines, plum leaf scald (the leaf scorch diseases being named in relation to their host plants, e.g. almond leaf scorch, coffee leaf scorch, oleander leaf scorch, olive leaf scorch, and pear leaf scorch)

Studies split *X. fastidiosa* into several subspecies (Schaad *et al.*, 2004; Scally *et al.*, 2005; Schuenzel *et al.*, 2005; Randall *et al.*, 2009; Yuan *et al.*, 2010; Nunney *et al.*, 2014). Currently, only the subspecies *fastidiosa* and *multiplex* are considered valid names by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (Bull *et al.*, 2012). Other additional *X. fastidiosa* subspecies proposed are “*pauca*” (Schaad *et al.*, 2004), “*sandyi*” (Schuenzel *et al.*, 2005) and “*morus*” (Nunney *et al.*, 2014). Recently, a revision of the *X. fastidiosa* subspecies has been proposed based on comparative genomic analysis (Marceletti and Scortichini, 2016; Denancé *et al.*, 2019; Kahn and Almeida, 2022), in which the subspecies *morus* and *sandyi* are merged with subspecies *fastidiosa* in the subspecies *fastidiosa sensu lato*. The strains associated with olive quick decline syndrome in Argentina, Brazil and Italy have been found to consistently belong to *X. fastidiosa* subsp. *pauca* but to different sequence types (Haelterman *et al.*, 2015; Giampetruzzi *et al.*, 2017; Safady *et al.*, 2019). The *Xylella* species associated with pear leaf scorch in the Taiwan Province of China (Leu and Su, 1993) is a new species, *X. taiwanensis* (Su *et al.*, 2016).

3. Detection

Plants infected with *X. fastidiosa* may be asymptomatic (Almeida and Purcell, 2003) or the symptoms may be similar to those associated with water stress or physiological disorders. Isolation methods are not recommended for detection because of the difficulty in isolating *X. fastidiosa* from plant tissue. Therefore, detection is generally based on inspection for symptoms and the use of specific serological or molecular tests on symptomatic plant material. There is limited information available on testing asymptomatic plants and the concentration of *X. fastidiosa* is likely to be lower than in symptomatic plants (Almeida and Nunney, 2015). Therefore, it is advisable to include molecular methods (e.g. polymerase chain reaction (PCR)) for testing asymptomatic plant material.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Symptoms

The presence of *X. fastidiosa* can have a broad impact on its host: from symptomless to plant death. Most host plants infected with *X. fastidiosa* do not show any symptoms, while some display symptoms that include leaf scorching, defoliation, chlorosis or bronzing along the leaf margin, and dwarfing. The bronzing may intensify before browning and drying. Symptoms are usually more pronounced in stressed plants (e.g. stressed by high temperature or drought) and they can vary according to the plant species or cultivar, *X. fastidiosa* strain and environmental conditions (Janse and Obradovic, 2010; CABI, n.d.).

The host range can be markedly different between *X. fastidiosa* subspecies; however, there is some uncertainty with regard to the potential host range for each subspecies. Each subspecies can be found in multiple host plants. For example, *X. fastidiosa* subsp. *fastidiosa* not only infects grapes but also causes alfalfa dwarf and overlaps with *X. fastidiosa* subsp. *multiplex* in causing almond leaf scorch (Yuan *et al.*, 2010). Symptoms can be confused with other biotic (e.g. several fungal diseases) or abiotic causes (environmental stresses, water deficiency, salt, air pollutants, nutritional problems, etc.): see, for example, <https://agriculture.gouv.fr/telecharger/85855>. Pictures of symptoms on various hosts can be found at <https://gd.eppo.int/taxon/XYLEFA/photos> and <https://nature.berkeley.edu/xylella>. The following descriptions provide examples of the more characteristic symptoms observed on some key hosts, and the associated subspecies of *X. fastidiosa*, that are widely acknowledged in the current literature.

3.1.1 Pierce's disease of grapevines

Symptoms of Pierce's disease vary depending on the *Vitis* species, cultivar and local climatic conditions. *X. fastidiosa* subsp. *fastidiosa* has been the only subspecies reported to cause disease in grapevines (Nunney *et al.*, 2010). Recently, Abdelrazek *et al.* (2023) detected *X. fastidiosa* subsp. *multiplex* (Xfm) in infected grapevines by multilocus sequencing typing (MLST) and metagenomic sequencing but this has not confirmed by isolation and pathogenicity assessment. *Vitis rotundifolia* (Muscadinia) and indigenous American cultivars display milder symptoms than those of *V. vinifera*. On *V. vinifera*, the initial symptoms are chlorotic spots on areas of the leaf blade, particularly along the margins, with a sudden drying of leaf edges often surrounded by a yellowish or a reddish halo (Hopkins and Purcell, 2002). In late summer and autumn, the necrotic leaf edges coalesce to form concentric rings that extend from the outer edge towards the centre. Subsequently, the leaf turns dry on the edges, but the leaf remains turgid and the whole lamina may shrivel and drop; the petiole remains attached to the branch (as so-called "match sticks"). The latter is a characteristic symptom of Pierce's disease late in the season. Fruit clusters shrivel or turn into raisins; branches and twigs usually start wilting from the tip; and infected stems mature irregularly, showing patches of green tissue called "green islands" (Kahn *et al.*, 2023). Buds on infected plants sprout later than those on healthy plants, and the new shoots grow slowly and are stunted. Severely affected plants may die within one or two years, although in several species and cultivars they may continue to live considerably longer. Symptoms on the twigs can be confused with those of fungal diseases such as rotbrenner and esca (EPPO, 2023b).

3.1.2 Citrus variegated chlorosis

The first symptoms of citrus variegated chlorosis (CVC) to appear on leaves are mottled variegations, with small chlorotic spots on the upper surface that correspond to small gummy brown spots on the underside of the leaf. Isolates within the *X. fastidiosa* subsp. *pauca* complex have been reported to cause CVC (Schaad *et al.*, 2004; Almeida *et al.*, 2008). Symptoms are most obvious on three- to six-year-old trees and mainly on *C. sinensis* cultivars. Affected trees show foliar interveinal chlorosis resembling zinc deficiency, but the symptoms are not symmetrical on opposite sides of the leaf. Symptoms of CVC can also be distinguished from zinc chlorosis by the presence of the gummy, brown necrotic regions on the underside of the leaf, which coincide with the chlorosis on the upper leaf surface (CABI, n.d.). Sectoring of symptoms in the canopy occurs on newly affected trees. However, the CVC syndrome generally develops throughout the entire canopy on older infected trees. Affected trees are stunted and the canopy has a thin appearance because of defoliation and dieback of twigs and branches. Flowering is abnormal; fruits ripen earlier and do not fill, being much smaller than normal and very firm. The growth rate of affected trees is greatly reduced and twigs and branches may wilt. The plants do not usually die, but the yield and quality of the fruit are severely reduced (Donadio and Moreira, 1998).

3.1.3 Coffee leaf scorch

Symptoms of coffee leaf scorch appear on young flushes of field plants as large marginal and apical scorched zones on recently matured leaves (EPPO, 2023b). Affected leaves drop prematurely, shoot growth is stunted, and apical leaves are small and chlorotic. Symptoms may progress to shoot dieback and overall plant stunting. Fruit size and yield are generally reduced (De Lima *et al.*, 1998). Side branches have no leaves and fruit, the exception being a tuft of leaves at the branch tip. Infection of coffee plants by *X. fastidiosa* can also lead to the "crespera" disease, which has been reported in Costa Rica (Montero-Astúa *et al.*, 2008). Symptoms range from mild to severe curling of leaf margins, chlorosis and deformation of leaves, asymmetry (Bergsma-Vlami *et al.*, 2015), stunting of plants, shortening of internodes and dieback of branches (Montero-Astúa *et al.*, 2008). Infected coffee plants may remain asymptomatic (De Lima *et al.*, 1998; Montero-Astúa *et al.*, 2008).

3.1.4 Olive leaf scorching and quick decline

In two different, distant regions around the world (southern Europe and South America), leaf scorching symptoms on *O. europaea* trees have been associated with *X. fastidiosa* (Saponari *et al.*, 2013; Haelterman *et al.*, 2015; Coletta-Filho *et al.*, 2016). The olive quick decline syndrome is characterized by leaf scorching and randomly distributed desiccation of twigs and small branches, which, in the early

stages of the infection, are mainly observed in the upper part of the canopy. Leaf tips and margins turn dark yellow to brown, eventually leading to desiccation. Over time, symptoms become increasingly severe and extend to the rest of the crown, which acquires a blighted appearance. 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 the death of the entire tree. *X. fastidiosa* has also been detected in young olive trees with leaf scorching and quick decline (EPPO, 2023b).

3.1.5 Almond leaf scorch disease

The most characteristic symptoms of almond leaf scorch disease are leaf scorching followed by decreased productivity and general decline. Strains of *X. fastidiosa* subsp. *fastidiosa* and subsp. *multiplex* have been reported to cause almond leaf scorch disease (Yuan *et al.*, 2010). In early summer, leaves appear with marginal leaf scorch (brown, necrotic (dead) leaf tissue). Usually, a narrow band of yellow (chlorotic) tissue occurs between the dead tissue and the part of the leaf that is still green, but 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 limbs die back from the tip (Mircetich *et al.*, 1976). Even highly susceptible varieties take many years to die completely, but nut production is severely reduced within a few years in most varieties (Mircetich *et al.*, 1976). Sisterson *et al.* (2008) also found that infected trees had lower yields but did not observe complete tree death in their three-year study. The disease is sometimes described as golden death, because of the golden yellow colour of the canopy of infected trees (Adaskaveg *et al.*, n.d.).

3.1.6 Bacterial leaf scorch of shade trees

Symptoms of bacterial leaf scorch of shade trees are similar on different shade tree hosts (e.g. *Acer* spp., *Platanus* spp., *Quercus* spp., *Ulmus americana* (Gould and Lashomb, 2007)). In most cases, the disease is identified by a characteristic marginal leaf scorch. Symptoms first appear in late summer to early autumn. Affected leaves have marginal necrosis, which may be surrounded by a chlorotic (yellow) or red halo. Generally, symptoms progress from older to younger leaves as the diseased branches die and the tree declines.

3.1.7 Bacterial leaf scorch of blueberry

The first symptom of bacterial leaf scorch of blueberry is marginal leaf scorching, and the scorched leaf zone may be bordered by a darker band (Brannen *et al.*, 2022; EPPO, 2023b). 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. Following leaf drop, the plant dies, typically during the second year after symptoms are observed (Chang *et al.*, 2009).

3.1.8 Phony peach disease and plum leaf scald

In phony peach disease and plum leaf scald, young shoots are stunted and bear greener, denser foliage than those on healthy trees. Strains associated with *X. fastidiosa* subsp. *multiplex* have been associated with phony peach disease. 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 longer than on healthy trees. Affected trees yield increasingly fewer and smaller fruits, becoming economically worthless after three to five years (Mizell *et al.*, 2008).

3.1.9 Alfalfa dwarf

The main symptom of alfalfa dwarf is stunted regrowth after cutting. This stunting may not be apparent until many months after initial infection. Leaflets on affected plants are smaller and often slightly darker in colour than those on uninfected plants, but not distorted, cupped, mottled or yellow. The tap-root is of a normal size, but the lignified tissue has an abnormally yellowish colour, with fine dark streaks of

dead tissue scattered throughout. In newly 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 (EPPO, 2023b). The inner bark is not discoloured, nor do large brown or yellow patches appear as in bacterial wilt (caused by *Clavibacter insidiosus*). Alfalfa dwarf progressively worsens over the first one to two years after the symptoms appear, and eventually kills infected plants.

3.1.10 Other hosts

X. fastidiosa has been detected on a number of different hosts in the recent European outbreaks. Most symptomatic plants display leaf scorching symptoms. On *N. oleander*, necrosis develops on the leaf margin and infection may lead to the death of entire plants (EPPO, 2023b). *P. myrtifolia* and *Spartium junceum* have been found to be two of the most susceptible hosts in the recent European outbreaks. Infected plants show scorched leaves, with desiccation starting from the tip and progressing to the entire blade (EPPO, 2023b). Symptoms can be seen at <https://gd.eppo.int/taxon/XYLEFA/photos>.

3.2 Sampling and sample preparation for symptomatic and asymptomatic material

Samples of necrotic and dead tissue or sections of the plant at an advanced stage of infection are unsuitable for *X. fastidiosa* diagnosis as saprophytes quickly colonize necrotic or dead tissue, interfering with the isolation or detection of the pathogen. *X. fastidiosa* is confined to the xylem tissue of its hosts. The petiole and the midrib recovered from leaf samples are therefore a good source for diagnosis, as they contain a greater number of xylem vessels (Hopkins, 1981). Other sources of tissue may include small twigs and roots of *P. persica* (Aldrich, Gould and Martin, 1992), stem and roots of *Vaccinium* (Holland *et al.*, 2014) and *Citrus* fruit petioles (Rossetti *et al.*, 1990). Samples should be taken close to the symptoms and preferably consist of stems that have mature symptomatic leaves with petioles and woody twigs. Individual leaves with petioles may also be sampled. Samples of branches or canes with attached leaves that include mature leaves generally provide the most reliable results. Young growing shoots should be avoided. For small plants, the entire plant may be sent to the laboratory. D'Onghia *et al.* (2022) 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).

Guidance on the number of leaves (including their petioles), twigs or stems and approximate weights to be used in the laboratory sample is presented in Table 1 for the testing of individual plants and in Table 2 and Table 3 for the testing of composite samples (EPPO, 2023b). For detailed guidance on the sampling of composite samples, see also Loconsole *et al.* (2021).

Table 1. 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 and type of tissue	Minimum number of leaves per laboratory sample	Approximate weight of laboratory sample
Sample from individual plant with leaves	Basal parts of leaves of large size (e.g. from <i>Citrus</i> spp., <i>Coffea</i> spp., <i>Nerium oleander</i> , <i>Ficus</i> spp., <i>Vitis</i> spp.)	5	0.5–1 g
	Basal parts of leaves of small size (e.g. from <i>Olea</i> spp., <i>Polygala myrtifolia</i>)	25	0.5–1 g
	Plant species without petioles or with small petiole and midrib (basal parts of leaves and midrib)	25	0.5–1 g
Dormant plant or cuttings	Xylem tissue [†]	n/a	0.5–1 g
Other cuttings	Stems	n/a	1 g

Notes: [†] The superficial bark should be removed and scrapings taken from the active tissues (youngest external ring).

n/a, not applicable.

Source: EPPO (European and Mediterranean Plant Protection Organization). 2023. Diagnostic protocol for *Xylella fastidiosa*. PM 7/24(5). *EPPO Bulletin*, 53: 205–276. <https://doi.org/10.1111/epp.12923>

3.2.1 Sampling period for symptomatic or asymptomatic plants

The distribution and concentration of *X. fastidiosa* within the plant can be variable and is dependent upon the plant species and seasonal and environmental factors. As a result, sampling during the period of active growth of the plants (Hopkins, 1981) maximizes the likelihood of detection. For tropical plant species grown indoors, such as coffee plants, sampling may be performed year-round when plants are exhibiting periods of active growth (EPPO, 2023b).

Experience in Europe (EPPO, 2023b) has shown the following:

- *O. europaea* and *N. oleander* sampling can be performed year-round, with no decrease in the diagnostic sensitivity throughout the year, except in the warmest and coldest periods (D’Onghia *et al.*, 2022). These observations are considered valid for areas with a Mediterranean climate.
- *P. myrtifolia* sampling can be performed from late spring to autumn with adequate diagnostic sensitivity.
- For deciduous plant species (e.g. *Prunus* spp.), symptoms have been consistently recorded, together with a detectable bacterium concentration, in leaves collected during summer (Olmo *et al.*, 2017; Zecharia *et al.*, 2021).
- If necessary, dormant plants can be sampled by taking mature branches (e.g. woody cuttings), from which the xylem tissue is recovered and processed.

In temperate zones of the world where *V. vinifera* or deciduous trees (e.g. *Prunus cerasus*, *P. dulcis*) have been infected for some time, the bacteria do not move into the new season’s growth until the middle of summer, when symptoms may also become visible (Kahn *et al.*, 2023). For example, the most suitable time for searching for symptoms in grapevine is late summer to early autumn when weather conditions are predominately hot and dry or when grape plants are exposed to drought stress (Galvez *et al.*, 2010).

In a tropical climate, such as in Brazil, *X. fastidiosa*-associated symptoms and detection occurs throughout the year for olive quick decline syndrome (Safady *et al.*, 2019) and mainly from February to June for CVC in the southern hemisphere (Bassanezi and Primiano, 2021). The vigorous sprout growth can make it difficult to identify symptoms during periods of rain and high temperatures.

3.2.2 Sampling of symptomatic plants

The sample should consist of branches or cuttings representative of the symptoms seen on the plant or plants and containing at least 5 to 25 leaves or twigs (or a combination of leaves and twigs) depending on leaf size. The approximate weight needed for laboratory samples is between 0.5 g and 1 g leaf petioles, midribs or other plant material from each individual plant (EPPO, 2023b) (Table 1). Symptomatic plant material should preferably be collected from a single plant; however, a pooled (i.e. composite) sample may also be collected. It is recommended that, when testing composite samples, the limit of detection for each detection test be confirmed.

3.2.3 Sampling of asymptomatic plants

For asymptomatic plants, the sample should be representative of the entire aerial part of the plant. Recent experimental data on detection of *X. fastidiosa* in monumental and ancient *O. europaea* trees showed that detection was more reliable when sampling the medium–upper part of the canopy (Valentini and Porcelli, 2016). As mentioned in the first paragraph of section 3.2, olive tree twigs have been found to be a better matrix than leaves for the detection of the bacterium. For testing individual asymptomatic plants, at least four to ten branches should be collected, depending on the host and plant size. Detailed guidance on collecting the minimum amount of tissue from a plant to achieve consistent and reliable detection can be found in Loconsole *et al.* (2021).

Further information on the number of samples to be collected per lot can be found in ISPM 31 (*Methodologies for sampling of consignments*). Sampling details for tests of composite samples composed of a small amount of tissue are presented in Table 2. Sampling details for tests of samples composed of a large amount of tissue are presented in Table 3. Validation data are available in the European and Mediterranean Plant Protection Organization (EPPO) database on diagnostic expertise (EPPO, n.d.(b)).

Table 2. Guidance for both sampling and laboratory testing of composite samples composed of a small amount of tissue

Host species	Minimum no. of leaves, twigs or stems to be collected per plant	No. of plants that may be pooled
<i>Olea europaea</i> (small-sized plants, i.e. in nurseries or consignments)	4 (leaf midribs, petioles or leaf basal part for each leaf)	5
<i>Olea europaea</i> (trees of large size, i.e. in orchards)	4 (pieces of 1–1.5 cm for each twig)	7
<i>Nerium oleander</i>	3 (petioles)	8
<i>Polygala myrtifolia</i>	6 (pieces of 1–1.5 cm for each twig)	8
<i>Prunus avium</i>	4 (0.1 g of xylem chips for each twig)	5

Note: The extraction procedure does not include a bacterial concentration step: the volume of extraction buffer is 1:10 w:v (EPPO, 2023).

EPPO (European and Mediterranean Plant Protection Organization). 2023. Diagnostic protocol for *Xylella fastidiosa*. PM 7/24(5). *EPPO Bulletin*, 53: 205–276. <https://doi.org/10.1111/epp.12923>

Source: Loconsole, G., Zicca, S., Manco, L., Hatib, O. El, Altamura, G., Potere, O., Elicio, V., Valentini, F., Boscia, D. & Saponari, M. 2021. Diagnostic procedures to detect *Xylella fastidiosa* in nursery stocks and consignments of plants for planting. *Agriculture*, 11(10): 922. <https://doi.org/10.3390/agriculture11100922>

Table 3. Guidance on sampling for lots of large composite samples (e.g. composite samples from consignments or places of production of plants for planting)

Host species	Minimum no. of leaves, plantlets, twigs or stems to be collected per plant	Maximum size of the composite sample	Maximum no. of plants that may be pooled
<i>Olea europaea</i> [†]	4 (leaves)	800–900 leaf midribs, petioles or basal parts (up to 20 g)	200 to 225
<i>Nerium oleander</i>	2 (leaves)	200 leaf petioles (up to 20 g)	100
Herbaceous plantlets	1 (plantlet)	200 stems (up to 40 g)	200
<i>Polygala myrtifolia</i> [‡]	2 (twigs)	250 shoot pieces of 1.5–2 cm (up to 2020 g)	125
<i>Lavandula</i> spp. [§]	2 (stems)	180–200 shoot pieces of 2.5–3 cm (up to 20 g)	90 to 100
<i>Prunus avium</i>	2 (twigs)	200 wood chips (up to 20 g)	100
<i>Coffea</i> spp.	2 (leaves)	100 petioles and midribs (up to 10 g)	50
<i>Helichrysum italicum</i>	2 (stems)	100 pieces of stems 2–3 cm (5 g)	50

Notes: The extraction procedure performed includes a bacterial concentration step: the volume of extraction buffer is 1:3 w:v for all samples except for herbaceous plantlets (1:1 or 1:1.5) and *Coffea* (1:4) (EPPO, 2023).

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

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

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

Sources: Bergsma-Vlami, M., van de Bilt, J.L.J., Tjou-Tam-Sin, N.N.A., Helderma, C.M., Gorkink-Smits, P.P.M.A., Landman, N.M., Nieuwburg, J.G.W., van Veen, E.J. & Westenberg, M. 2017. Assessment of the genetic diversity of *Xylella fastidiosa* in imported ornamental *Coffea arabica* plants. *Plant Pathology*, 66: 1065–1074. <https://doi.org/10.1111/ppa.12696>

EPPO (European and Mediterranean Plant Protection Organization). 2023. Diagnostic protocol for *Xylella fastidiosa*. PM 7/24(5). *EPPO Bulletin*, 53: 205–276. <https://doi.org/10.1111/epp.12923>

Loconsole, G., Zicca, S., Manco, L., Hatib, O. El, Altamura, G., Potere, O., Elicio, V., Valentini, F., Boscia, D. & Saponari, M. 2021. Diagnostic procedures to detect *Xylella fastidiosa* in nursery stocks and consignments of plants for planting. *Agriculture*, 11(10): 922. <https://doi.org/10.3390/agriculture11100922>

3.2.4 Plant sample transport and storage in the laboratory

Once samples are collected, they should be kept cool (e.g. 4–15 °C) and transported to the laboratory as quickly as possible (preferably within no more than two days) in clean, transparent, plastic sample bags. Lower temperatures can reduce sample deterioration. However, *X. fastidiosa* does not survive well in cold temperatures and so 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 that the samples are checked for the presence of insects before opening the sample bags. If any insects are present, samples should be stored in the refrigerator for approximately 12 h.

Samples for isolation (see section 4.1) should be processed immediately or stored refrigerated (e.g. 4 °C) for up to three days. For isolation, samples should be surface disinfected. For other tests, samples may be refrigerated for up to one week. For longer term storage, plant samples may be stored at –20 °C or –80 °C for up to one year for molecular or serological detection.

3.3 Sampling and testing of vectors

Insects collected with sweeping nets (adults), light trapping or aspirators or from sticky traps may be used for testing. Vectors can be removed from the sticky traps using small forceps (pincers) and a suitable solvent. After removal from the traps, insects should be rinsed in ethanol or acetone. Sampling for insects can be carried out from late spring until autumn to maximize the likelihood of detecting the bacterium. If insects cannot be processed immediately, they should be stored in 95–99% ethanol at –20 °C or at –80 °C with or without ethanol. Sticky traps with captured insects can also be stored at

–20 °C. A video on collecting spittlebugs on olive in Europe has been published by the European Food Safety Authority.¹ Identification resources are available (Purcell *et al.*, 2014; EPPO, 2020).

Insects collected may be analysed by PCR to detect *X. fastidiosa*. Enzyme-linked immunosorbent assay (ELISA: see section 3.4) is not sensitive enough, as the bacterium only colonizes the insect foregut where, despite its multiplication, it is generally present at low levels (Purcell *et al.*, 2014). According to Cornara *et al.* (2016), there is a saturation point for the number of cells detected in the mouthparts of spittlebug (*Philaenus spumarius*) and the population size of *X. fastidiosa* may be limited to fewer than 10³ cells. In France, the bacterial load of *P. spumarius* collected in the Corsica region was estimated by droplet digital PCR and ranged from 10¹ to 10⁶ cells per insect (Cunty *et al.*, 2020). Amoia *et al.* (2023) further validated the droplet digital PCR method and were able to detect down to 10² cells per insect in *P. spumarius* samples. In Italy, the bacterial load can be about 10⁴ cells per insect (Cavaliere *et al.*, 2019). In the United States of America, the number of cells can be higher than 10⁴ cells per insect in sharpshooter vectors (Retchless *et al.*, 2014; Ranieri *et al.*, 2020).

3.4 Serological detection

A number of serological methods have been developed for the detection of *X. fastidiosa*, including methods using ELISA (Sherald and Lei, 1991), membrane entrapment immunofluorescence (Hartung *et al.*, 1994), dot immunobinding assay (Lee *et al.*, 1992), western blotting (Chang *et al.*, 1993) and immunofluorescence (Carbajal, Morano and Morano, 2004). More recently, direct tissue blot immunoassay has been reported as an alternative means of rapidly screening *O. europaea* samples for *X. fastidiosa* (Djelouah *et al.*, 2014). Instructions for performing an ELISA (including tissue print, squash or dot ELISA) or an immunofluorescence test can be found in EPPO (2009, 2010). Serological methods are not sensitive enough for use early in the growing season, when no symptoms of the disease are observed, because of the low concentration of bacteria likely to be present in young asymptomatic tissue. Enzyme-linked immunosorbent assay works well for samples with symptoms and tissue that contains high concentrations of *X. fastidiosa*. The leaf petiole and mid-veins of symptomatic leaves are the best sources of tissue for ELISA. The technique can also be used on twigs and canes but is unsuitable for use on necrotic or dead tissue.

3.4.1 Double-antibody sandwich ELISA

Positive and negative controls should be included in each test and these are normally provided in commercial kits. Positive controls should consist of a reference *X. fastidiosa* strain resuspended in healthy host-plant extract (for detection in plant material) or in phosphate-buffered saline (PBS) (for identification of bacterial cultures). Negative controls should consist of healthy host-plant extract (for detection in plant material) or a suspension of a non-target bacterial species (for identification of bacterial cultures). For plant materials, the healthy plant-extract control should be of the same species, variety or cultivar to allow for comparison with the test samples and to check for potential background- or cross-reactions.

Samples should be processed following the general procedure recommended for the specific serological method being used. In general, plant tissue is macerated in extraction buffer (polyvinylpyrrolidone (PVP)-10, 20 g; mannitol, 10 g; ascorbic acid, 1.76 g; reduced glutathione, 3 g; PBS, 10 mM, 1 litre; pH 7.2) or in PBS (NaCl, 8 g; KCl, 0.2 g; Na₂HPO₄·12 H₂O, 2.9 g; KH₂PO₄, 0.2 g; distilled water to 1 litre; pH 7.2) (1:10 w/v) using either a mortar and pestle or a tissue homogenizer (e.g. Polytron, Homex) or is pulverized in liquid nitrogen (Loconsole *et al.*, 2014; EPPO, 2023b).² Further information on using double-antibody sandwich ELISA (DAS-ELISA) to detect plant pathogenic bacteria is available in EPPO (2010).

¹ <https://www.youtube.com/watch?v=Rjh7FFQCtg8>

² The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

Kits for the serological detection of *X. fastidiosa* are commercially available from Agritest, Agdia and Loewe Biochemica.² These kits detect a wide range of *X. fastidiosa* strains isolated from different hosts. When using them, the manufacturer's instructions should be followed. The sensitivity of detection when using DAS-ELISA is approximately 10⁴ colony-forming units (c.f.u.)/mL; however, test sensitivity can vary depending on the plant species matrix being tested (Loconsole *et al.*, 2014; EPPO, 2023b).

The specificity and sensitivity of DAS-ELISA to detect *X. fastidiosa* on *O. europaea*, using a kit from Loewe, were evaluated by Loconsole *et al.* (2014).² Additionally, a test performance study at the Institute for Sustainable Plant Protection (Bari, Italy) was conducted on serological kits from Agritest, Agdia and Loewe.² These studies showed that these kits achieved 100% diagnostic sensitivity and specificity when testing naturally infected samples. The data on the test performance study are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

3.4.2 Interpretation of ELISA results

The reactions of the controls should be verified. Negative ELISA readings in positive control wells indicate that the test has not been performed correctly or that it has been inhibited. Positive ELISA readings in negative control wells indicate that cross-contamination or non-specific antibody binding has occurred. In these cases, the test should be performed again.

Once the reactions of the controls have been verified, the results for each sample are interpreted as follows:

- The ELISA is negative if the average absorbance reading of duplicate wells containing tissue macerate is <2× the average absorbance reading of the negative control wells containing healthy host-tissue macerate.
- The ELISA is positive if the average absorbance reading of duplicate wells is ≥2× the average absorbance reading of the negative control wells containing healthy host-tissue macerate.
- The manufacturer's instructions should also be checked for interpretation of test results.

3.5 Molecular detection

Various molecular methods have been developed for the detection of *X. fastidiosa* directly on plant tissue and insect vectors (Firrao and Bazzi, 1994; Minsavage *et al.*, 1994; Pooler and Hartung, 1995; Oliveira *et al.* 2002; Schaad, Opgenorth and Gaush, 2002; Rodrigues *et al.*, 2003; Francis *et al.*, 2006; Harper, Ward and Clover, 2010; Li *et al.*, 2013; Ouyang *et al.*, 2013; Bonants *et al.*, 2019; Dupas *et al.*, 2019a; Hodgetts *et al.*, 2021). The latter two tests allow subspecies assignment. A digital PCR (Dupas *et al.*, 2019b) and isothermal amplification for use in the field without prior extraction steps (loop-mediated isothermal amplification (LAMP) (Yaseen *et al.*, 2015) or recombinase polymerase amplification (Cesbron, Dupas and Jacques, 2023)) have also been developed or evaluated.³ The conventional PCRs developed by Minsavage *et al.* (1994), Pooler and Hartung (1995) and Rodrigues *et al.* (2003), two real-time PCRs (Harper, Ward and Clover, 2010; Li *et al.*, 2013), and the two real-time PCRs allowing subspecies assignment (Dupas *et al.*, 2019a; Hodgetts *et al.*, 2021) are described in this protocol for the detection of *X. fastidiosa*. The real-time methods using isothermal amplification such as LAMP are also described in this protocol.³ The PCR methods described hereafter are as described in the original publications.

3.5.1 DNA extraction from plant material

A number of methods have been described for the extraction of the DNA of *X. fastidiosa* from plant material (Minsavage *et al.*, 1994; Pooler and Hartung, 1995; Francis *et al.*, 2006; Huang, Bentz and Sherald, 2006; Harper, Ward and Clover, 2010; Li *et al.*, 2013). Extraction can be achieved using

³ When using LAMP on a regular basis in an area which has a patent system such as Japan (Patent Nos. 3,313,358, 3,974,441 and 4,139,424), the United States of America (US6,410,278, US6,974,670 and US7,494,790), the European Union (Nos. 1,020,534, 1,873,260, 2,045,337 and 2,287,338), China (ZL008818262), the Republic of Korea (Patent No. 10-0612551), Australia (No. 779160), and the Russian Federation (No. 2,252,964), it is necessary for users to receive a licence from Eiken Chemical Co., Ltd. before use.

standard commercial kits (e.g. Huang, Bentz and Sherald, 2006; Bextine and Child, 2007). The following methods are a selection of those widely used in laboratories. Many other similar DNA extraction kits will also readily extract *Xylella* DNA from plant material. Validation data on the sensitivities associated with the different nucleic acid extraction methods can be found in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

The analytical sensitivity of PCR tests can be improved when an additional ultrasonication (1 min at 35–40 kHz) is performed on the plant extract before DNA extraction (Bergsma-Vlami *et al.*, 2017; Dupas *et al.*, 2019a). This has improved the release of bacteria from biofilms, in particular with difficult matrices such as *O. europaea* and *Quercus* spp. Validation data from the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

CTAB-based extraction (EPPO, 2023b). In brief, 0.5–1 g midrib, petiole or twig tissue is placed into an extraction bag with 5 mL extraction buffer (hexadecyltrimethylammonium bromide (CTAB) buffer: 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 10 mM ethylenediaminetetraacetic acid (EDTA); 2% CTAB; 3% PVP-40) and homogenized using a homogenizer (e.g. Homex, Polytron).² The homogenate (1 mL) is transferred to a microcentrifuge tube and incubated at 65 °C for 30 min. After cooling, the tube is centrifuged at 16 000 g for 10 min. The supernatant (1 mL) is transferred to a new tube and mixed with the same volume of chloroform:isoamylalcohol (24:1, v/v), vortexed and then centrifuged at 3 000 g for 15 min. The aqueous layer (the upper layer – approximately 700 µL) is carefully transferred to a new tube and mixed with 490 µL ice-cold isopropanol. The suspension is mixed gently and incubated for at least 20 min at –20 °C. After this DNA precipitation step, the suspension is centrifuged at 16 000 g for 15 min and the supernatant is then discarded, taking care not to disturb the pellet. The pellet is washed with 1 mL ethanol (70%) by repeating the last centrifugation step. After washing and decanting the supernatant, the pellet is air-dried and suspended in 100 µL Tris-EDTA buffer or deoxyribonuclease-free water.

DNeasy® Plant Mini Kit (QIAGEN).² DNA is extracted from at least 0.1–0.2 g plant tissue (leaf midrib, petiole or twig tissue) and macerated in lysis buffer using homogenizing equipment (e.g. Homex, Polytron).² Alternatively, plant tissue can be ground to a fine powder in liquid nitrogen before extraction. These extracts are then treated according to the manufacturer's instructions.

DNeasy® mericon® Food Kit (Standard Protocol) (QIAGEN) and Maxwell® RSC PureFood GMO and Authentication Kit (Promega).² In a performance study, the first of these kits was adapted to recover high-quality DNA from a wide range of plant species and both kits performed well with DNA extracts from a large amount of tissue (EPPO, 2023b). Validation data are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

QuickPick™ SML Plant DNA Kit (QRET Technologies).² Plant tissue (200 mg leaf midrib, petiole or twig tissue) is homogenized using any of the available methods (e.g. mechanical grinding with bead mills or with liquid nitrogen, tissue grinder). The plant tissue should be sufficiently homogenized before starting the purification procedure. Appropriate volumes of plant DNA lysis buffer and proteinase K solution, as specified in the manufacturer's instructions, are added to the plant tissue. The sample is thoroughly vortex-mixed and then incubated at 65 °C for 15–30 min. After the lysis step, DNA purification is performed according to the manufacturer's instructions. Alternatively, a larger sample size can be processed by crushing 0.5–1 g fresh small pieces of midribs, petioles, basal leaf parts or twigs in 5 mL sterile water and leaving to soak for 15 min with gentle shaking. The plant extract (250 µL) is centrifuged for 20 min at 20 000 g. The pellet is then suspended in 75 µL lysis buffer with 5 µL proteinase K and the manufacturer's instructions followed. This method can be performed either manually or with the KingFisher™ mL (15 samples) or KingFisher™ Flex (96 samples) Purification System (Thermo Fisher Scientific).² Validation data are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)). Caution is needed for users who are not familiar with this method, if performing manually, because the risk of cross-contamination between samples is high.

InviMAG® Plant DNA Mini Kit (Invitex Molecular) using the **KingFisher™ Flex Purification System** (96 samples) (Thermo Scientific).² This automated magnetic bead extraction procedure is ideal for high-throughput testing. Samples are homogenized in the lysis kit buffer (or CTAB buffer) at a tissue to buffer ratio of 1:5. The plant extracts are incubated at 60 °C for 30 min and then treated according to the manufacturer's instructions.

3.5.2 DNA extraction from insect vectors

DNA may be extracted from a single insect head or a pool of up to ten heads (EPPO, 2023b). Only the heads of insects are used, because they contain the foregut and mouthparts where *X. fastidiosa* resides (Bextine *et al.*, 2004). For DNA extraction from insects with larger heads (e.g. *Cicadella viridis*, *Cicada orni*), only a single head should be used. The removal of the eye tissue, a potential source of PCR inhibitors, is recommended as it has been reported that this increases sensitivity (Bextine *et al.*, 2004; Purcell *et al.*, 2014). Insect tissue can be ground in lysis buffer, or homogenized using a bead-beater system such as MagNA Lyser (Roche) or by vacuum application and release (Bextine *et al.*, 2004, 2005; Huang, Bentz and Sberald, 2006).² A number of DNA extraction methods have been evaluated for the detection of *X. fastidiosa* in insect vectors. The following methods are a selection of those widely used in laboratories.

CTAB-based extraction for insects. The homogenization of the insect heads can be performed in a microcentrifuge tube using a microhomogenizer or tungsten carbide beads. For the DNA extraction of insect samples, 500 µL CTAB buffer is used. The incubation and centrifugation steps are similar to those used for plant samples (see section 3.5.1), but with proportional volumes.

DNeasy® Blood & Tissue Kit (QIAGEN).² A DNA extraction method using this kit has been shown to reliably detect 50–500 *X. fastidiosa* cells in *Homalodisca coagulata* (Bextine *et al.*, 2004, 2005; Huang, Bentz and Sberald, 2006).

QuickPick™ SML Plant DNA Kit (QRET Technologies).² The homogenization of individual insect heads or groups of up to ten heads can be performed in 200 µL sterile distilled water using a bead-beater system such as the Mixer Mill MM400 (Retsch).² Samples are homogenized for 2 min at 30 Hertz using ten stainless steel beads (diameter 3 mm) per 2 mL microcentrifuge tube. The microcentrifuge tube is placed on a magnet and the supernatant is transferred to a new microcentrifuge tube. The extract is centrifuged for 20 min at 20 000 g. The pellet is then suspended in 37.5 µL lysis buffer with 2.5 µL proteinase K and the manufacturer's instructions followed. This kit can be used either manually or with the KingFisher™ mL (15 samples) or KingFisher™ Flex (96 samples) Purification System (Thermo Scientific) (Cunty *et al.*, 2020).²

Maxwell® RSC PureFood GMO and Authentication Kit. A DNA extraction method using this kit has been shown to reliably detect *X. fastidiosa* cells in insect vectors (EPPO, 2023b).

3.5.3 Conventional PCR using the primers of Minsavage *et al.* (1994)

This PCR was designed by Minsavage *et al.* (1994) to target part of the *rpoD* gene, producing an amplicon of 733 base pairs (bp). It is widely used in laboratories for the detection of *X. fastidiosa* in different host plants and vectors. Analytical specificity was validated later by Harper, Ward and Clover (2010) with 22 different *X. fastidiosa* strains from 11 different hosts and 12 closely related or host-related non-target bacterial strains. In their study, American *X. fastidiosa* strains from *Quercus rubra* and *Quercus laevis* and several strains from grapevines were not detected with this PCR. The analytical sensitivity of the method as stated by Minsavage *et al.* (1994) is 1×10^2 c.f.u./mL on *V. vinifera* and *P. persica*. Further validation data on other hosts are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

The master mix composition and cycling parameters described by Minsavage *et al.* (1994) for this PCR are presented in Table 4, but variations on these have also been developed and validated in European laboratories (EPPO, 2023b).

The oligonucleotide primers used are:

RST31 (forward): 5'-GCG TTA ATT TTC GAA GTG ATT CGA TTG C-3'

RST33 (reverse): 5'-CAC CAT TCG TAT CCC GGT G-3'

Table 4. Master mix composition, cycling parameters and amplicons for conventional PCR using the primers of Minsavage *et al.* (1994)

Reagents	Final concentration
PCR-grade water	–†
PCR buffer (Invitrogen)‡	1×
dNTPs	200 µM
MgCl ₂	1.5 mM
Primer RST31 (forward)	0.5 µM
Primer RST33 (reverse)	0.5 µM
Taq DNA polymerase (Invitrogen)‡	1.25 U
DNA volume	2 µL bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	95 °C for 1 min
Number of cycles	40
- Denaturation	95 °C for 30 s
- Annealing	55 °C for 30 s
- Elongation	72 °C for 45 s
Final elongation	72 °C for 5 min
Expected amplicons	
Size	733 bp

Notes: † For a final reaction volume of 20 µL.

‡ See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Source: Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, R.M.V.B.C. & Stall, R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology*, 84: 456–461.

<https://doi.org/10.1094/Phyto-84-456>

3.5.4 Conventional PCR using the primers of Pooler and Hartung (1995)

This PCR was designed by Pooler and Hartung (1995) with primers that target a specific, random amplified polymorphic DNA (RAPD) fragment present in *X. fastidiosa*. The primers 272-1-int and 272-2-int are known to detect all known strains of *X. fastidiosa*. Analytical specificity has been validated with 57 different *X. fastidiosa* strains collected from different regions of Brazil and the United States of America (Huang, 2009; Reisenzein, 2017).

The oligonucleotide primers used are:

272-1-int (forward): 5'-CTG CAC TTA CCC AAT GCA TCG-3'

272-2-int (reverse): 5'-GCC GCT TCG GAG AGC ATT CCT-3'

The master mix used for this PCR is described in Table 5.

Table 5. Master mix composition, cycling parameters and amplicons for conventional PCR using the primers of Pooler and Hartung (1995)

Reagents	Final concentration
PCR-grade water	–†
PCR buffer (Invitrogen)‡	1×
dNTPs	200 µM
MgCl ₂	1.5 mM
Primer 272-1-int (forward)	0.4 µM
Primer 272-2-int (reverse)	0.4 µM
Taq DNA polymerase (Invitrogen)‡	1.0 U
DNA volume	2 µL bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 1 min
Number of cycles	40
- Denaturation	94 °C for 1 min
- Annealing	67 °C for 1 min
- Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	500 bp

Notes: † For a final reaction volume of 20 µL.

‡ See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Source: Pooler, M.R. & Hartung, J.S. 1995. Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Current Microbiology*, 31: 377–381. <https://doi.org/10.1007/BF00294703>

3.5.5 Real-time PCR using the primers and probes of Harper, Ward and Clover (2010)

This PCR, developed by Harper, Ward and Clover (2010), is designed to amplify part of the 16S ribosomal (r)RNA processing protein *rimM* gene. DNA can be amplified from bacterial cultures, infected leaves, cane tissue or insect vectors. This PCR can be performed, with internal positive controls, either as a simplex PCR on plants and cultures or as a duplex PCR on plants and vectors (EPPO, 2023b).

Harper, Ward and Clover (2010) evaluated analytical specificity with 95 strains of *X. fastidiosa* from 20 different hosts and 26 non-target bacterial strains. Only *X. fastidiosa* was detected. *X. taiwanensis* from the Taiwan Province of China was not detected. The PCR was further validated by Li *et al.* (2013). The diagnostic specificity and sensitivity, as determined using citrus and grape hosts, are both 100% (EPPO, 2023b). For *O. europaea* hosts when using CTAB extraction methods, the diagnostic specificity is 100% and the diagnostic sensitivity is 91% (EPPO, 2023b). Further validation data are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)). The analytical sensitivity varies depending on the host plant and the DNA extraction protocol (EPPO, 2023b).

The oligonucleotide primers and probes used are:

XF-F (forward primer): 5'-CAC GGC TGG TAA CGG AAG A-3'

XF-R (reverse primer): 5'-GGG TTG CGT GGT GAA ATC AAG-3'

XF-P (hydrolysis probe): 5'-6-FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ-1-3'

The master mix for the Harper, Ward and Clover (2010) primers and probes is described in Table 6.

Table 6. Master mix composition and cycling parameters for real-time PCR using the primers and probes of Harper, Ward and Clover (2010)

Reagents	Final concentration
PCR-grade water	–†
PCR mix (2x Supermix-UDG (Invitrogen))‡	1x
MgCl ₂	4 mM
Bovine serum albumin (non-acetylated)	300 ng/μL
Primer XF-F (forward)	0.3 μM
Primer XF-R (reverse)	0.3 μM
Probe XF-P	0.1 μM
DNA volume	2 μL bacterial suspension or DNA extract
Cycling parameters	
Pre-incubation	50 °C for 2 min
Initial denaturation	94 °C for 2 min
Number of cycles	40
- Denaturation	94 °C for 10 s
- Annealing and elongation	62 °C for 40 s

Notes: † For a final reaction volume of 20 μL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Harper, S.J., Ward, L.I. & Clover, G.R.G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology*, 100: 1282–1288. [Incorporates the correction of an erratum in 2013.] <https://doi.org/10.1094/PHYTO-06-10-0168>

3.5.6 Real-time PCR using the primers and probes of Li *et al.* (2013)

This PCR, developed by Li *et al.* (2013), is designed to amplify part of the 16S rRNA gene. DNA can be amplified from bacterial cultures, infected leaves, cane tissue or insect vectors.

Li *et al.* (2013) evaluated analytical specificity with 77 strains of *X. fastidiosa* from 15 different hosts and 14 non-target bacterial strains. Only *X. fastidiosa* was detected. The diagnostic specificity and sensitivity, as determined using *Citrus* hosts, were both 100%. The analytical sensitivity (detection limit) was between two and ten cells of *X. fastidiosa* per reaction for *Citrus* samples.

The oligonucleotide primers and probes used are:

XF16Sf (forward primer): 5'-CGG CAG CAC GTT GGT AGT AA-3'

XF16Sr (reverse primer): 5'-CCG ATG TAT TCC TCA CCC GTC-3'

XF16Sp (hydrolysis probe): 5'-6-FAM-CA TGG GTG GCG AGT GGC-BHQ-1-3'

The master mix for the Li *et al.* (2013) real-time PCR is described in Table 7.

Table 7. Master mix composition and cycling parameters for real-time PCR using the primers and probes of Li *et al.* (2013)

Reagents	Final concentration
PCR-grade water	–†
PCR buffer (Invitrogen)‡	1×
dNTPs	240 µM
MgCl ₂	6 mM
Primer XF16Sf (forward)	0.240 µM
Primer XF16Sr (reverse)	0.240 µM
Probe XF16Sp	0.12 µM
Platinum Taq (Invitrogen)‡	1 U
DNA volume	2 µL bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	95 °C for 20 s
Number of cycles	40
- Heating ramp speed	5 °C/s
- Denaturation	95 °C for 1 s
- Annealing and elongation	60 °C for 40 s

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Li, W.B., Teixeira, D.C., Hartung, J.S., Huang, Q., Duan, Y., Zhou, L., Chen, J. *et al.* 2013. Development and systematic validation of qPCR assays for rapid and reliable differentiation of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Journal of Microbiological Methods*, 92: 79–89. <https://doi.org/10.1016/j.mimet.2012.10.008>

3.5.7 Real-time PCR using the primers and probes of Dupas *et al.* (2019a)

This tetraplex real-time PCR is suitable for the detection and identification of *X. fastidiosa* and the assignment of subspecies on plant samples and cell cultures. The target sequences are:

- a gene coding for a ketol-acid reductoisomerase (WP_004084873) (M23) to identify *Xylella fastidiosa* (*Xf*);
- a gene coding for a restriction modification system (ACB93575) (M23) to identify *X. fastidiosa* subsp. *fastidiosa* (*Xff*);
- a genome region with unknown function (M23) to identify *X. fastidiosa sensu lato* (*Xfsl*);
- a gene coding for an S24 peptidase (AIC14009) (MUL0034) to identify *X. fastidiosa* subsp. *morus* (*Xfmo*);
- a gene coding for a DNA adenine methylase (WP_004083560) (M12) to identify *X. fastidiosa* subsp. *multiplex* (*Xfm*); and
- a gene coding for a histidine kinase and an ABC transporter substrate (ARO67912, ARO69620) (De Donno) to identify *X. fastidiosa* subsp. *pauca* (*Xfp*).

Dupas *et al.* (2019a) evaluated analytical specificity with 39 strains of *X. fastidiosa* from different subspecies and 30 non-target bacterial strains. Only *X. fastidiosa* was detected. The diagnostic specificity and sensitivity were 100% and 92%, respectively, on pure DNA extract and 100% on tenfold diluted DNA. The analytical sensitivity (detection limit) in a range of spiked matrices from 13 different plant species ranged from 10³ to 10⁵ cells/mL.

To allow simultaneous detection of *X. fastidiosa* and identification at the subspecies level, primer and probe combinations were designed in six different triplex and tetraplex quantitative PCR sets. The oligonucleotide primers and probes are used in the following sets:

- Set 1: XF–XFFSL–XFM–XFP
- Set 2: XF–XFF–XFM–XFP
- Set 3: XF–XFF–XFM–XMO
- Set 4: XFFSL–XFM–XFP
- Set 5: Harper XF–XFFSL–XFM–XFP
- Set 6: Ioos 18S–XFFSL–XFM–XFP

The sequences for the Dupas *et al.* (2019a) primers and probes are presented in Table 8 and the corresponding master mix is described in Table 9.

Table 8. Oligonucleotide primers and probes used in the tetraplex real-time PCR of Dupas *et al.* (2019)

Target species or subspecies	Primer name	Sequence (5' → 3')	Reference
<i>Xylella fastidiosa</i>	XF-F	AAC CTG CGT GAC TCT GGT TT	Dupas <i>et al.</i> (2019)
	XF-R	CAT GTT TCG CTG CTT GGT CC	
	XF-P	FAM-GCT CAG GCT GAC GGT TTC ACA GTG CA-BHQ1	
<i>X. fastidiosa</i>	Harper XF-F	CAC GGC TGG TAA CGG AAG A	Harper Ward & Glover (2010)
	Harper XF-R	GGG TTG CGT GGT GAA ATC AAG	
	Harper XF-P	FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ1	
<i>X. fastidiosa</i> subsp. <i>fastidiosa</i>	XFF-F	TTA CAT CGT TTT CGC GCA CG	Dupas <i>et al.</i> (2019)
	XFF-R	TCG GTT GAT CGC AAT ACC CA	
	XFF-P	HEX-CCC GAC TCG GCG CGG TTC CA-BHQ1	
<i>X. fastidiosa</i> subsp. <i>fastidiosa sensu lato</i>	XFFSL-F	TAG TAT GCG TGC GAG CGA C	Dupas <i>et al.</i> (2019)
	XFFSL-R	CGC AAT GCA CAC CTA AGC AA	
	XFFSL-P	HEX-CGC GTA CCC ACT CAC GCC GC-BHQ1	
<i>X. fastidiosa</i> subsp. <i>morus</i>	XFMO-F	TAA CGC TAT CGG CAG GTA GC	Dupas <i>et al.</i> (2019)
	XFMO-R	GCA TCA GCT TCA CGT CTC CT	
	XFMO-P	CY5-GGT TCC GCA CCT CAC ATA TCC GCC C-BHQ2	
<i>X. fastidiosa</i> subsp. <i>multiplex</i>	XFM-F	ACG ATG TTT GAG CCG TTT GC	Dupas <i>et al.</i> (2019)
	XFM-R	TGT CAC CCA CTA CGA AAC GG	
	XFM-P	ROX-ACG CAG CCC ACC ACG ATT TAG CCG-BHQ2	
<i>X. fastidiosa</i> subsp. <i>pauca</i>	XFP-F	TGC GTT TTC CTA GGT GGC AT	Dupas <i>et al.</i> (2019)
	XFP-R	GTT GGA ACC TTG AAT GCG CA	
	XFP-P	CY5-CCA AAG GGC GGC CAC CTC GC-BHQ2	
18S	loos_18S_F	GCA AGG CTG AAA CTT AAA GGA A	loos <i>et al.</i> (2009)
	loos_18S_R	CCA CCA CCC ATA GAA TCA AGA	
	loos_18S_P	FAM-ACG GAA GGG CAC CAC CAG GAG T-BHQ1	

Sources: Dupas, E., Briand, M., Jacques, M.-A. & Cesbron, S. 2019. Novel tetraplex quantitative PCR assays for simultaneous detection and identification of *Xylella fastidiosa* subspecies in plant tissues. *Frontiers in Plant Science*, 10: 1732. <https://doi.org/10.3389/fpls.2019.01732>

Harper, S.J., Ward, L.I. & Glover, G.R.G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. [Incorporates the correction of an erratum in 2013.] *Phytopathology*, 100: 1282–1288. <https://doi.org/10.1094/PHYTO-06-10-0168>

loos, R., Fourrier, C., Iancu, G. & Gordon, T.R. 2009. Sensitive detection of *Fusarium circinatum* in pine seed by combining an enrichment procedure with a real-time polymerase chain reaction using dual-labeled probe chemistry. *Phytopathology*, 99: 582–590. <https://doi.org/10.1094/PHYTO-99-5-0582>

Table 9. Master mix composition and cycling parameters for tetraplex real-time PCR using the primers and probes of Dupas *et al.* (2019)

Reagents	Final concentration
PCR-grade water	–†
PCR mix (SsoAdvanced™ Universal Probe Supermix (Bio-Rad))‡	1×
Each primer F (forward)	575 nM
Each primer R (reverse)	575 nM
Each probe	200 nM
Bovine serum albumin (non-acetylated)	600 ng/μL
DNA volume	2 μL (1 μL DNA + 1 μL water)
Cycling parameters	
Initial denaturation	95 °C for 3 min
Number of cycles	40
- Denaturation	95 °C for 15 s
- Annealing and elongation	60 °C for 30 s

Notes: † For a final reaction volume of 10 μL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Dupas, E., Briand, M., Jacques, M.-A. & Cesbron, S. 2019. Novel tetraplex quantitative PCR assays for simultaneous detection and identification of *Xylella fastidiosa* subspecies in plant tissues. *Frontiers in Plant Science*, 10: 1732. <https://doi.org/10.3389/fpls.2019.01732>

3.5.8 Real-time PCR using the primers and probes of Hodgetts *et al.* (2021)

This simplex real-time PCR is suitable for the detection and the identification of *X. fastidiosa* and assignment of subspecies on plant samples and cell cultures. Hodgetts *et al.* (2021) evaluated analytical specificity with eight strains of *X. fastidiosa* from different subspecies (*X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *morus*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *sandyi*) and 50 non-target bacterial strains. Only *X. fastidiosa* was detected. The diagnostic specificity and sensitivity were both 100%. The analytical sensitivity (detection limit) was 124 fg DNA of *X. fastidiosa* subsp. *fastidiosa*, 59.2 fg DNA of *X. fastidiosa* subsp. *morus*, 182 fg DNA of *X. fastidiosa* subsp. *multiplex*, 84.2 fg DNA of *X. fastidiosa* subsp. *pauca*, and 908 fg DNA of *X. fastidiosa* subsp. *sandyi*.

The sequences for the Hodgetts *et al.* (2021) primers and probes are presented in Table 10 and the corresponding master mix is described in Table 11.

Table 10. Oligonucleotide primers and probes used in the simplex real-time PCR of Hodgetts *et al.* (2021)

Target subspecies	Primer	Sequence (5' → 3')
<i>X. fastidiosa</i> subsp. <i>fastidiosa</i>	Xff_3-Fw-a_122365	TCG TTG TGG GAT TAC CGT TAA A
	Xff_3-Rv_122365	ACC TGA GAA TTG CCC TTA ATC G
	Xff_3-Pr_122365	FAM-TCG AAA ACA CCG GAC TTG CCA ACA-BHQ1
<i>X. fastidiosa</i> subsp. <i>morus</i>	Xfmo_7_Fw_67218	CCA CCT CGC TTT AGT TAC GTG ATT
	Xfmo_7_Rva_67218	GGA GTT TAT TTG GCT GAA CTG AGT G
	Xfmo_7_Pr_67218	FAM-AAG CGT GAT ACT ACT CC-MGB-NFQ
<i>X. fastidiosa</i> subsp. <i>multiplex</i>	Xfmu_3-Fw_15747	CAA TCG CTT TTG AGG TCA TCC
	Xfmu_3-Rv_15747	GCG ATT GTT TCT TCT CTA CAC CAA G
	Xfmu_3-Pr_15747	FAM-TCT GCA AAC GCT TTA AAA ACT GCT CGC C-BHQ1
<i>X. fastidiosa</i> subsp. <i>pauca</i>	Xfp_1-Fw_28775	GCA TCC TCA CCA CCG AAG G
	Xfp_1-Rw_28775	TCC ACA TCC AGC AAG GTG AC
	Xfp_1-Pr_28775	FAM-CCT TGG ACG CGG ATA CCC GCA-BHQ1
<i>X. fastidiosa</i> subsp. <i>sandyi</i>	Xfs_4_Fwb_112076	CCC CGC TGT GGC AGA A
	Xfs_4_Rv_112076	GGT CCG AGC CAT ACG GC
	Xfs_4_Pr_112076	FAM-CAG CGC CTT CAA TC-MGB-NFQ

Source: Hodgetts, J., Glover, R., Cole, J., Hall, J. & Boonham, N. 2021. Genomics informed design of a suite of real-time PCR assays for the specific detection of each *Xylella fastidiosa* subspecies. *Journal of Applied Microbiology*, 131 (2): 855–872. <https://doi.org/10.1111/jam.14903>

Table 11. Master mix composition and cycling parameters for simplex real-time PCR using the primers and probes of Hodgetts *et al.* (2021)

Reagents	Final concentration
PCR-grade water	–†
TaqMan® Fast Universal PCR Master Mix no AmpErase® UNG (Applied Biosystems)‡	1×
Primer F (forward)	300 nM
Primer R (reverse)	300 nM
Probe	100 nM
Bovine serum albumin (non-acetylated)	6 µg
DNA volume	2 µL
Cycling parameters	
Initial denaturation	95°C for 10 min
Number of cycles	40
- Denaturation	95°C for 10 s
- Annealing and elongation	62°C for 40 s

Notes: † For a final reaction volume of 18 µL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Hodgetts, J., Glover, R., Cole, J., Hall, J. & Boonham, N. 2021. Genomics informed design of a suite of real-time PCR assays for the specific detection of each *Xylella fastidiosa* subspecies. *Journal of Applied Microbiology*, 131 (2): 855–872. <https://doi.org/10.1111/jam.14903>

3.5.9 LAMP using the primers of Harper, Ward and Clover (2010)³

3.5.9.1 LAMP of Harper, Ward and Clover (2010)³

This LAMP method was developed by Harper, Ward and Clover (2010) and can be used on crude plant tissue and insect extracts or with the DNA extraction methods described in section 3.5.1 and section 3.5.2.³ Hydroxynaphthol blue can be used as a means of detecting the end-point (Harper, Ward and Clover, 2010). Hydroxynaphthol blue or other dyes that can be added before amplification are recommended as they allow the LAMP to be performed as a closed-tube system.³ This avoids the risk of opening tubes after amplification, which could lead to aerosol contamination because of the high titre of the LAMP amplicon.³ The LAMP can also be performed in a real-time PCR thermocycler.³

The LAMP,³ which targets the *rimM* gene, can detect 250 copies of the gene. In validation, only *X. fastidiosa* was detected among 95 strains of *X. fastidiosa* from 20 different hosts and 26 non-target bacterial strains.³ All strains of *X. fastidiosa* were detected.

The primers used are:

XF-F3 (external primer): 5'-CCG TTG GAA AAC AGA TGG GA-3'

XF-B3 (external primer): 5'-GAG ACT GGC AAG CGT TTG A-3'

XF-FIP (internal primer): 5'-ACC CCG ACG AGT ATT ACT GGG TTT TTC GCT ACC GAG AAC CAC AC-3'

XF-BIP (internal primer): 5'-GCG CTG CGT GGC ACA TAG ATT TTT GCA ACC TTT CCT GGC ATC AA-3'

XF-LF (loop primer): 5'-TGC AAG TAC ACA CCC TTG AAG-3'

XF-LB (loop primer): 5'-TTC CGT ACC ACA GAT CGC T-3'

The master mix for the Harper, Ward and Clover (2010) LAMP is described in Table 12.³

Table 12. Master mix composition and incubation parameters for LAMP, according to Harper, Ward and Clover (2010)³

Reagents	Final concentration
PCR-grade water	–†
ThermoPol buffer (New England Biolabs)‡	1×
MgSO ₄ (to reach a final concentration)	8 mM
Betaine	0.8 M
Each dNTP	1.4 mM
External primer XF-F3	0.2 µM
External primer XF-B3	0.2 µM
Internal primer XF-FIP	1.6 µM
Internal primer XF-BIP	1.6 µM
Loop primer XF-LF	0.8 µM
Loop primer XF-LB	0.8 µM
Hydroxynaphthol blue (Sigma Aldrich)‡	150 µM
<i>Bst</i> DNA polymerase	8 U
DNA volume	2 µL DNA extract
Incubation parameters	
Incubation	65 °C for 60 min
Enzyme inactivation	80 °C for 2 min

Notes: † For a final reaction volume of 25 µL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Harper, S.J., Ward, L.I. & Clover, G.R.G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology*, 100: 1282–1288. [Incorporates the correction of an erratum in 2013.] <https://doi.org/10.1094/PHYTO-06-10-0168>

A colour change from purple to a light blue is considered a positive result. Negative samples in which no amplification occurs remain violet (Figure 1).

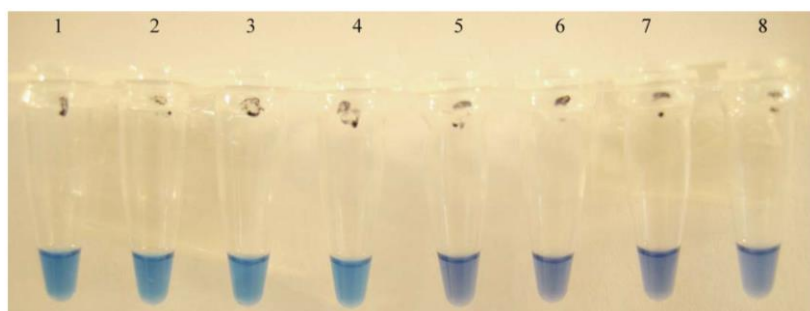


Figure 1. Successful *rimM* loop-mediated isothermal amplification, visualized using hydroxynaphthal blue dye. Samples that are positive for *Xylella fastidiosa* change to a blue colour (tubes 1 to 4); negative samples in which no amplification occurs remain violet (tubes 5 to 8).

Source: Harper, S.J., Ward, L.I. & Clover, G.R.G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. [Incorporates the correction of an erratum in 2013.] *Phytopathology*, 100: 1282–1288. <https://doi.org/10.1094/PHYTO-06-10-0168>

3.5.9.2 LAMP of Harper, Ward and Clover (2010) modified by Yaseen et al. (2015)³

This method is based on the above LAMP primers developed by Harper, Ward and Clover (2010), and was modified by Yaseen *et al.* (2015).³ The modifications consist of a simplified extraction method and reduced incubation times. Ready-to-use kits for the method are commercially available and they are

performed in real time on a specific device or by using a standard real-time thermocycler (e.g. Enbiotech, Qualiplante, OptiGene).² The kits should be used as per the manufacturer's instructions. Diagnostic sensitivity and specificity using the Enbiotech and Qualiplante kits have been determined as being between 83% and 92%, respectively.² The analytical sensitivity (detection limit) of these kits is between 10^2 and 10^3 c.f.u./mL for *Citrus* spp., *V. vinifera* and *O. europaea*. Validation data are available in the EPPO database on diagnostic expertise (EPPO, n.d.(b)).

3.5.10 Controls for molecular testing

For the test result to be considered reliable, appropriate controls – which will depend on the type of tests used and the level of certainty required – should be considered for each series of nucleic acid isolations and amplifications of the target pest or target nucleic acid.

For PCR, a positive nucleic acid (*X. fastidiosa*) control, an internal (host gene) control and a negative amplification control (no template control) are the minimum controls that should be used.

For LAMP, a positive nucleic acid (*X. fastidiosa*) control and a negative amplification control (no template control) are the minimum controls that should be used.³

Additional controls may be used for both LAMP and PCR as described below.³

Positive nucleic acid control. This control is used to monitor the efficiency of PCR amplification. Pre-prepared (stored) nucleic acid, whole genomic DNA or a synthetic control (e.g. cloned PCR product) may be used. For this protocol, genomic DNA (50 ng/ μ L) extracted from either a culture of *X. fastidiosa* or naturally infected tissue is recommended as a positive nucleic acid control.

Internal control. For conventional and real-time PCR, a plant housekeeping gene such as *COX* (Weller *et al.*, 2000; Li, Hartung and Levy, 2006), the 18S rRNA gene (Ioos *et al.*, 2009) or *GADPH* (Mafra *et al.*, 2012) should be used as an internal control to eliminate the possibility of PCR false negatives resulting either from nucleic acid extraction failure or degradation or from the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives resulting from contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture, or sterile PBS, is added at the amplification stage.

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

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

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

3.5.11 Interpretation of results from conventional and real-time PCR

3.5.11.1 Conventional PCR

A pathogen-specific PCR will be considered valid only if both these criteria are met:

- the positive control produces the correct size amplicon for the bacterium; and
- no amplicon of the correct size for the bacterium is produced in the negative extraction control and the negative amplification control.

If internal control primers targeted at the 18S rRNA gene are also used, then the negative (healthy plant tissue) control (if used), the positive control, and each of the test samples must produce an approximately 1.6 kilobase (kb) band (the amplicon size will depend on which 18S rRNA primers are used (Ioos *et al.*, 2009)). Note that synthetic and plasmid positive controls will not produce a 1.6 kb band. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.

A sample will be considered positive if it produces an amplicon of the correct size.

3.5.11.2 Real-time PCR

A pathogen-specific, real-time PCR will be considered valid only if both of these criteria are met:

- the positive control produces an amplification curve with the pathogen-specific primers and probe; and
- no amplification curve is seen with the negative extraction control and the negative amplification control.

If the *COX* or 18S rRNA-gene internal control primers are also used, then the negative control (if used), the positive control, and each of the test samples must produce an amplification curve. Failure of the internal control amplification, however, does not affect the validation of the sample results in the case of target amplification. In the case of negative results with the target, failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the nucleic acid has degraded.

A sample will be considered positive if it produces a typical amplification curve. A cycle cut-off value may be established in each laboratory when implementing the method for the first time. Guidance on how to determine the cycle cut-off value can be found in Chandelier, Planchon and Oger (2010).

3.5.11.3 LAMP³

A LAMP will be considered valid only if both of these criteria are met:³

- the positive nucleic acid control produces a specific reaction (the type of reaction varies with the technology used in the LAMP method (e.g. fluorescence, coloration, amplification curve); the specific reaction is described in the instructions of the kit providers or in the specific section of the protocol describing the LAMP method);³ and
- the negative amplification control does not produce a specific reaction.

A test will be considered positive if it produces a specific reaction as defined for the control reactions (see above). A test will be considered negative if it produces no specific reaction. Tests should be repeated if any contradictory or unclear results are obtained.

4. Identification

The minimum requirements for identification are positive results from two tests based on different biological principles or from two molecular tests that amplify different genetic loci. However, if the

outcome is critical (e.g. post-entry quarantine samples, new host record, new country record), it is recommended that the bacterium is isolated and the requirements for Koch's postulates fulfilled.

Further tests may be done in instances where the national plant protection organization (NPPO) requires additional confidence in the identification of the *X. fastidiosa* subspecies or sequence type. Sequencing of the complete genome (Simpson *et al.*, 2000; Van Sluys *et al.*, 2003), or MLST (Sally *et al.*, 2005; Yuan *et al.*, 2010), is recommended for subspecies or sequence-type identification or when atypical or undescribed strains are suspected (section 4.5).

4.1 Isolation

Isolation is a preliminary step that is taken before performing identification methods. It is not an identification method in itself, nor is it necessary to perform it in all cases.

X. fastidiosa strains are difficult to isolate, even from symptomatic plants, and difficult to grow in axenic culture. They do not grow on most common bacterial media and require specialized media such as PD2 (Davis, Purcell and Thomson, 1980), BCYE (Wells *et al.*, 1981) or PWG (modified from Hill and Purcell, 1995; EPPO, 2023b). It is recommended that at least two different media be used for isolation (EPPO, 2023b).

Midrib, petiole, twig or stem tissue from symptomatic samples are considered the best sources for reliable isolation of *X. fastidiosa*. However, other sources of infected plant tissue from which the bacterium can be isolated include root sections (Hopkins, 2001). Also, it is technically possible to isolate *X. fastidiosa* from insect vectors (Hill and Purcell, 1995), but very few data are available on the performance of this method.

It is very important to surface sterilize the sample in order to avoid contaminants, because *X. fastidiosa* grows very slowly (up to 30 days) and can be readily overgrown by other microorganisms. Midrib, petiole, twig or stem samples are surface sterilized by immersion in 70% ethanol for 1 min. A piece of tissue, 1 cm in length, is then cut and transferred quickly into 96% ethanol, flamed rapidly, and placed in sterile demineralized water. This piece of tissue is then cut into very small pieces and subjected to gentle agitation for 15 min before plating. Alternatively, samples can be placed in 1% bleach for 2 min, followed by two rinses in sterile distilled water. Surface-sterilized plant-tissue segments are cut in the middle, squeezed with flame-sterilized needle-nose pliers, and the sap that exudes can be blotted directly onto media (Hopkins, 2001); or tissue is cut in small pieces in PBS at ratios of 1:10 and 1:100, or ground with a mortar and pestle or a homogenizer (e.g. Homex), and then plated onto two different types of specific media (e.g. PD2, BCYE, PWG).

The application of ultrasonication during the process has been shown to improve isolation from asymptomatic *Coffea arabica* plants (Bergsma-Vlami *et al.*, 2017). After tissue is ground in PBS, the crushed plant tissue is ultrasonicated for 30–60 s at 40 kHz.

Insect vectors are surface sterilized as above and the heads are severed from the body and homogenized in 2 mL PBS. Drops of the insect tissue are plated onto specific media as above (Hill and Purcell, 1995).

The plates should be incubated at 28 °C for 8–45 days, in plastic bags or sealed with Parafilm® or equivalent to prevent desiccation.² Plates are observed regularly for colony development using, if necessary, a binocular microscope. Colonies visible to the unaided eye within the first two days should be regarded as contaminants and can be carefully excised from the plate under aseptic conditions.

4.1.1 Culture media

The culture media described in this protocol are as described in the original publications. There are other modifications of these culture media available that have been observed to produce reliable results (EPPO, 2023b). All media are autoclaved at 121 °C for 15 min.

PD2 medium (Table 13). All components except bovine serum albumin and hemin chloride stock solution are added to 980 mL of distilled water before autoclaving. The pH is adjusted to 7.0 after

dissolving the agar. After autoclaving, the bovine serum albumin (dissolved in distilled water) and hemin chloride stock solution are filter sterilized (0.2 µm membrane) and added to the cooled (45–50 °C) sterile basal medium.

Table 13. PD2 medium

Reagents	Per litre
Phytone peptone (e.g. BD BBL) [†]	2.0 g
Bacto tryptone (e.g. Oxoid) [†]	4.0 g
Trisodium citrate	1.0 g
Disodium succinate	1.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 mL
Bovine serum albumin (20% w/v) (e.g. Sigma) [†]	10 mL
MgSO ₄ ·7H ₂ O	1.0 g
K ₂ HPO ₄	1.5 g
KH ₂ PO ₄	1.0 g
Bacto agar (e.g. BD Difco) [†]	15 g
Distilled water to a final volume of 1 litre	

Note: [†] See page footnote 2.

Source: Davis, M.J., Purcell, A.H. & Thomson, S.V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology*, 70: 425–429. <https://doi.org/10.1094/Phyto-70-425>

BCYE medium (Table 14). To overcome the difficulties of dissolving and resuspending the individual components, it is recommended that ingredients are dissolved in the following order. The ACES (2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid) buffer is first rehydrated in 500 mL distilled water at 50 °C before addition of the yeast extract, activated charcoal and agar. Before adding the agar, the pH is adjusted to 6.9 by the addition of approximately 40 mL 1 M KOH. The medium is autoclaved and then cooled to 50 °C. Both the cysteine hydrochloride (0.4 g) and ferric pyrophosphate (0.25 g) are resuspended in 10 mL distilled water, filter sterilized and added to the cooled sterile medium. The ferric pyrophosphate needs to be heated, under agitation, at 75 °C for approximately 15–20 min (EPPO, 2023b).

Table 14. BCYE medium

Reagents	Per litre
ACES buffer (e.g. Sigma) [†]	10.0 g
Yeast extract	10.0 g
Activated charcoal (e.g. Norit) [†]	2.0 g
L-cysteine hydrochloride-1-hydrate (e.g. Sigma) [†]	0.4 g
Ferric pyrophosphate (e.g. Sigma) [†]	0.25 g
Bacto agar (e.g. BD Difco) [†]	17 g
Distilled water to a final volume of 1 litre	

Notes: [†] See page footnote 2.

ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid.

Source: Wells, J.M., Raju, B.C., Nyland, G. & Lowe, S.K. 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. *Applied and Environmental Microbiology*, 42(2): 357–363. <https://doi.org/10.1128/aem.42.2.357-363.1981>

Modified PWG medium (Table 15). All constituents except L-glutamine and bovine serum albumin are added before autoclaving. Bovine serum albumin (3 g) is dissolved in 15 mL distilled water, and 4 g L-glutamine is dissolved in 50 mL distilled water over a low heat (approximately 50 °C). These two solutions are filter sterilized (0.2 µm membrane) and added to the cooled sterile basal medium.

Hemin chloride stock is 0.1 % bovine hemin chloride dissolved in 0.05 N NaOH.

Table 15. Modified PWG medium

Reagents	Per litre
Gelrite gellan gum (e.g. Sigma) [†]	9.0 g
Phytone peptone (e.g. BD BBL) [†]	4.0 g
Bacto tryptone (e.g. Oxoid) [†]	1.0 g
Phenol red stock solution (0.2%)	10 mL
L-glutamine (e.g. Sigma) [†]	4 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 mL
Bovine serum albumin (e.g. Sigma) [†]	3.0 g
MgSO ₄ ·7H ₂ O	1.0 g
K ₂ HPO ₄	1.5 g
KH ₂ PO ₄	1.0 g
Distilled water to a final volume of a 1 litre	

Note: [†] See page footnote 2.

Sources: Based on Hill and Purcell (1995) and information provided in EPPO (2023).

EPPO (European and Mediterranean Plant Protection Organization). 2023. List of validation data. In: *EPPO database on diagnostic expertise*. Paris. [Cited 12 February 2018]. <http://dc.eppo.int/validationlist.php>

Hill, B.L. & Purcell, A.H. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology*, 85: 209–212. <https://doi.org/10.1094/Phyto-85-209>

4.1.2 Colony morphology

X. fastidiosa colony morphology can be variable (Davis, French and Schaad, 1981; Chen *et al.*, 2005), but on most selective media colonies are convex, either smooth or rough, and with entire or finely undulate margins (Bradbury, 1991). The comparison of colony morphology with a reference culture of *X. fastidiosa* (Table 16) may help a correct identification to be reached. Colonies reach a diameter of approximately 1 mm after two weeks, depending on the subspecies.

Table 16. Reference *Xylella fastidiosa* strains

Strain	Source
CFBP 7969 (ST2), 8073 (ST75), 7970 ^T (ST2), 8173 ^T (ST41)	International Center for Microbial Resources – French Collection for Plant-Associated Bacteria, Beaucauzé, France https://cirm-cfbp.fr/
LMG 17159 (ST2)	Belgian Coordinated Collection of Microorganisms, Ghent, Belgium https://bccm.belspo.be/
ICMP 11140, 15197	International Collection of Microorganisms from Plants, Auckland, New Zealand https://www.landcareresearch.co.nz/tools-and-resources/collections/icmp-culture-collection/
NCPPB 4432	National Collection of Plant Pathogenic Bacteria, York, United Kingdom of Great Britain and Northern Ireland https://www.fera.co.uk/ncppb
DSM 10026	Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany https://www.dsmz.de/

Notes: ^T, type strain; ST – sequence type.

4.1.3 Interpretation of isolation results

The isolation is negative if no bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* (see Figure 24 to Figure 28 in EPPO, 2023b) are observed after 14–45 days on any medium and typical *X. fastidiosa* colonies are found in the positive controls, if included.

The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed after 14–45 days on at least one medium. In some cases, the incubation time can be up to 45 days because of the fastidious growth requirements of *X. fastidiosa*. The presumptive identification of *X. fastidiosa* colonies should be confirmed by serological- or molecular-based methods.

4.2 Description and biochemical characteristics

X. fastidiosa is a fastidious, Gram-negative, straight, rod-shaped bacterium measuring 0.25–0.35 µm by 0.9–3.5 µm. It is strictly aerobic, non-flagellate, and does not form spores (Davis, Purcell and Thompson, 1978; Wells *et al.*, 1987; Bradbury, 1991). Some of the key biochemical and physiological characteristics for *X. fastidiosa* are listed in Table 17.

The reference *X. fastidiosa* strains available from different collections are listed in Table 16. These strains are suggested for use as positive controls in biochemical, serological and molecular tests.

Table 17. Key biochemical and physiological characteristics of *Xylella fastidiosa*

Parameter assessed	Result
Catalase	+
Oxidase reaction	–
Gelatin liquefaction	+
Indol production	–
H ₂ S production	–
DL-lactate	+
Glucose fermentation	–
Temperature optimum	26 to 28 °C
pH optimum (<i>X. fastidiosa</i> is very sensitive to variations in pH)	6.5 to 6.9

Sources: Bradbury, J.F. 1991. *Xylella fastidiosa*. IMI Descriptions of Fungi and Bacteria, Sheet 1049.

Davis, M.J., Purcell, A.H. & Thompson, S.V. 1978. Pierce's disease of grapevines: isolation of the causal bacterium. *Science*, 199: 75–77. <https://doi.org/10.1126/science.199.4324.75>

Wells, J.M., Raju, B.C., Hung, H.-Y., Weisburg, W.G., Mandelco-Paul, L. & Brenner, D.J. 1987. *Xylella fastidiosa* gen. nov., sp. nov: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *International Journal of Systematic Bacteriology*, 37: 136–143. <https://doi.org/10.1099/00207713-37-2-136>

4.3 Pathogenicity assessment

Pathogenicity assessment is recommended when additional information on strain aggressiveness or potential host range is required or to fulfil the requirements of Koch's postulates.

Actively growing, susceptible plants need to be maintained in a greenhouse or growth chamber at 25–28 °C. Inoculation techniques should deliver the inoculum directly into the xylem vessels for development of symptoms. The most widely used method for plant inoculation is by needle puncture into the stem at the insertion of the petiole (Hill and Purcell, 1995; Almeida *et al.*, 2001). A general inoculation procedure is described below.

Pathogenicity assessment should use plants of the same host from which the suspect *X. fastidiosa* was isolated. When possible, the most susceptible cultivars should be used. Some recommended examples include: for *V. vinifera*, the cultivars 'Cabernet sauvignon', 'Chardonnay', 'Chenin Blanc' and 'Pinot Noir'; for *C. sinensis*, 'Hamlin', 'Natal', 'Pera' and 'Valencia'; and for *O. europaea*, 'Cellina di Nardò', 'Coratina' and 'Frantoio' (EPPO, 2023b). *Catharanthus roseus* (Madagascar periwinkle) is a

herbaceous plant that is easily grown in a greenhouse and is susceptible to *X. fastidiosa* (Monteiro *et al.*, 2001).

To facilitate the rapid uptake of the inoculum by the transpiration system, inoculated plants should be young and should be grown in pots with dry soil. Growing conditions may vary according to the plant species (e.g. for grape, a lower night temperature should be applied). Cultures of bacteria grown for 8–10 days on suitable media should be used for pathogenicity assessments. Bacteria are removed from solid media and suspended in PBS to produce a turbid suspension of approximately 10^8 c.f.u./mL ($Ab_{S_{600nm}} = 0.2$). A drop (20–50 μ L) of inoculum is placed in a leaf axil and punctured through several times with a fine needle until the liquid is completely absorbed. Control plants are treated in the same way except that the suspending medium (PBS) is used instead of bacterial suspension. Plants must be maintained in the greenhouse or growing chamber at 25–28 °C (EPPO, 2023b).

An alternative method of inoculation is to raise a flap of stem tissue by cutting upwards with a razor blade to expose the wood. A few drops of bacterial suspension are placed under the flap and the flap is then replaced and wrapped with grafting tape.

Symptom development usually appears 60–80 days after inoculation; however, this is known to be variable and could be up to 24 months depending on the host and strain combination (Hopkins, 2001).

For both methods of inoculation, if possible the bacterium should be re-isolated to fulfil the requirements for Koch's postulates.

In addition, a bioassay can be performed on *Nicotiana tabacum* (tobacco) plants by inoculating the petioles with suspensions of *X. fastidiosa* (Francis, Civerolo and Bruening, 2008). *N. tabacum* cultivar 'SR1 (Petite Havana)' has been shown to be a better bioassay host for *X. fastidiosa* than *N. tabacum* cultivars 'Havana', 'RP1' and 'TNN' (Francis, Civerolo and Bruening, 2008). Leaf scorch symptoms develop 10–14 days after inoculation.

4.4 Serological identification

Enzyme-linked immunosorbent assay (described in section 3.4) can be used for the identification of suspect *X. fastidiosa* cultures or symptomatic plant material.

4.5 Molecular identification

Polymerase chain reaction (described in section 3.5) can be used for the identification of suspect *X. fastidiosa* isolated from diseased plant material. If only PCR is being performed, to allow rapid diagnosis, it is recommended that identification is confirmed by using two different sets of primers targeting two different genes. For interpretation of conventional and real-time PCR results, see section 3.5.11. For conventional PCR tests, the amplicons can be sequenced to further support the identification. Sequence data can be analysed using the Standard Basic Local Alignment Search Tool for nucleotides (BLASTN), available at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>).

A PCR can be readily conducted on boiled or heated preparations (e.g. suspensions of 10^8 c.f.u./mL heated at 95 °C for 15 min or 100 °C for 5 min) of bacterial colonies, or on DNA extracts purified using the methods in section 3.5.1.

4.5.1 Subspecies identification

Methods to identify subspecies of *X. fastidiosa* include PCR (Pooler and Hartung, 1995; Hernandez-Martinez *et al.*, 2006), Sanger sequencing, real-time PCR (Dupas *et al.*, 2019a; Hodgetts *et al.*, 2021), and MLST (see section 4.5.2). The PCR described by Hernandez-Martinez *et al.* (2006) can allow the identification of cultures of subspecies *fastidiosa*, *multiplex* and *sandyi*. Pooler and Hartung (1995) developed a conventional PCR that identifies subspecies *pauca*.

It is recommended that MLST be used to analyse *X. fastidiosa* strains detected in new areas or on new host associations.

The methods described in this section (section 4.5.1) have mainly been developed on pure cultures but can be used on DNA extracts from plants, except for the PCR by Hernandez-Martinez *et al.* (2006). However, it is recognized that the quantity and quality of target DNA, or the occurrence of possible mixed infections, may mean that not all amplicons are obtained or may prevent clear assignment of subspecies.

4.5.2 Multilocus sequence typing

An MLST approach has been described for the identification of *X. fastidiosa* subspecies and is recommended for the characterization of new strains (Scally *et al.*, 2005; Yuan *et al.*, 2010; Jacques *et al.*, 2016; Bergsma-Vlami *et al.*, 2017). This approach can be used on DNA extracted from either bacterial cultures or infected plants that have tested positive for *X. fastidiosa* (Loconsole *et al.*, 2016). For amplification of DNA direct from plant tissue, it has been observed that the quality of the target DNA may not always be suitable for obtaining all amplicons (EPPO, 2023b). Primers and parameters for the sequencing and analysis of seven housekeeping genes (*cysG*, *gltT*, *holC*, *leuA*, *malF*, *nuoL* and *petC*) are described by Yuan *et al.* (2010) and further details regarding analysis can be found on the *X. fastidiosa* MLST website (<http://pubmlst.org/xfastidiosa>) and in Appendix 16 of EPPO (2023b).

Expected amplicon sizes for the different housekeeping genes are: 642 bp for *cysG*, 698 bp for *gltT*, 422 bp for *holC*, 749 bp for *leuA*, 773 bp for *malF*, 599 bp for *nuoL* and 576 bp for *petC*.

The targeted regions are amplified by PCR, and if the amplicons are of good quality and the expected size they should be sequenced directly using forward and reverse primers (Table 18). Sequences are concatenated by following the alphabetical order of the genes and analysis should be performed as per advice on the MLST website (<http://pubmlst.org/xfastidiosa>). The results of the sequencing should be compared with the reference sequences for the housekeeping genes that can be found on the MLST website.

Table 18. Primer sequences for multilocus sequence typing

Gene	Forward primer	Reverse primer
<i>cysG</i>	5'-GCC GAA GCA GTG CTG GAA G-3'	5'-GCC ATT TTC GAT CAG TGC AAA AG-3'
<i>gltT</i>	5'-TCA TGA TCC AAA TCA CTC GCT T-3'	5'-ACT GGA CGC TGC CTC GTA AAC C-3'
<i>holC</i>	5'-ATG GCA CGC GCC GAC TTC T-3'	5'-ATG TCG TGT TTG TTC ATG TGC AGG-3'
<i>leuA</i>	5'-GGT GCA CGC CAA ATC GAA TG-3'	5'-GTA TCG TTG TGG CGT ACA CTG-3'
<i>malF</i>	5'-TTG CTG GTC CTG CGG TGT TG-3'	5'-GAC AGC AGA AGC ACG TCC CAG AT-3'
<i>nuoL</i>	5'-TAG CGA CTT ACG GTT ACT GGG C-3'	5'-ACC ACC GAT CCA CAA CGC AT-3'
<i>petC</i>	5'-GCT GCC ATT CGT TGA AGT ACC T-3'	5'-GCA CGT CCT CCC AAT AAG CCT-3'

Source: Yuan, X., Morano, L., Bromley, R., Spring-Pearson, S., Stouthamer, R. & Nunnery, L. 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. *Phytopathology*, 100: 601–611. <https://doi.org/10.1094/PHYTO-100-6-0601>

A protocol for nested MLST, which has the same targets as in Yuan *et al.* (2010) but is more sensitive than MLST, has been described (Cesbron *et al.*, 2020) and is appropriate when the MLST analysis (Yuan *et al.*, 2010) is not successful. For Sanger sequencing (Scally *et al.*, 2005; Yuan *et al.*, 2010), the PCR product of at least two housekeeping genes, either *rpoD* (Minsavage *et al.*, 1994) and *malF* (MLST analysis) or *cysG* and *malF* (MLST analysis), should be sequenced in both directions. The combination of these two genes is statistically equivalent to MLST for the determination of the subspecies. Sequencing a combination of at least two genes may also allow possible recombinant strains to be detected. To determine the sequence type, however, the PCR products of all the seven housekeeping genes listed above for MLST are needed. Sequence data for *rpoD* and *malF* can be analysed using BLASTN, available at the National Center for Biotechnology Information

(<https://www.ncbi.nlm.nih.gov/>). *CysG* and *malF* sequences can be compared with data available in the pubMLST database for MLST genes (<https://pubmlst.org/organisms/xylella-fastidiosa>). The nested MLST protocol described by Cesbron *et al.* (2020) can be used with *cysG* and *malF*. In the case of inconsistent results for the two sequenced genes, or atypical or new patterns, complete MLST analysis of the seven genes should be performed and sequences compared with data available in the pubMLST database as indicated.

5. Records

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

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where the bacterium is found in an area for the first time, the following records and evidence should be kept for at least one year in a manner that ensures traceability:

- Photographs of symptoms and signs, printouts of ELISA plate results, photographs of DNA agarose gels and real-time records should be retained.
- Cultures can be stored at -80°C or stored in an international culture collection.
- The original sample (labelled appropriately) should be kept frozen if possible at -80°C , or freeze-dried and kept at room temperature.
- If relevant, DNA extracts should be kept at -80°C and PCR amplification products at -20°C .

6. Contact points for further information

Further information on this protocol can be obtained from:

Citrus Center Sylvio Moreira (CCSM), Agronomic Institute (IAC), São Paulo, Brazil (Helvecio Coletta; email: hdcoletta@ccsm.br, helvecio.coletta@sp.gov.br; tel.: (+55) 19 3546 1399).

French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Plant Health Laboratory, Bacteriology, Virology and GMO Unit, Angers, France (Bruno Legendre; email: bruno.legendre@anses.fr; tel.: (+33) 2 4120 7440).

NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Narellan, New South Wales 2568, Australia (Toni Chapman; email: toni.chapman@dpi.nsw.gov.au; tel.: (+61) 2 4640 6219).

University of Angers, Institut Agro, INRAE, IRHS, SFR QUASAV, F-49000 Angers, France (Sophie Cesbron; email: sophie.cesbron@inrae.fr; tel.: (+33) 2 4122 5746).

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

7. Acknowledgements

This protocol was revised by Helvecio Coletta (CCSM, Brazil (see preceding section)), Bruno Legendre (ANSES, France (see preceding section)), Toni Chapman (NSW Department of Primary Industries, Australia (see preceding section)) and Sophie Cesbron (University of Angers, France (see preceding section)). The first version of this protocol, adopted in 2018, was written by Marta Francis (University of California Davis, United States of America), Robert Taylor (Biosecurity New Zealand, New Zealand), Helga Reisenzein (Austrian Agency for Health and Food Safety, Austria), John Hartung (United States Department of Agriculture (USDA), United States of America) and Wenbin Li (USDA Animal Plant Health and Inspection Service, United States of America). Ed Civerolo (formerly USDA) was also involved in the development of the originally adopted protocol.

8. Figures

One figure is included in this protocol (Figure 1). Pictures of symptoms are accessible on the EPPO global database website at <https://gd.eppo.int/taxon/XYLEFA/photos> and at <https://nature.berkeley.edu/xylella>.

9. References

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

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Publication history

This is not an official part of the standard

- 2004-11 Standards Committee (SC) added topic to work programme.
- 2016 Draft substantially rewritten by the authoring team to include information on the latest molecular methods.
- 2016-07 Technical Panel on Diagnostic Protocols (TPDP) revised.
- 2016-10 Expert consultation.
- 2016-12 Diagnostic Protocol (DP) drafting group revision.
- 2017-03 TPDP recommended to SC for approval for consultation (2017_eTPDP_Apr_01).
- 2017-04 SC approved draft DP for consultation (2017_eSC_May_11).
- 2017-07 First consultation.
- 2017-11 Revised by the DP lead based on the consultation comments.
- 2018-02 TPDP approved draft to submit to SC for approval for adoption.
- 2018-03 SC approved draft to be submitted to the 45-day DP notification period (2018_eSC_May_07).
- 2018-07 DP notification period (no objections received).
- 2018-08 SC adopted DP on behalf of CPM.
- ISPM 27. Annex 25.** 2018. *Xylella fastidiosa*. Rome, IPPC, FAO.
- 2021-03 SC added revision of DP 25 (*Xylella fastidiosa*) to work programme (2021-003).
- 2022-11 TPDP revised.
- 2023-01 Expert consultation.
- 2023-06 SC approved for consultation (2023_eSC_Nov_07).
- 2023-07 Consultation.
- 2023-11 TPDP revised.
- 2024-02 TPDP approved responses to consultation comments and recommended the draft DP to the SC for approval for adoption.
- 2024-06 SC approved draft to be submitted to the 45-day notification period (2024_eSC_Nov_03).
- 2024-07 DP notification period (no objections raised).
- 2024-08 SC adopted DP on behalf of CPM.
- ISPM 25. Annex 25.** 2024. *Xylella fastidiosa*. IPPC Secretariat. Rome, FAO.

Publication history last updated: 2024-12