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ISPM 27
Diagnostic protocols for regulated pests

DP 25: *Xylella fastidiosa*

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CONTENTS

1. Pest Information

Xylella fastidiosa Wells *et al.*, 1987 is a xylem-limited bacterium and is the causal agent of many economically important plant diseases of agronomic and horticultural 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, 2016; European Commission, 2018). *X. fastidiosa* is also expanding its geographical range. Until recently, it was mainly distributed throughout the Americas (Almeida and Nunney, 2015), but there have now been reports of outbreaks in Asia and Europe (EPPO, 2018a).

X. fastidiosa is genetically diverse and consists of several subspecies. *X. fastidiosa* subsp. *fastidiosa* causes Pierce's disease and infects a large host range including *V. vinifera*, *P. dulcis*, *Medicago sativa* and *Acer* spp. (Schuenzel *et al.*, 2005). *X. fastidiosa* subsp. *multiplex* is associated with scorch diseases of a range of trees that include *P. dulcis*, *Prunus persica*, *Quercus* spp. and *Platanus occidentalis*. *X. fastidiosa* subsp. *sandyi* causes oleander leaf scorch in *Nerium oleander* (Schuenzel *et al.*, 2005). *X. fastidiosa* subsp. *tashke* has been isolated from the ornamental tree *Chitalpa tashkentensis* (Randall *et al.*, 2009). *X. fastidiosa* subsp. *morus* (Nunney *et al.*, 2014) infects *Morus* spp. Finally, *X. fastidiosa* subsp. *pauca* (Schadd *et al.*, 2004) infects most *Citrus* and *Coffea* species, and *O. europaea.*

X. fastidiosa is a Gram-negative bacterium with fastidious growth requirements. The bacterial cells are non-motile, non-flagellate, rod-shaped cells, 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 *et al.*, 1992; He *et al.*, 2000; Li *et al.*, 2003). The pathogen overwinters in the xylem of the host plant. Insect transmission is considered the main factor for localized spread of *X. fastidiosa*. The vectors belong to the order Hemiptera, sub-order Auchenorrhyncha, families Cicadellidae (sharpshooter leafhopper), Cercopidae (spittlebugs) (Redak *et al*., 2004; Chatterjee *et al*., 2008), Aphrophoridae 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 other healthy plant hosts. While nymphs are able to acquire (and transmit) the bacterium, they lose it at each moult, so only continue to be infected if they reacquire the bacterium by feeding on infected plants after moulting (Almeida *et al.*, 2014). Once adults acquire the bacterium, they have it for life (as they do not moult). Once infected, adults can transmit throughout their whole lifetime, as the bacterium multiplies and persists in the vector foregut (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 planting material (e.g. budwood, seedlings) is assumed to be responsible for the long distance spread of the disease and its entry into new areas.

2. Taxonomic Information

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

Synonyms: None

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

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 are

named in relation to their host plants; for example, almond leaf scorch, oleander leaf scorch, olive leaf scorch, pear leaf scorch.

Recent studies have 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), "morus" (Nunney *et al.*, 2014) and "taskhe" (Randall *et al.*, 2009). The *Xylella* species associated with pear leaf scorch in Taiwan Province of China (Leu and Su, 1993) is a new species, *X. taiwanensis* (Su *et al*., 2016). Recently, a revision of the *X. fastidiosa* subspecies has been proposed (Marceletti and Schortichini, 2016) based on comparative genomic analysis.

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 due to the difficulty in isolating *X. fastidiosa* from plant tissue. Therefore, detection is based on inspection for symptoms and the use of specific serological and 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 & Nunney, 2015). Therefore, it is advisable to include molecular methods 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 or low temperature, or by drought) and they can vary according to the plant species or cultivar and environmental conditions (Janse and Obradovic, 2010; CABI, 2016).

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.). Pictures of symptoms on various hosts can be found at https://gd.eppo.int/taxon/XYLEFA/photos and https://nature.berkeley.edu/xylella. Symptoms may vary depending on the host and *X. fastidiosa* subspecies combination. The host range can be markedly different between subspecies; however, there is some uncertainty with regards to the potential host range for each subspecies. Each subspecies can be found in multiple host plants. For example, *X. fastidiosa* subspecies *fastidiosa* not only infects grapes but it also causes alfalfa dwarf and overlaps with *X. fastidiosa* subsp. *multiplex* in causing almond leaf scorch (Yuan *et al.*, 2010). 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). Muscadinia and native American cultivars display milder symptoms than those of *V. vinifera*. On *V. vinifera*, the initial symptoms are chlorotic spots on areas of the leaf lamina, in particular 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". 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 are rarely seen in one-year-old plants. Symptoms on the twigs can be confused with those of fungal diseases such as rotbrenner and esca (EPPO, 2018b).

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, 2016). 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, 2018b). 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 fruits except for 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 from 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). *Coffea* plants may remain asymptomatic (De Lima *et al.*, 1998; Montero-Astúa *et al.*, 2008).

3.1.4 Olive leaf scorching and quick decline

In three different distant regions around the world (the southern region of Italy, Argentina and Brazil), 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 strains associated with this disease in Italy are recombinants of strains within *X. fastidiosa* subsp. *pauca* (Loconsole *et al.*, 2014). 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 death of the entire tree. *X. fastidiosa* has also been detected in young olive trees with leaf scorching and quick decline (EPPO, 2018b).

3.1.5 Almond leaf scorch disease

The most characteristic symptoms of almond leaf scorching 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.

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 a marginal leaf scorching, and the scorched leaf zone may be bordered by a darker band (Brannen *et al.*, 2016; EPPO, 2018b). 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, this typically occurring 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.*, 2015).

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 steaks of dead tissue scattered throughout. In recently infected plants, the yellowing is mostly in a ring beginning under the bark, with a normal white-coloured cylinder of tissue inside the yellowed outer layer of wood (EPPO, 2018b). The inner bark is not discoloured, nor do large brown or yellow patches appear as in bacterial wilt (caused by *Clavibacter michiganensis* subsp. *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 typical leaf scorching symptoms. On *N. oleander*, necrosis develops on the

leaf margin and infection may lead to death of entire plants (EPPO, 2018b). *Polygala myrtifolia* has been found to be one 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, 2018b). 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. Samples should be taken from close to the symptoms and preferably consist of stems that have mature symptomatic leaves with petioles and woody twigs. Individual leaves with petioles can also be sampled. The best plant material to test for the presence of *X. fastidiosa* is the leaf petiole and the midrib of mature leaves from either asymptomatic or symptomatic plant material. Guidance on the number of leaves (including their petioles) and approximate weights to be used in the laboratory sample is provided in Table 1 (EPPO, 2018b).

Table 1. Number of leaves (including their petioles) to be used and approximate weight of the laboratory sample. Data from EPPO (2018b).

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	Petioles or midribs, or both, of leaves of large size (e.g. from *Coffea* spp., *Ficus* spp., *Vitis* spp., *Nerium* spp.)	5	0.5–1 g
	Petioles or midribs, or both, of leaves of small size (e.g. *Polygala myrtifolia* and *Olea* spp.)	25	0.5–1 g
	Plant species without petioles or with small petiole and midrib	25	0.5–1 g
Dormant plant or cuttings	Xylem tissue	n/a[†]	0.5–1 g
Composite sample from several coffee plants from a single lot with leaves	Samples of asymptomatic plants (e.g. collected from imported consignments or nursery monitoring)	100–200	10–50 g

[†] n/a, not applicable.

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 plant species type, seasonal and environmental factors. To maximize the likelihood of detection, sampling should be performed during the period of active growth of the plants (Hopkins, 1981). This is usually from late spring to autumn in temperate zones. For asymptomatic plants, sampling is also possible during the period of active growth. However, sampling after warm periods (e.g. late summer–early autumn) increases the probability of accurate bacterial detection (European Commission, 2015).

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. 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). For tropical plant species grown indoors such as coffee plants, sampling may be performed all year round when plants are exhibiting periods of active growth (EPPO, 2018b).

3.2.2 Plant sample collection

X. fastidiosa is confined to the xylem tissue of its hosts. The petiole and the midrib recovered from leaf samples are therefore the best sources for diagnosis, as they contain a greater number of xylem vessels (Hopkins, 1981). Other sources of tissue can include small twigs and roots of *P. persica* (Aldrich *et al.*, 1992), stem and roots of *Vaccinium* (Holland *et al.*, 2014) and *Citrus* fruit petioles (Rossetti *et al.*, 1990). 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 can be sent to the laboratory.

3.2.3 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 10 to 25 leaves depending on leaf size. The approximate weight needed for laboratory samples is between 0.5 g and 1 g leaf petioles or midribs from each individual plant (EPPO, 2018b). Symptomatic plant material should preferably be collected from a single plant; however, a pooled sample may also be collected. It is recommended that, when testing pooled samples, the limit of detection for each detection test should be confirmed.

3.2.4 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). For testing individual asymptomatic plants, the number of branches to be collected is at least four to ten, depending on the host and plant size. There is limited experience of testing samples composed of leaves (including their petioles) collected from several asymptomatic plants. Further information on number of samples to be collected per lot can be found in ISPM 31 (*Methodologies for sampling of consignments*).

3.2.5 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. Lower temperatures can reduce sample deterioration. However, *X. fastidiosa* does not survive well in cold temperatures and for culture isolation work it is better to process samples immediately rather than refrigerate. Samples should be processed as soon as possible after arrival at the laboratory. If necessary, however, samples for isolation (see section 4.1) may be kept refrigerated (e.g. 4 °C) for up to three days. For other tests, samples may be refrigerated for up to one week. For longer term storage, samples may be stored at −20 °C or −80 °C for molecular or serological detection.

3.2.6 Sampling of vectors

Vectors should preferably be collected with sweeping nets (adults) or aspirators. Sticky traps are usually not effective for xylem feeders (Purcell *et al.*, 2014), but insects may be trapped accidentally and specimens collected from sticky traps can be used for testing. Vectors can be removed from the 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 should preferably be carried out from late spring until early autumn to maximize the likelihood of detecting the bacterium. If insects cannot be processed immediately, they should be stored in 95–99% ethanol, or at −20 °C or −80 °C with or without ethanol. Sticky traps with captured insects can also be stored at −20 °C.

Insects collected from the field or from the wild can be analysed by polymerase chain reaction (PCR) to detect *X. fastidiosa*. Enzyme-linked immunosorbent assay (ELISA: see section 3.3) is not sensitive enough, as the bacterium only colonizes the insect foregut where, in spite of its multiplication, it is generally present at low levels (Purcell *et al.*, 2014). On the aphrophorid *Philaenus spumarius*, the population size of *X. fastidiosa* may be limited to fewer than 10^3 cells (Cornara *et al*., 2016).

3.3 Serological detection

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.

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 *et al*., 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, due to the low concentration of bacteria likely to be present in young asymptomatic tissue.

3.3.1 Preparation of material

ELISA 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.

3.3.2 Double-antibody sandwich ELISA (DAS-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; $Na_2HPO_4 \cdot 12\ H_2O$, 2.9 g; KH_2PO_4, 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[1], Homex[1]) or is pulverized in liquid nitrogen (Loconsole *et al*., 2014; EPPO, 2018a). Further information on using DAS-ELISA to detect plant pathogenic bacteria is available in EPPO (2010).

Kits for the serological detection of *X. fastidiosa* are commercially available from Agritest[1], Agdia[1] and Loewe Biochemica[1]. 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 10^4 colony-forming units (cfu)/ml; however, test sensitivity can vary depending on the plant species matrix being tested (Loconsole *et al.*, 2014; EPPO, 2018b).

The specificity and sensitivity of DAS-ELISA to detect *X. fastidiosa* on *O. europaea*, using a kit from Loewe[1], were evaluated by Loconsole *et al.* (2014). Additionally, a test performance study performed at the Institute for Sustainable Plant Protection (Bari, Italy) was conducted on serological kits from Agritest[1], Agdia[1] and Loewe[1]. These studies showed that these kits achieved 100% diagnostic sensitivity

[1] 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.

and specificity when testing naturally infected samples. The data on the test performance study are available in the EPPO database on diagnostic expertise (EPPO, 2018c).

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

3.4 Molecular detection

Various molecular methods have been developed for the detection and identification of *X. fastidiosa* directly on pure cultures, plant tissue and insect vectors (Firraro and Bazzi., 1994; Minsavage *et al*., 1994; Pooler and Hartung, 1995; Schaad *et al.*, 2002; Rodrigues *et al.*, 2003; Francis *et al.*, 2006; Harper *et al.*, 2010, erratum 2013; Li *et al.*, 2013; Ouyang *et al.*, 2013). The conventional PCR developed by Minsavage *et al.* (1994) and Rodrigues *et al*. (2003), and two real-time PCRs (Harper *et al.*, 2010, erratum 2013, and Li *et al.*, 2013) are described in this protocol for the detection and identification of *X. fastidiosa*. The PCR methods described hereafter are as described in the original publications; however, some modifications (e.g. variations in PCR conditions or the use of other mixes) can be applied for optimization purposes.

3.4.1 DNA extraction from plant material

A number of methods have been described for the extraction of the DNA of *X. fastidiosa* from bacterial colonies and from plant tissue (Minsavage *et al.*, 1994; Pooler and Hartung, 1995; Francis *et al.*, 2006; Huang *et al.*, 2006; Harper *et al.*, 2010, erratum 2013; Li *et al.*, 2013). Extraction can be achieved using a number of standard commercial kits (e.g. Bextine and Child, 2007; Huang, 2009). The following methods are a selection of those widely used in several laboratories. There are many other similar DNA extraction kits that 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, 2018c). A PCR can be readily conducted on boiled or heated preparations (e.g. suspensions of 10^8 cfu/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 below.

CTAB-based extraction. 0.5–1 g midrib, petiole or twig tissue is placed into an extraction bag with 5 ml CTAB buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 10 mM ethylenediaminetetraacetic acid (EDTA); 2% hexadecyltrimethylammonium bromide (CTAB); 3% polyvinylpyrrolidone (PVP)-40) and homogenized using a homogenizer (e.g. Homex[1], Polytron[1]). 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 5 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 3000 *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 30 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 deoxyribonuclease-free water.

DNeasy Plant Mini Kit (Qiagen)[1]. DNA is extracted from 0.5–1.0 g plant tissue (leaf midrib, petiole or twig tissue) and macerated in lysis buffer using homogenizing equipment (e.g. Homex[1], Polytron[1]). Alternatively, plant tissue can be ground to a fine powder in liquid nitrogen prior to extraction. These extracts are then treated according to the manufacturer's instructions.

QuickPick SML Plant DNA Kit (Bio-Nobile)[1]. 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 material 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[1] (15 samples) or KingFisher Flex[1] (96 samples) purification system (Thermo Scientific)[1]. Validation data are available in the EPPO database on diagnostic expertise (EPPO, 2018c). 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.

KingFisher (Thermo Scientific)[1] **using InviMAG Plant DNA Kit** (Stratec Molecular)[1]. This automated magnetic bead extraction procedure is ideal for high-throughput testing and uses the InviMAG Plant DNA Mini Kit (Stratec Molecular)[1] with the KingFisher 96 system (Thermo Scientific)[1]. 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.

3.4.2 DNA extraction from insect vectors

DNA may be extracted from a single insect head or a pool of five heads (Bextine *et al.*, 2004; Purcell *et al.*, 2014; EPPO, 2018b). 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 big 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)[1] or by vacuum application and release (Bextine *et al.*, 2004, 2005; Huang *et al.*, 2006). 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 several laboratories.

DNeasy Tissue Kit (Qiagen)[1]. 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 *et al.*, 2006).

QuickPick SML Plant DNA Kit (Bio-Nobile)[1] **for insects**. The homogenization of individual insect heads can be performed in 200 µl sterile distilled water using a bead-beater system such as the Retsch MM400[1]. Samples are homogenized for 2 min at 30 Hertz using ten stainless steel beads (diameter 3 mm) per 2 ml microtube. The microtube is placed on a magnet and the supernatant is transferred to a new microtube. 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[1] (15 samples) or KingFisher Flex[1] (96 samples) system (Thermo Scientific)[1].

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 3.4.1), but with adapted volumes.

3.4.3 Conventional polymerase chain reaction (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 many laboratories for the detection of *X. fastidiosa* in different host plants and vectors. Analytical specificity was validated by Harper *et al.* (2010, erratum 2013) 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 red oak and turkey oak 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 cfu/ml on *V. vinifera* and *P. persica*. Further validation data on other hosts are available in the EPPO database on diagnostic expertise (EPPO, 2018c).

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′

The master mix used for this PCR developed by Minsavage *et al.* (1994) is described in Table 2.

Table 2. 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]	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]	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

[†] For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

3.4.4 Conventional polymerase chain reaction (PCR) using the primers of Pooler and Hartung (1995)

This PCR was designed by Pooler and Hartung (1995) by developing PCR primers that target a specific randomly amplified polymorphic DNA 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 3.

Table 3. 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]	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]	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

† For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

3.4.5 Conventional PCR using the primers of Rodrigues *et al.* (2003)

The PCR based on primers for the 16S ribosomal (r)RNA and *gyrB* genes was developed by Rodrigues *et al.* (2003). The 16S rRNA gene-targeted primers (sets A, B, C), the *gyrB* gene-targeted primers (FXYgyr499 and RXYgyr907) and the multiplex PCR (16SrRNA and *gyrB* primers combined) were evaluated using 30 *X. fastidiosa* strains from different plant hosts and 36 closely related or host related non-target bacterial strains. The specific sets of primers for the 16S rRNA or *gyrB* genes can be used as either single or multiplex PCR. The analytical sensitivity for the multiplex PCR is similar to the singleplex reactions, which is approximately 10^2 cfu/ml.

The 16S rRNA gene-targeted primers are as follows.

Set A:

S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′

S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′

Primer set A amplifies a product of 1348 bp.

Set B:

S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′

S-S-X.fas-0838-a-A-21 (reverse): 5′-CGA TAC TGA GTG CCA ATT TGC-3′

Primer set B amplifies a product of 745 bp.

Set C:

S-S-X.fas-0838-a-S-21 (forward): 5′-GCA AAT TGG CAC TCA GTA TCG-3′

S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′

Primer set C amplifies a product of 603 bp.

The master mix and PCR conditions for the Rodrigues *et al*. primers (sets A, B, C) are described in Table 4. Multiplex PCR conditions are maintained as described except with 0.2 and 0.4 µM concentrations of each 16S rRNA and *gyrB* primer, respectively.

Table 4. Master mix composition, cycling parameters and amplicons for conventional PCR using the 16S rRNA gene-targeted primers of Rodrigues *et al.* (2003)

Reagents	**Final concentration**
PCR grade water	–[†]
PCR buffer	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer (forward set A, or B or C)	0.2 µM
Primer (reverse set A, or B or C)	0.2 µM
Taq DNA polymerase (Invitrogen)[1]	2.0 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	30
- Denaturation	94 °C for 1 min
- Annealing	55 °C for 30 s
- Elongation	72 °C for 2 min
Final elongation	72 °C for 7 min
Expected amplicons	
Size	Primer set A: 1348 bp Primer set B: 745 bp Primer set C: 603 bp

[†] For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

The *gyrB* primers used are:

FXYgyr499 (forward): 5′-CAG TTA GGG GTG TCA GCG-3′

RXYgyr907 (reverse): 5′-CTC AAT GTA ATT ACC CAA GGT-3′

The *gyrB* primer set produces an amplicon of 429 bp.

The master mix for the *gyr*B gene-targeting primers is described in Table 5.

Table 5. Master mix composition, cycling parameters and amplicons for conventional PCR using the *gyr*B gene-targeting primers of Rodrigues *et al.* (2003)

Reagents	**Final concentration**
PCR grade water	–[†]
PCR buffer	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer FXYgyr499 (forward)	0.4 µM
Primer RXYgyr907 (reverse)	0.4 µM
Taq DNA polymerase (Invitrogen)[1]	2.5 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	30
- Denaturation	94 °C for 1 min
- Annealing	60 °C for 1 min
- Elongation	72 °C for 2 min
Final elongation	72 °C for 7 min
Expected amplicons	
Size	429 bp

[†] For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

3.4.6 Real-time PCR using the primers and probes of Harper *et al.* (2010, erratum 2013)

This PCR, developed by Harper *et al.* (2010, erratum 2013), is designed to amplify part of the 16S rRNA processing protein *rimM* gene. DNA can be amplified from bacterial cultures, infected leaves, cane tissue or insect vectors.

Harper *et al.* (2010, erratum 2013) evaluated analytical specificity with 95 strains of *X. fastidiosa* from 20 different hosts and 26 non-target bacterial strains. Only *X. fastidiosa* was detected. *Xylella taiwanensis* from Taiwan Province of China was not detected. The PCR was further validated by Li *et al.* (2013). Diagnostic specificity and sensitivity, as determined using citrus and grape hosts, are 100% (EPPO, 2018b). For *O. europaea* hosts when using CTAB extraction methods, diagnostic specificity is 100% and diagnostic sensitivity is 91% (EPPO, 2018b). Further validation data are available in the EPPO database on diagnostic expertise (EPPO, 2018c). The analytical sensitivity (detection limit) is between 10^2 cfu/ml for *Citrus* spp. and *V. vinifera* and 10^5 cfu/ml for *O. europaea*.

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 *et al.* (2010, erratum 2013) 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 *et al.* (2010, erratum 2013)

Reagents	Final concentration
PCR grade water	–[†]
PCR mix (2× Supermix – UDG Invitrogen)[1]	1×
$MgCl_2$ (to a final concentration of)	4 mM
BSA	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
Heating ramp speed	5 °C/s
Denaturation	94 °C for 10 s
Annealing and elongation	62 °C for 40 s

[†] For a final reaction volume of 20 µl.

BSA, bovine serum albumin; PCR, polymerase chain reaction.

3.4.7 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 rDNA 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. Diagnostic specificity and sensitivity, as determined using *Citrus* hosts, were both 100%. The analytical sensitivity (detection limit) is between 2 and 10 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 GT-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]	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]	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

[†] For a final reaction volume of 25 µl.

PCR, polymerase chain reaction.

3.4.8 LAMP[2] using the primers of Harper *et al.* (2010, erratum 2013)

3.4.8.1 The LAMP[2] of Harper et al. *(2010, erratum 2013)*

This loop-mediated isothermal amplification (LAMP[2]) method was developed by Harper *et al.* (2010, erratum 2013) and can be used on crude plant tissue and insect extracts or with the DNA extraction methods described in section 3.4.1. Hydroxynaphthol blue can be used as a means of detecting the endpoint (Harper *et al.*, 2010, erratum 2013). Hydroxynaphthol blue or other dyes that can be added prior to amplification are recommended as they allow the LAMP[2] to be performed as a closed-tube system. This avoids the risk of opening tubes post amplification, which could lead to aerosol contamination due to the high titre of the LAMP[2] amplicon.

Analytical specificity using hydroxynaphthol blue for endpoint detection is similar to that reported for the real-time PCR (Harper *et al.*, 2010, erratum 2013). In validation, only *X. fastidiosa* was detected among 95 strains of *X. fastidiosa* from 20 different hosts and 26 non-target bacterial strains. 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′

[2] 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 license from Eiken Chemical Co., Ltd. before use.

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 *et al.* (2010, erratum 2013) LAMP[2] is described in Table 8.

Table 8. Master mix composition and test conditions for LAMP[2], according to Harper *et al.* (2010, erratum 2013)

Reagents	Final concentration
PCR grade water	–[†]
ThermoPol buffer (New England Biolabs)[1]	1×
$MgSO_4$ (additional to a final concentration)	8 mM
Betaine	0.8 M
BSA	300 ng/µl
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)[1]	150 µM
Bst 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

[†] For a final reaction volume of 25 µl.

BSA, bovine serum albumin; PCR, polymerase chain reaction.

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

3.4.8.2 Real-time LAMP[2]

This method is based on the above LAMP[2] primers developed by Harper *et al*. (2010, erratum 2013), and was modified by Yaseen *et al*. (2015). 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[1], Qualiplante[1], Optigene[1]). The kits should be used as per the manufacturer's instructions. Diagnostic sensitivity and specificity using the Enbiotech[1] and Qualiplante[1] kits have been determined as being between 83% and 92%. The analytical sensitivity (detection limit) of these kits is between 10^2 and 10^3 cfu/ml for *Citrus* spp., *V. vinifera* and *O. europaea*. Validation data are available in the EPPO database on diagnostic expertise (EPPO, 2018c).

3.4.9 Controls for molecular testing

For the test result to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid 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[2], a positive nucleic acid (*X. fastidiosa*) control and a negative amplification control (no template control) are the minimum controls that should be used.

Additional controls may be used for both LAMP[2] and PCR as described below.

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/µl) 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 *et al*., 2006), the 16S rDNA gene (Weisburg *et al.*, 1991) 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 near the concentration considered the detection limit of the test.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. 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 should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor 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 previously tested negative for *X. fastidiosa*. It is recommended that multiple controls be included when large numbers of positive samples are expected.

3.4.10 Interpretation of results from conventional and real-time PCR

3.4.10.1 Conventional PCR

The 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
- no amplicons of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

If 16S rDNA internal control primers 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 (amplicon size will depend on which 16S rDNA primers are used (Weisburg *et al.*, 1991)). 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.

3.4.10.2 Real-time PCR

The real-time PCR will be considered valid only if both these criteria are met:

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

If the *COX* 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 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. The cycle cut-off value needs to be verified 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 *et al.* (2010).

3.4.10.3 Real time LAMP

The real time PCR LAMP will be considered valid only if both these criteria are met:

- the positive nucleic acid control produces a specific reaction (the type of reaction varies with the technology of the LAMP test (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 test)
- 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 NPPO requires additional confidence in the identification of the *X. fastidiosa* subspecies or strain type. Sequencing of the complete genome (Simpson *et al.*, 2000; Van Sluys *et al.*, 2003), or multilocus sequence analysis (MLSA or MLST) (Scally *et al.*, 2005; Yuan *et al.*, 2010), is recommended for subspecies identification or when atypical or undescribed strains are suspected (section 4.5.1).

4.1 Isolation

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 *et al.*, 1980), BCYE (Wells *et al.*, 1981) or PWG (modified from Hill and Purcell, 1995; EPPO, 2018b). It is recommended that at least two different media be used for isolation.

Midrib and petiole tissue from symptomatic leaf 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 small twigs, stem and root sections (Hopkins, 2001). *X. fastidiosa* can also be isolated from insect vectors (Hill and Purcell, 1995).

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. Petiole or midrib samples are surface sterilized by immersion in 70% ethanol for 1 min and flaming, or 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). Alternatively, tissue is ground in PBS at ratios of 1:10 and 1:100 with a mortar and pestle or a homogenizer (e.g. Homex)[1] and then plated onto two different types of specific media (e.g. PD2, BCYE, PWG). The application of ultrasonication during the extraction 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 material 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.

The plates should be incubated at 28 °C for 8–30 days, in plastic bags or sealed with parafilm[1] to prevent desiccation. Plates are observed regularly for colony development using a binocular microscope. Colonies visible to the unaided eye within two days should be regarded as contaminants.

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, 2018b). All media are autoclaved at 121 °C for 15 min.

PD2 medium (Table 9). All components except BSA (bovine serum albumin) and hemin chloride stock solution are added to 980 ml of distilled water prior to autoclaving. The pH is adjusted to 7.0 after dissolving the agar. After autoclaving, the BSA (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 9. PD2 medium (Davis *et al.*, 1980)

Reagents	Per litre
Phytone peptone (BD BBL)[1]	2.0 g
Bacto tryptone (Oxoid)[1]	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
BSA (20% w/v) (Sigma)[1]	10 ml
$MgSO_4 \cdot 7H_2O$	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Bacto agar (e.g. BD Difco)[1]	15 g
Distilled water to a final volume of 1 litre	

BSA, bovine serum albumin.

BYCE medium (Table 10). Due to the difficulty of dissolving and re-suspending the individual components it is recommended that ingredients are dissolved in the following order. ACES 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, 2018b).

Table 10. BCYE medium (Wells *et al.*, 1981)

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

Modified PWG medium (Table 11). All constituents except L-glutamine, hemin chloride stock solution and BSA are added prior to 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 (c. 50 °C). Hemin chloride stock is 0.1 % bovine hemin chloride dissolved in 0.05 N NaOH. These three solutions are filter sterilized (0.2 µm membrane) and added to the cooled sterile basal medium.

Table 11. Modified PWG medium (based on Hill and Purcell (1995) and information provided in EPPO (2018b))

Reagents	Per litre
Gelrite gellan gum (Sigma)[1]	9.0 g
Phytone peptone (e.g. BD BBL)[1]	4.0 g
Bacto tryptone (e.g. Oxoid)[1]	1.0 g
Phenol red stock solution (0.2%)	10 ml
L-glutamine (Sigma)[1]	4 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 ml
BSA (Sigma)[1]	3.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Distilled water to a final volume of a 1 litre	

BSA, bovine serum albumin.

4.1.2 Colony morphology

X. fastidiosa colony morphology can be variable (Davis *et al.*, 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 12) may help a correct identification to be reached.

Table 12. Reference *X. fastidiosa* strains

Strain	Source
CFBP 7969, 8073	International Center for Microbial Resources – French Collection for Plant-associated Bacteria, Beaucouze, France
LMG 17159	Belgium Co-ordinated Collection of Micro-organisms, Ghent, Belgium
ICMP 11140, 15197	International Collection of Microorganisms from Plants, Auckland, New Zealand
NCPPB 4432	National Collection of Plant Pathogenic Bacteria, York, United Kingdom of Great Britain and Northern Ireland
DSM 10026	Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

4.1.3 Interpretation of isolation results

The isolation is negative if no bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed after 14–30 days on any medium and typical *X. fastidiosa* colonies are found in the positive controls.

The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed after 14–30 days on at least one medium. In some cases, the incubation time can be up to 30 days due to 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, non-motile, and does not form spores (Davis *et al.*, 1978; Wells *et al.*, 1987; Bradbury, 1991). Some of the key biochemical and physiological characteristics for *X. fastidiosa* are listed in Table 13.

The reference *X. fastidiosa* strains available from different collections are listed in Table 12. These strains are suggested for use as positive controls in biochemical and molecular tests.

Table 13. Key biochemical and physiological characteristics of *X. fastidiosa* (Davis ***et al.***, 1978; Wells ***et al.***, 1987; Bradbury, 1991)

Test	Result
Catalase	+
Oxidase reaction	−
Gelatin liquefaction	+
Indol production	−
H_2S production	−
DL-lactate	+
Glucose fermentation	−
Temperature optimum	26 to 28 °C
pH optimum (*X. fastidiosa* is very sensitive to variations in pH)	6.5 to 6.9

4.3 Pathogenicity tests

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

Actively growing, susceptible plants need to be maintained in a greenhouse or growth chamber at 26–28 °C. Inoculation techniques should deliver 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 tests should use plants of the same host from which the suspect *X. fastidiosa* was isolated. Where possible, the most susceptible cultivars should be used. Some recommended examples include: for *V. vinifera*, the cultivars 'Chardonnay', 'Cabernet sauvignon', 'Chenin Blanc' and 'Pinot Noir'; for *C. sinensis*, 'Pera', 'Hamlin', 'Natal' and 'Valencia'; and for *O. europaea*, 'Cellina di Nardo', 'Frantoio' and 'Leccino' (EPPO, 2018b). *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. Cultures of bacteria grown for 8–10 days on suitable media should be used for pathogenicity tests. Bacteria are removed from solid media and suspended in PBS to produce a turbid suspension of approximately 10^8–10^9 cfu/ml ($Abs_{600nm} = 0.2$). A drop (20–50 µl) 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 chambers at 26–28 °C.

An alternative method of inoculation is to raise a flap of stem tissue by cutting upward with a razor blade to expose the wood. A few drops of bacterial suspension are placed under the flap and the flap 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 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 *et al*., 2008). Leaf scorch symptoms develop 10–14 days after inoculation.

4.4 Serological identification

ELISA (described in section 3.3) can be used for the identification of suspect *X. fastidiosa* strains isolated from diseased plant material.

4.5 Molecular identification

PCR (described in section 3.4) can be used for the identification of suspect *X. fastidiosa* strains 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 3.4.9. For conventional PCR tests, the amplicons can be sequenced to further support the identification. Sequence data can be analysed using the Standard Nucleotide Basic Local Alignment Search Tool (BLASTN), available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

4.5.1 Multilocus sequence typing

A multilocus sequence typing (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 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, 2018b). Primers and conditions 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/). If erratic amplification occurs, the following PCR parameters can be adjusted: the DNA extract can be diluted (to limit inhibition) or the amount of DNA added to the PCR can be increased, different Taq polymerases or master mixes can be used, the annealing temperature can be decreased from 65 °C to 60 °C or 58 °C, or the primer concentration can be increased from 0.3 to 0.5 µM (EPPO, 2018b).

Expected amplicon sizes for the different housekeeping genes are: 708 bp for *leuA*, 533 bp for *petC*, 600 bp for *cysG*, 654 bp for *gltT*, 379 bp for *holC*, 730 bp for *malF*, and 557 bp for *nuoL*.

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. 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 reference sequences for the different housekeeping genes that can be found on the MLST website.

Although different methods are available for subspecies identification (see section 4.5.2), it is recommended that MLST be used to analyse *X. fastidiosa* strains detected in new areas or on new host associations.

4.5.2 Subspecies- and strain-specific PCR

There are a number of specific methods using PCR that enable *X. fastidiosa* subspecies determination (Pooler and Hartung 1995; Hernandez-Martinez *et al.*, 2006; Li *et al.*, 2013). The PCR described by Hernandez-Martinez *et al.* (2006) can allow the identification of cultures of subspecies *fastidiosa*, *multiplex* and *sandyi*. The methods described above have mainly been developed on pure cultures but can be used on DNA extract from plants except for the multiplex 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. Pooler and Hartung (1995) developed a conventional PCR that identifies subspecies *pauca*. The CVC strains of *X. fastidiosa* can be identified by using either a conventional PCR (Pooler and Hartung, 1995) or a real-time PCR (Li *et al.*, 2013). The oleander leaf scorch strains of *X. fastidiosa* can be specifically detected and differentiated from other strains by PCR (Huang, 2009) or real-time PCR (Guan *et al*., 2013). Recently, a PCR has been developed to allow specific detection and identification of American mulberry-infecting strains of *X. fastidiosa* and the newly discovered strains of *X. fastidiosa* associated with Italian olive trees (Guan *et al*., 2015).

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 additional material should be kept for at least one year in a manner that ensures traceability:

- Photographs of symptoms and signs, printouts of ELISA plate results, and photographs of DNA agarose gels 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:

Austrian Agency for Health and Food Safety (AGES), Plant Health Laboratory, Spargelfeldstraße 191, 1220 Vienna, Austria (Helga Reisenzein; email: Helga.reisenzein@ages.at).

Ministry for Primary Industries, Plant Health and Environment Laboratory, PO Box 2095, Auckland 1140, New Zealand (Robert Taylor; email: Robert.taylor@mpi.govt.nz).

United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Molecular Plant Pathology Laboratory, Beltsville Agriculture Research Center-West, 10300 Baltimore Avenue, Beltsville, MD 20705, United States of America (John Hartung; email: John.hartung@ars.usda.gov).

USDA Animal Plant Health and Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Phytosanitary Issues Management, 4700 River Road, Riverdale, MD 20737, United States of America (Wenbin Li; email: Wenbin.li@aphis.usda.gov).

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

7. Acknowledgements

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8. Figures

No figures are included in the protocol itself. Pictures of symptoms are accessible on the EPPO global database website at https://gd.eppo.int/taxon/XYLEFA/photos.

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 Lead based on the consultation comments.

2018-02 TPDP approved draft to submit to SC for adoption.

2018-03 SC approved draft to be submitted to the 45-day DP notification period (2018_eSC_May_07).

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2018-08 SC adopted DP on behalf of CPM.

ISPM 27. Annex 25. *Xylella fastidiosa* (2018). Rome, IPPC, FAO.

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