

[1] **DRAFT ANNEX to ISPM 27– *Xylella fastidiosa* (2004-024)**

[2] Status box	
[3] <i>This is not an official part of the standard and it will be modified by the IPPC Secretariat after adoption</i>	
[4] Date of this document	[5] 2017-06-09
[6] Document category	[7] Draft new annex to ISPM 27:2006 (<i>Diagnostic protocols for regulated pests</i>)
[8] Current document stage	[9] For consultation
[10] Origin	[11] Work programme topic: Bacteria, CPM-1 (2006) [12] Original subject: <i>Xylella fastidiosa</i> (2004-024)
[13] Major stages	[14] 2004-11 Standards Committee added topic to work programme [15] 2016 Draft substantially rewritten by the authoring team to include information on the latest molecular methods [16] 2016-07 Technical Panel on Diagnostic Protocols (TPDP) revised in meeting [17] 2016-10 Expert consultation [18] 2016-12 Diagnostic Protocol (DP) drafting group revision [19] 2017-03 TPDP recommended to SC for approval for consultation via e-decision (2017_eTPDP_Apr_01) [20] 2017-04 SC approved draft DP for consultation (2017_eSC_May_11)
[21] Discipline leads history	[22] 2016-07 Geraldine ANTHOINE (FR, Discipline lead) [23] 2012-11 Robert TAYLOR (NZ, Discipline lead) [24] 2012-11 Brendan RODONI (AUS, Referee)
[25] Consultation on technical level	[26] The first draft of this diagnostic protocol was prepared by: <ul style="list-style-type: none"> - [27] Marta Francis (formerly United States Department of Agriculture (USDA), USA) with consultation from Ed Civerolo (formerly USDA, USA), Helga Reisenzen (Plant Health Laboratory, Austrian Agency for Health and Food Safety, Vienna, Austria) and John Hartung (Molecular Plant Pathology Laboratory, USDA Agricultural Research Service, Beltsville, MD, USA). - [28] Marta Francis resigned as lead author and Wenbin Li (Plant Protection and Quarantine, USDA Animal Plant Health Inspection Service, Riverdale, MD, USA) joined the DP drafting group. - [29] Robert Taylor (TPDP member, New Zealand) was selected as lead author in 2016-07.
[30] Main discussion points during development of the diagnostic protocol [to be updated throughout DP development]	- [31] Scope of the protocol is for the detection and identification of <i>X. fastidiosa</i> . Some discussion on whether the protocol should focus on identification of specific strains of <i>X. fastidiosa</i> , e.g. the <i>X. fastidiosa</i> citrus variegated chlorosis strains. Some information has been included to enable identification of subspecies. - [32] The symptoms and sampling sections were updated using information obtained from the recently revised European and

	<p>Mediterranean Plant Protection Organization (EPPO) diagnostic protocol 2016, in agreement with EPPO and with suitable acknowledgement.</p> <ul style="list-style-type: none"> - [33]Discussion around the most suitable molecular methods for inclusion. - [34]Discussion regarding what was appropriate for the minimum identification requirements.
[35]Notes	<p>[36]This is a draft document. The final formatting will be adjusted at later stage.</p> <p>[37]2017-03 Edited</p>

[38]Contents

[39][to be added later]

[40]Adoption

[41]This diagnostic protocol was adopted by the Commission on Phytosanitary Measures in ----. [to be completed after adoption]

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

[43]1. Pest Information

[44]*Xylella fastidiosa* Wells *et al.* (1987) is a xylem-limited bacterium that 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 at

http://www.ec.europa.eu/food/plant/plant_health_biosecurity/legislation/emergency_measures/xylella-fastidiosa/susceptible_en.htm and <https://www.efsa.europa.eu/fr/efsajournal/pub/4378>. *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, 2015).

[45]*X. fastidiosa* is genetically diverse and consists of six sub-species. *X. fastidiosa* subsp. *fastidiosa* causes Pierce's disease and infects a large host range including *Vitis vinifera*, *Prunus 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 *Prunus dulcis*, *Prunus persica*, *Quercus* spp. and *Platanus occidentalis*. *X. fastidiosa* subsp. *sandyi* causes oleander leaf scorch (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 *Olea europaea*. A different *Xylella* species is associated with pear leaf scorch in Taiwan Province of China (Leu and Su, 1993) and is now classified as *X. taiwanensis* (Su *et al.*, 2016). *X. fastidiosa* is also present in Taiwan Province of China on *Vitis vinifera* (Su *et al.*, 2013).

[46]*X. fastidiosa* is a Gram-negative, xylem-limited 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* do not display any symptoms (Almeida and Purcell, 2003). The bacterium proliferates in the xylem of an infected host and invades the plant systemically, including the roots of infected plants as well as all above-ground plant parts (Aldrich *et al.*, 1992; He *et al.*, 2000; Li *et al.*, 2003). The pathogen overwinters in the xylem of the host plant as well as in weeds. Insect transmission is considered the main factor for *X. fastidiosa* spread. The vectors belong to the order Hemiptera, sub-order Auchenorrhyncha, and the families of 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 then to transmit the pathogen to a healthy host. 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). 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.

[47]2. Taxonomic Information

[48]**Name:** *Xylella fastidiosa* Wells *et al.*, 1987

[49]**Synonyms:** None

[50]**Taxonomic position:** Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae

[51]**Common names:** Pierce's disease of grapevines, citrus variegated chlorosis, olive quick decline syndrome, alfalfa dwarf, phony peach disease, plum leaf scald, dwarf lucerne, periwinkle wilt and bacterial leaf scorch disease. 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.

[52]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.

[53]3. Detection

[54]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. Detection is therefore based on inspection for symptoms and the use of specific serological and molecular tests.

[55]3.1 Symptoms

[56]The presence of *X. fastidiosa* can have a broad impact on its host: from causing no symptoms to plant death. Most host plants infected with *X. fastidiosa* do not display 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 plant species and cultivars and environmental conditions (Janse and Obradovic, 2010; CABI, 2016).

[57]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.). Symptoms on various hosts can be seen 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. 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 it also causes alfalfa dwarf and overlaps with *X. fastidiosa* subsp. *multiplex* in causing almond leaf scorch (Yuan *et al.* 2010). Some examples of the subspecies of *X. fastidiosa* that are linked to the below disease descriptions are provided when widely acknowledged in the current literature. The following descriptions are some of the more characteristic symptoms observed on some key hosts.

[58]3.1.1 Pierce's disease of grapevines

[59]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 *Vitis 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, 2016).

[60]3.1.2 Citrus variegated chlorosis

[61]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 citrus variegated chlorosis (Schaad *et al.* 2004; Almeida *et al.* 2008). Symptoms are most obvious on three- to six-year-old trees and mainly on *Citrus 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).

[62]3.1.3 Coffee leaf scorch

[63]Symptoms of coffee leaf scorch appear on young flushes of field plants as large marginal and apical scorched zones on recently matured leaves (EPPO, 2016). 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 the Republic of

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).

[64]3.1.4 Olive leaf scorching and quick decline

[65]In three different distant regions around the world (the southern region of the Republic of Italy, the Argentine Republic and the Federative Republic of Brazil), leaf scorching symptoms on *Olea 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 a recombinant of alleles within the *X. fastidiosa* subspecies *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, 2016).

[66]3.1.5 Almond leaf scorch disease

[67]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.

[68]3.1.6 Bacterial leaf scorch of shade trees

[69]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.

[70]3.1.7 Bacterial leaf scorch of blueberry

[71]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.*, 2008; EPPO, 2016). In the early stages of disease progression, symptoms may be localized, but over time, symptoms can become uniformly distributed throughout the foliage. Newly developed shoots can be abnormally thin with a reduced number of flower buds. Leaf drop occurs, and twigs and stems have a distinct “skeletal” yellow appearance. Following leaf drop, the plant dies, this typically occurring during the second year after symptoms are observed (Chang *et al.*, 2009).

[72]3.1.8 Phony peach disease and plum leaf scald

[73]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).

[74]3.1.9 Alfalfa dwarf

[75]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 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, 2016). 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.

[76]3.2 Sampling and sample preparation for symptomatic and asymptomatic material

[77]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. Once samples are collected, they should be kept cool and transported to the laboratory as soon 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 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.

[78]3.2.1 Sampling period for symptomatic or asymptomatic plants

[79]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.

[80]In temperate zones of the world where *Vitis vinifera* or deciduous trees (e.g. *Prunus cerasus*, *Prunus 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, 2016).

[81]3.2.2 Sample collection

[82]*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 *Prunus 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.

[83]3.2.3 Sampling of symptomatic plants

[84]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, 2016). 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.

[85]3.2.4 Sampling of asymptomatic plants

[86]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 *Olea 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*).

[87]3.2.5 Sampling of vectors

[88]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).

[89]3.2.6 Vector sample collection

[90]Adult 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 done 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 $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$, or in 95–99% ethanol at $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$. Sticky traps can also be stored at $-20\text{ }^{\circ}\text{C}$.

[91]3.2.7 Sample storage in the laboratory

[92]Samples should be processed as soon as possible after arrival. For isolation (see section 4.1), samples may be kept refrigerated for up to three days. For other tests, samples may be refrigerated for up to one week.

[93]3.3 Serological detection

[94]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

[95]A number of serological tests have been developed for the detection of *X. fastidiosa*, these including tests using enzyme-linked immunosorbent assay (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 *Olea europaea* samples for *X. fastidiosa* (Djelouah *et al.*, 2014).

[96]3.3.1 Preparation of material

[97]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. ELISA can also be used on twigs and canes but is unsuitable for use on necrotic or dead tissue.

[98]3.3.2 Double antibody sandwich ELISA

[99]Positive and negative controls should be included in each test and these are normally provided in commercial kits. Positive controls can 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 or variety to allow for comparison with the test samples and to check for potential background- or cross-reactions.

[100]Samples should be processed following the general procedure recommended for the specific serological test 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; Na₂HPO₄·12H₂O, 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. Polytron¹, Homex¹) or by pulverising in liquid nitrogen (EPPO, 2016; Loconsole *et al.*, 2014). Further information on using ELISA to detect plant pathogenic bacteria is available in EPPO (2010).

[103]Kits for the serological detection of *X. fastidiosa* are commercially available from Agritest¹, Agdi¹ and Loewe Biochemica¹. 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 ELISA is approximately 10⁴ colony-forming units (c.f.u.)/ml (Loconsole *et al.*, 2014; EPPO, 2016). The technique is not sensitive enough for use early in the growth season, when no symptoms of the disease are observed, due to the low concentration of bacteria likely to be present in young asymptomatic tissue.

[104]The specificity and sensitivity of ELISA to detect *X. fastidiosa* on *Olea europaea*, using a kit from Loewe¹, 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 Agritest¹, Agdia¹ and Loewe¹. 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 at <http://dc.eppo.int/validationlist.php>.

[105]3.3.3 Interpretation of ELISA results

[106]The reaction 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.

[107]Once the reaction of the controls has been verified, then the results for each sample are interpreted as follows:

□

[101]¹ In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. 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. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

[102]

- [108]The ELISA is negative if the average absorbance readings of duplicate wells containing tissue macerate is $<2\times$ the average absorbance of the negative control wells containing healthy host tissue macerate.
- [109]The ELISA is positive if the average absorbance readings of duplicate sample wells is $\geq 2\times$ the average absorbance readings of the negative control wells containing healthy host tissue macerate.

[110]3.4 Molecular detection

[111]Various molecular methods have been developed for the detection and identification of *X. fastidiosa* directly on pure cultures, plant tissue and insect vectors (Ferraro 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 PCR's (Harper *et al.*, 2010, and Li *et al.*, 2013) are described in this protocol for the detection and identification of *X. fastidiosa*.

[112]3.4.1 Nucleic acid extraction and purification for bacterial colonies and plant material

[113]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; 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. Validation data on the sensitivities associated with the different nucleic acid extraction methods can be found at <http://dc.eppo.int/validationlist.php>. 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) of bacterial colonies, or on DNA extracts purified using the methods below.

[114]*CTAB-based extraction.* 200 mg 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 (EDTA); 2% hexadecyltrimethylammonium bromide (CTAB); 3% polyvinylpyrrolidone (PVP-40)) and homogenized using a homogenizer (e.g. Homex¹, Polytron¹). 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 (c. 750 µl) is carefully transferred to a new tube and mixed with the same volume of 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 above centrifugation step. After washing and decanting the supernatant, the pellet is air dried and suspended in 100 µl deoxyribonuclease-free water.

[115]*DNeasy Plant Mini Kit* (Qiagen)¹. DNA is extracted from 200 mg plant tissue (leaf midrib, petiole or twig tissue) and macerated in lysis buffer using homogenizing equipment (e.g. Homex¹, Polytron¹). 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.

[116]*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 part 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 mL¹ (15 samples) or KingFisher Flex¹ (96 samples) purification system (Thermo Scientific)¹ (validation data available at <http://dc.eppo.int/validationlist.php>).

[117]KingFisher (Thermo Scientific)¹ using InviMAG Plant DNA Kit¹. This automated magnetic bead extraction procedure is ideal for high-throughput testing and uses the InviMAG Plant DNA Mini Kit (Strattec Molecular)¹ with the KingFisher 96 system (Thermo Scientific)¹. Samples are homogenized in the lysis kit buffer (or CTAB buffer) at a ratio of 1:5 tissue to buffer. The plant extracts are incubated at 60 °C for 30 min and then treated according to the manufacturer's instructions.

[118]3.4.2 Nucleic acid extraction and purification for insect vectors

[119]DNA may be extracted from a single insect head or a pool of five heads (Bextine *et al.*, 2004; Purcell *et al.*, 2014; EPPO, 2016). Only insect heads 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.

[120]Qiagen's DNeasy Tissue Kit¹. A DNA extraction method using this kit has been shown to reliably detect 50–500 *X. fastidiosa* cells in *Homalidisca coagulata* (Bextine *et al.*, 2004, 2005; Huang *et al.*, 2006).

[121]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 MM400¹. Samples are homogenized for two minutes 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 mL¹ (15 samples) or KingFisher Flex¹ (96 samples) system (Thermo Scientific)¹.

[122]CTAB-based extraction for insects. The homogenization of the insect heads can be performed in a microcentrifuge tube using a micro-homogenizer 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.

[123]3.4.3 Conventional polymerase chain reaction (PCR) using the primers of Minsavage *et al.*, (1994)

[124]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) 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 grape vines were not detected with this PCR. The analytical sensitivity of the test is 1 × 10² c.f.u./ml. Further validation data are available at <http://dc.eppo.int/validationlist.php>.

[125]The oligonucleotide primers used are:

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

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

[128]The master mix used for this PCR developed by Minsavage *et al.* (1994) is described in Table 1.

[129]**Table 1.** Master mix composition for PCR and cycling conditions for a final reaction volume of 20 μ l.

[130]Reagents	[131]Final concentration
[132]PCR grade water	[133]N.A.
[134]PCR buffer (Invitrogen) ¹	[135]1x
[136]dNTPs	[137]200 μ M
[138]MgCl ₂	[139]1.5 mM
[140]Primer RST31 (forward)	[141]0.5 μ M
[142]Primer RST33 (reverse)	[143]0.5 μ M
[144]Taq DNA polymerase (Invitrogen ¹)	[145]1.0 U
[146]DNA volume	[147]2 μ l bacterial suspension or DNA extract
[148]Cycling parameters	[149]
[150]Initial denaturation	[151]95 °C for 1 min
[152]Number of cycles	[153]40
[154] Denaturation	[155]95 °C for 30 s
[156] Annealing	[157]55 °C for 30 s
[158] Elongation	[159]72 °C for 45 s
[160]Final elongation	[161]72 °C for 5 min
[162]Expected amplicons	[163]
[164]Size	[165]733 bp

[166]bp, base pairs; N.A., not applicable;.

[167]3.4.4 Conventional PCR using the primers of Rodrigues *et al.* (2003)

[168]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 analytical sensitivity for the multiplex PCR is approximately 10² c.f.u./ml.

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

[170]Set A:

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

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

[173] Primer set A amplifies a product of 1348 bp.

[174]Set B:

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

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

[177] Primer set B amplifies a product of 745 bp.

[178]Set C:

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

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

[181] Primer set C amplifies a product of 603 bp.

[182]The master mix for Rodrigues *et al.*'s primers (sets A, B, C) is described in Table 2.

[183]**Table 2.** Master mix composition for PCR and cycling conditions for a final reaction volume of 20 μ l

[184]Reagents	[185]Final concentration
[186]PCR grade water	[187]N.A.
[188]PCR Buffer	[189]1 \times
[190]dNTPs	[191]200 μ M
[192]MgCl ₂	[193]1.5 mM
[194]Primer (forward set A, or B or C)	[195]0.2 μ M
[196]Primer (reverse set A, or B or C)	[197]0.2 μ M
[198]Taq DNA polymerase (Invitrogen ¹)	[199]2.0 U
[200]DNA volume	[201]2 μ l bacterial suspension or DNA extract
[202]Cycling parameters	[203]
[204]Initial denaturation	[205]94 °C for 3 min
[206]Number of cycles	[207]30
[208] Denaturation	[209]94 °C for 1 min
[210] Annealing	[211]55 °C for 30 s
[212] Elongation	[213]72 °C for 2 min
[214]Final elongation	[215]72 °C for 7 min
[216]Expected amplicons	[217]
[218]Size	[219]Primer set A: 1348 bp [220]Primer set B: 745 bp [221]Primer set C: 603 bp

[222]bp, base pairs; N.A., not applicable; PCR, polymerase chain reaction.

[223]The *gyrB* primers used are:

[224] FXY*gyr499* (forward): 5'-CAG TTA GGG GTG TCA GCG-3'

[225] RXY*gyr907* (reverse): 5'-CTC AAT GTA ATT ACC CAA GGT-3'

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

[227]The master mix for the *gyrB* gene-targeting primers is described in Table 3.

[228]**Table 3.** Master mix composition for *gyrB* gene-targeting PCR and cycling conditions for a final reaction of 20 μ l

[229]Reagents	[230]Final concentration
[231]PCR grade water	[232]N.A.
[233]PCR Buffer	[234]1 \times
[235]dNTPs	[236]200 μ M
[237]MgCl ₂	[238]1.5 mM

[239]Primer FXYgyr499 (forward)	[240]0.4 µM
[241]Primer RXYgyr907 (reverse)	[242]0.4 µM
[243]Taq DNA polymerase (Invitrogen ¹)	[244]2.5 U
[245]DNA volume	[246]2 µl bacterial suspension or DNA extract
[247]Cycling parameters [248]	
[249]Initial denaturation	[250]94 °C for 3 min
[251]Number of cycles	[252]30
[253] Denaturation	[254]94 °C for 1 min
[255] Annealing	[256]60 °C for 1 min
[257] Elongation	[258]72 °C for 2 min
[259]Final elongation	[260]72 °C for 7 min
[261]Expected amplicons [262]	
[263]Size	[264]429 bp

[265]bp, base pairs; N.A., not applicable; PCR, polymerase chain reaction.

[266]3.4.5 Real-time PCR using the primers and probes of Harper *et al.* (2010, erratum 2013)

[267]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.

[268]Harper *et al.* (2010, erratum 2013) evaluated specificity (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, 2016). For *Olea europaea* hosts when using CTAB extraction methods, diagnostic specificity is 100% and diagnostic sensitivity is 91% (EPPO, 2016). Further validation data are available at <http://dc.eppo.int/validationlist.php>. The sensitivity (analytical sensitivity; detection limit) is between 10² c.f.u./ml for *Citrus* spp. and *Vitis vinifera* and 10⁵ c.f.u./ml for *Olea europaea*.

[269]The oligonucleotide primers and probes used are:

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

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

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

[273]The master mix for the Harper *et al.* (2010, erratum 2013) primers and probes is described in Table 4.

[274]**Table 4.** Master mix composition for real-time PCR and cycling conditions for a final reaction volume of 20 µl

[275]Reagents	[276]Final concentration
[277]PCR grade water	[278]N.A.
[279]PCR Mix (2 × Supermix – UDG Invitrogen ¹)	[280]1×
[281]MgCl ₂ (to a final concentration of)	[282]4 mM
[283]BSA	[284]300 ng/µl
[285]Primer XF-F (forward)	[286]0.3 µM
[287]Primer XF-R (reverse)	[288]0.3 µM

[289]Probe XF-P	[290]0.1 µM
[291]DNA volume	[292]2 µl bacterial suspension or DNA extract
[293]Cycling parameters	[294]
[295]Pre-incubation	[296]50 °C for 2 min
[297]Initial denaturation	[298]94 °C for 2 min
[299]Number of cycles	[300]40
[301]Heating ramp speed	[302]5 °C/sec
[303]Denaturation	[304]94 °C for 10 sec
[305]Annealing - Elongation	[306]62 °C for 40 sec

[307]BSA, bovine serum albumin; N.A., not applicable;.

[308]Harper *et al.* (2010, erratum 2013) considered that a cycle cut-off value was 38 using the Bio-Rad CFX-96 real-time thermocycler (Bio-rad Laboratories¹) and materials and reagents used as described above. It should be noted that for the use of this test in the described conditions, a Ct cut off value was defined as 38. Provided that the relevant positive and negative controls (described in section 3.4.8) give the correct reaction, below this value the sample is considered positive; at and above it, the sample is considered negative.

[309]3.4.6 Real-time PCR using the primers and probes of Li *et al.* (2013)

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

[311]Li *et al.* (2013) evaluated specificity (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 sensitivity (analytical sensitivity; detection limit) is between 2 and 10 cells of *X. fastidiosa* per reaction for *Citrus* samples.

[312]The oligonucleotide primers and probes used are:

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

[314] XF16Sr (reverse primer): 5'-CCG ATG TAT TCC TCA CCC GT-3'

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

[316]The master mix for the Li *et al.* (2013) real-time PCR is described in Table 5.

[317]**Table 5.** Master mix composition for real-time PCR and cycling conditions for a final reaction volume of 25 µl

[318]Reagents	[319]Final concentration
[320]PCR grade water	[321]N.A.
[322]PCR buffer (Invitrogen)	[323]1x
[324]dNTPs	[325]240 µM
[326]MgCl ₂	[327]6 mM
[328]Primer XF16Sf (forward)	[329]0.240 µM
[330]Primer XF16Sr (reverse)	[331]0.240 µM
[332]Probe XF16Sp	[333]0.12 µM
[334]Platinum Taq (Invitrogen ¹)	[335]1 U
[336]DNA volume	[337]2 µl bacterial suspension or DNA extract
[338]Cycling parameters	[339]

[340]Initial denaturation	[341]95 °C for 20 sec
[342]Number of cycles	[343]40
[344]Heating ramp speed	[345]5 °C/sec
[346]Denaturation	[347]95 °C for 1 sec
[348]Annealing - Elongation	[349]60 °C for 40 sec

[350]N.A., not applicable;.

[351]The cycle cut-off value of 40 was obtained using the SmartCycler II real-time PCR system (Cepheid¹) and materials and reagents used as described above. It should be noted that:

- [352]The amplification curve should be exponential.
- [353]A sample will be considered positive if it produces a Ct value of <40, provided the contamination controls are negative.
- [354]A sample will be considered negative if it produces a Ct value of ≥ 40 , provided the assay and extraction inhibition controls are positive.

[355]The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

[356]3.4.7 LAMP² using the primers of Harper *et al.* (2010, erratum 2013)

[358]3.4.7.1 The LAMP² of Harper *et al.* (2010, erratum 2013)

[359]This loop-mediated isothermal amplification (LAMP²) test 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).

[360]Method specificity (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, except for the *Xylella* strain from Taiwan Province of China.

[361]The primers used are:

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

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

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

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

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

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

[368]The master mix for the Harper *et al.* (2010, erratum 2013) LAMP² is described in Table 6.

□—————

[357]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.

[369] **Table 6.** Master mix composition for LAMP² and test conditions for a final reaction volume of 25 µl

[370] Reagents	[371] Final concentration
[372] PCR grade water	[373] N.A.
[374] ThermoPol buffer (New England Biolabs ¹)	[375] 1×
[376] MgSO ₄ (additional to a final concentration)	[377] 8 mM
[378] Betaine	[379] 0.8 M
[380] BSA	[381] 300 ng/µl
[382] Each dNTP	[383] 1.4 mM
[384] External primer XF-F3/	[385] 0.2 µM
[386] External primer XF-B3	[387] 0.2 µM
[388] Internal primer XF-FIP	[389] 1.6 µM
[390] Internal primer XF-BIP	[391] 1.6 µM
[392] Loop primer XF-LF	[393] 0.8 µM
[394] Loop primer XF-LB	[395] 0.8 µM
[396] Hydroxynaphthol blue (Sigma Aldrich ¹)	[397] 150 µM
[398] <i>Bst</i> DNA polymerase	[399] 8 U
[400] DNA volume	[401] 2 µl DNA extract
[402] Incubation parameters	[403]
[404] Incubation	[405] 65 °C for 60 min
[406] Enzyme inactivation	[407] 80 °C for 2 min

[408] BSA, bovine serum albumin; N.A., not applicable;

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

[410] 3.4.7.2 *Real-time LAMP²*

[411] This test is based on the above LAMP 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. The test is commercially available as ready-to-use kits and is performed in real-time on a specific device or by using a standard real-time thermocycler (e.g. Enbiotech¹, Qualiplate¹, Optigene¹). The kits should be used as per the manufacturer's instructions. Diagnostic sensitivity and specificity using the Enbiotech¹ and Qualiplate¹ kits have been determined as being between 83% and 92%. The sensitivity of these kits (analytical sensitivity; detection limit) is between 10² and 10³ c.f.u./ml for citrus, grape and olive. Validation data are available at <http://dc.eppo.int/validationlist.php>.

[412] 3.4.8 **Controls for molecular testing**

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

[414] 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.

[415] For LAMP², a positive nucleic acid (*X. fastidiosa*) control and a negative amplification control (no template control) are the minimum controls that should be used.

[416] Additional controls may be used for both LAMP² and PCR as described below.

[417]*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.

[418]*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.

[419]*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.

[420]*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.

[421]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.

[422]*Negative extraction control.* This control is used to monitor contamination during nucleic acid extraction or 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*. Multiple controls are recommended to be included when large numbers of positive samples are expected.

[423]3.4.9 Interpretation of results from conventional and real-time PCR

[424]3.4.9.1 Conventional PCR

[425]The pathogen-specific PCR will be considered valid only if both these criteria are met:

- [426]the positive control produces the correct size amplicon for the bacterium
- [427]no amplicons of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

[428]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.

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

[430]3.4.9.2 Real-time PCR

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

- [432]the positive control produces an amplification curve with the pathogen-specific primers and probe
- [433]no amplification curve is seen (i.e. Ct value is 40) with the negative extraction control and the negative amplification control.

[434]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.

[435]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 test for the first time. Guidance on how to determine the cycle cut-off value can be found in Chandelier *et al.* (2010).

[436]4. Identification

[437]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.

[438]Further tests may be done in instances where the NPPO requires additional confidence for 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).

[439]4.1 Isolation

[440]*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, 2016). It is recommended that at least two different media are used for isolation.

[441]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).

[442]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 micro-organisms. Petiole or midrib samples are surface sterilized by immersion in 70% ethanol for 1 min and then with 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).

[443]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 on specific media as above.

[444]The plates should be incubated at 28 °C for 8–30 days, in plastic bags or sealed with parafilm¹ 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 discarded as contaminants.

[445]4.1.1 Culture media

[446]All media are sterilized at 121 °C for 15 min.

[447]Table 7. PD2 medium (Davis *et al.*, 1980)

[448]Reagents	[449]per litre
[450]Phytone Peptone (BD BBL ¹)	[451]2.0 g
[452]Bacto tryptone (Oxoid ¹)	[453]4.0 g
[454]Trisodium citrate	[455]1.0 g
[456]Disodium succinate	[457]1.0 g
[458]Hemin chloride stock solution (0.1% in 0.05 N NaOH)	[459]10 ml
[460]BSA (20% w/v) (Sigma ¹)	[461]10 ml
[462]MgSO ₄ ·7H ₂ O	[463]1.0 g
[464]K ₂ HPO ₄	[465]1.5 g
[466]KH ₂ PO ₄	[467]1.0 g
[468]Bacto agar (Difco ¹)	[469]15 g
[470]Distilled water to a final volume of 1 litre	[471]

[472]BSA, bovine serum albumin.

[473]PD2 medium (Table 7). All constituents 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.

[474]Table 8. BCYE medium (Wells *et al.*, 1981)

[475]Reagents	[476]per litre
[477]ACES buffer (Sigma ¹)	[478]10.0 g
[479]Yeast extract	[480]10.0 g
[481]Activated charcoal (Norit ¹)	[482]2.0 g
[483]L-cysteine hydrochloride-1-hydrate (Sigma ¹)	[484]0.4 g
[485]Ferric pyrophosphate (Sigma ¹)	[486]0.25 g
[487]Bacto agar (Difco ¹)	[488]17 g
[489]Distilled water to a final volume of a 1 litre	[490]

[491]BYCE medium (Table 8). 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 sterilised 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, 2016).

[492]Table 9. Modified PWG medium (based on Hill and Purcell, 1995 and information provided in EPPO (2016)

[493]Reagents	[494]per litre
[495]Gelrite gellan gum (Sigma ¹)	[496]9.0 g
[497]Phytone Peptone (BD BBL ¹)	[498]4.0 g

[499]Bacto tryptone (Oxoid ¹)	[500]1.0 g
[501]Phenol red stock solution (0.2%)	[502]10 ml
[503]L-glutamine (Sigma ¹)	[504]4 g
[505]Hemin chloride stock solution (0.1% in 0.05 N NaOH)	[506]10 ml
[507]BSA (Sigma ¹)	[508]3.0 g
[509]MgSO ₄ ·7H ₂ O	[510]1.0 g
[511]K ₂ HPO ₄	[512]1.5 g
[513]KH ₂ PO ₄	[514]1.0 g
[515]Bacto agar (Difco ¹)	[516]15 g
[517]Distilled water to a final volume of a 1 litre	[518]

[519]BSA, bovine serum albumin.

[520]*Modified PWG medium* (Table 9). 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.

[521]4.1.2 Colony morphology

[522]*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 10) may help a correct identification to be reached.

[523]**Table 10.** Reference *X. fastidiosa* strains

[524]Strain	[525]Source
[526]CFBP 7969, 8073	[527]International Center for Microbial Resources – French Collection for Plant-associated Bacteria, Beaucouze, the French Republic
[528]LMG 17159	[529]Belgium Co-ordinated Collection of Microorganisms, Ghent, the Kingdom of Belgium
[530]ICMP 11140, 15197	[531]International Collection of Microorganisms from Plants, Auckland, New Zealand
[532]NCPBP 4432	[533]National Collection of Plant Pathogenic Bacteria, York, the United Kingdom of Great Britain and Northern Ireland
[534]DSM 10026	[535]Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, the Federal Republic of Germany

[536]4.1.3 Interpretation of isolation results

[537]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.

[538]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.

[539]4.2 Description and biochemical characteristics

[540]*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 11.

[541]The reference *X. fastidiosa* strains available from different collections are listed in Table 10. These strains are suggested for use as positive controls in biochemical and molecular tests.

[542]**Table 11.** Key biochemical and physiological characteristics of *X. fastidiosa* (Davis *et al.*, 1978; Wells *et al.*, 1987; Bradbury, 1991)

[543]Test	[544]Result
[545]Catalase	[546]+
[547]Oxidase reaction	[548]-
[549]Gelatin liquefaction	[550]+
[551]Indol production	[552]-
[553]H ₂ S production	[554]-
[555]DL-lactate	[556]+
[557]Glucose fermentation	[558]-
[559]Temperature optimum	[560]26 to 28 °C
[561]pH optimum (<i>X. fastidiosa</i> is very sensitive to variations in pH)	[562]6.5 to 6.9

[563]4.3 Pathogenicity tests

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

[565]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.

[566]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 *Vitis vinifera*, the cultivars Chardonnay, Cabernet sauvignon, Chenin Blanc and Pinot Noir; for *Citrus sinensis*, Pera, Hamlin, Natal and Valencia; and for *Olea europaea*, Cellina di Nardo, Frantoio and Leccino (EPPO, 2016). Madagascar periwinkle (*Catharanthus roseus*) is a herbaceous-grown plant that is easily grown in a greenhouse and is susceptible to *X. fastidiosa* (Monteiro *et al.*, 2001).

[567]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 medium should be used for pathogenicity tests. Bacteria are removed from solid media and suspended in PBS to produce a turbid suspension of approximately 10⁸–10⁹ c.f.u./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.

[568]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.

[569]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).

[570]For both pathogenicity tests, if possible the bacterium should be re-isolated to fulfil the requirements for Koch's postulates.

[571]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.

[572]4.4 Serological identification

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

[574]4.5 Molecular identification

[575]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 Basic Local Alignment Search Tool (BLASTN), available at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

[576]4.5.1 Multilocus sequence typing

[577]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). 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, 2016). 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/>). The annealing temperature described in Yuan *et al.* (2010) is 65 °C but other researchers have observed that an annealing temperature of 60 °C improves the reliability of the method (French National Institute for Agricultural Research–Research Institute of Horticulture and Seeds, personal communication, 2016).

[578]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*.

[579]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 reverse and forward primers. Sequences are concatenated by following the alphabetic order of the genes and analysis should be performed as per advice on the MLST website. The results of the sequencing should be compared with reference sequences for the different housekeeping genes that can be found on the MLST website.

[580]It is recommended that this test be used to analyse *X. fastidiosa* strains detected in new areas or on new host associations.

[581]4.5.2 Subspecies- and strain-specific PCR

[582]There are a number of specific tests 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*. Pooler and Hartung (1995) developed a conventional PCR that identifies subspecies *pauca*. The citrus variegated chlorosis 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 *X. fastidiosa* associated with Italian olives (Guan *et al.*, 2015).

[583]5. Records

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

[585]In cases where other contracting parties may be affected by the results of the diagnosis, records and evidence of the results (in particular cultures, photographs of symptoms and signs, ELISA plate results printouts, DNA extracts, photographs of DNA agarose gels) should be retained for at least one year in a manner that ensures traceability.

[586]6. Contact Points for Further Information

[587]Further information on this protocol can be obtained from (in alphabetical order):

[588]Austrian Agency for Health and Food Safety (AGES), Plant Health Laboratory, Spargelfeldstraße 191, 1220 Vienna, the Republic of Austria (Helga Reisenzein; email: Helga.reisenzein@ages.at; tel: +43 50 555 33340).

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

[590]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, the United States of America (John Hartung; email: John.hartung@ars.usda.gov).

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

[592]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).

[593]7. Acknowledgements

[594]This diagnostic protocol was drafted by Marta Francis (formerly USDA), Robert Taylor (Plant Health and Environment Laboratory, Ministry for Primary Industries, New Zealand (see preceding section)), Helga Reisenzein (Austrian Agency for Health and Food Safety, Plant Health Laboratory, the Republic of Austria (see preceding section)), John Hartung (USDA ARS, Molecular Plant Pathology Laboratory, Beltsville Agriculture Research Center-West, the United States of America (see preceding section)), Wenbin Li (USDA APHIS-PPQ, the United States of America (see preceding section)).

[595]In addition, Ed Civerolo (formerly USDA) was involved in the development of this protocol. The diagnostic protocol developed for the detection of *X. fastidiosa* in the European and Mediterranean Plant Protection Organization (EPPO) region (EPPO, 2016) was used as an important contribution to the drafting of this protocol.

[596]8. Figures

[597]No figures are included in the protocol itself. Pictures of symptoms are accessible at <https://gd.eppo.int/taxon/XYLEFA/photos>.

[598]9. References

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

[600]**Aldrich, J.H., Gould, A.B. & Martin, F.G.** 1992. Distribution of *Xylella fastidiosa* within roots of peach. *Plant Disease*, 76: 885–888.

[601]**Almeida, R.P.P., Blua, M.J., Lopes, J.R.S. & Purcell, A.H.** 2005. Vector transmission of *Xylella fastidiosa*: Applying fundamental knowledge to generate disease management strategies. *Annals of the Entomological Society of America*, 98: 775–786.

[602]**Almeida, R. P., Nascimento, F. E., Chau, J., Prado, S. S., Tsai, C. W., Lopes, S. A., & Lopes, J. R.** (2008). Genetic structure and biology of *Xylella fastidiosa* strains causing disease in citrus and coffee in Brazil. *Applied and Environmental Microbiology*, 74: 3690–3701.

[603]**Almeida, R.P.P. & Nunney, L.** 2015. How do plant diseases caused by *Xylella fastidiosa* emerge? *Plant Disease*, 99:1457–1467.

[604]**Almeida, R.P.P., Pereira, E.F., Purcell, A.H. & Lopes, J.R.S.** 2001. Multiplication and movement of a citrus strain of *Xylella fastidiosa* within sweet orange. *Plant Disease*, 85: 382–386.

[605]**Almeida, R.P.P. & Purcell, A.H.** 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. *Applied and Environmental Microbiology*, 69: 7447–7452.

[606]**Bergsma-Vlami, M., van de Bilt, J.L.J., Tjou-Tam-Sin, N.N.A., van de Vossenbergh, B.T.L.H. & Westenberg, M.** 2015. *Xylella fastidiosa* in *Coffea arabica* ornamental plants imported from Costa Rica and Honduras in the Netherlands. *Journal of Plant Pathology*, 97, 395.

[607]**Bextine, B., Blua, M., Harshman, D. & Miller, T.A.** 2005. A SYBR green-based real-time polymerase chain reaction protocol and novel DNA extraction technique to detect *Xylella fastidiosa* in *Homalodisca coagulata*. *Journal of Economic Entomology*, 98: 667–672.

[608]**Bextine, B. & Child, B.** 2007. *Xylella fastidiosa* genotype differentiation by SYBR® Green-based QRT-PCR. *FEMS Microbiology Letters*, 276(1): 48–54.

[609]**Bextine, B., Tuan, S.J., Shaikh, H., Blua, M. & Miller, T.A.** 2004. Evaluation of methods for extracting *Xylella fastidiosa* DNA from the glassy-winged sharpshooter. *Journal of Economic Entomology*, 97: 757–763.

[610]**Bradbury, J.F.** 1991. *Xylella fastidiosa*. IMI Descriptions of Fungi and Bacteria, Sheet 1049.

[611]**Brannen, P.M., Krewer, G., Boland, B., Horton, D. & Chang, C.J.** 2016. *Bacterial leaf scorch of blueberry*. University of Georgia Agriculture (UGA) Extension Circular 922. Athens, GA. 6 pp. Available at http://extension.uga.edu/publications/files/pdf/C%20922_4.PDF (last accessed 7 October 2016).

[612]**Brlansky, R.H., Timmer, L.W., French, W.J. & McCoy, R.E.** 1983. Colonization of the sharpshooter vectors, *Oncometopia nigricans* and *Homalodisca coagulata*, by xylem limited bacteria. *Phytopathology*, 73: 530–535.

[613]**Bull, C.T., De Boer, S.H., Denny, T.P., Firrao, G., Fischer-Le Saux, M., Saddler, G.S., Scortichini, M., Stead, D.E. & Takikawa, Y.** 2012. List of new names of plant pathogenic bacteria (2008–2010). *Journal of Plant Pathology*, 94: 21–27.

[614]**CABI.** 2016. *Xylella fastidiosa*. Crop Protection Compendium. Wallingford, UK, CABI. Available at <http://www.cabi.org/cpc/datasheet/57195> (last accessed 07 October 2016).

- [615] Carbajal, D., Morano, K.A. & Morano, L.D. 2004. Indirect immunofluorescence microscopy for direct detection of *Xylella fastidiosa* in xylem sap. *Current Microbiology*, 49: 372–375.
- [616] Chandelier, A., Planchon, V. & Oger, R. 2010. Determination of cycle cut off in real-time PCR for the detection of regulated plant pathogens. *EPPO Bulletin*, 40: 52–58.
- [617] Chang, C.-J., Donaldson, R., Brannen, P., Krewer, G. & Boland, R. 2009. Bacterial leaf scorch, a new blueberry disease caused by *Xylella fastidiosa*. *HortScience*, 44(2): 413–417.
- [618] Chang C.J., Garnier M., Zreik L., Rossetti V. & Bove J.M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology*, 27: 137–142.
- [619] Chatterjee, S., Almeida, R.P.P. & Lindow, S. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. *Annual Review of Phytopathology*, 46: 243–271.
- [620] Chen, J., Groves, R., Civerolo, E.L., Viveros, M., Freeman, M. & Zheng, Y. 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. *Phytopathology*, 95: 708–714.
- [621] Coletta-Filho, H.D., Francisco, C.S., Lopes, J.R.S., De Oliveira, A.F. & Da Silva, L.F.O. 2016. First report of olive leaf scorch in Brazil, associated with *Xylella fastidiosa* subsp. pauca. *Phytopathologia Mediterranea*, 55: 130–135.
- [622] Cornara, D., Sicard, A., Zeilinger, A.R., Porcelli, F., Purcell, A.H. & Almeida, R.P.P. 2016. Transmission of *Xylella fastidiosa* to grapevine by the meadow spittlebug. *Phytopathology*, 106, 1285–1290.
- [623] Davis, M.J., French, W.J. & Schaad, N.W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Current Microbiology*, 6: 309–314.
- [624] Davis, M.J., Purcell, A.H. & Thompson, S.V. 1978. Pierce's disease of grapevines: Isolation of the causal bacterium. *Science*, 199: 75–77.
- [625] Davis, M.J., Purcell, A.H. & Thomson, S.V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology*, 70: 425–429.
- [626] De Lima, J.E.O., Miranda, V.S., Hartung, J.S., Brlansky, R.H., Coutinho, A., Roberto, S.R. & Carlos, E.F. 1998. Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison with *Xylella fastidiosa* of citrus. *Plant Disease*, 82: 94–97.
- [627] Djelouah, K., Frasher, D., Valentini, F., D'Ongnia, A.M. & Digiaro, M. 2014. Direct tissue blot immunoassay for detection of *Xylella fastidiosa* in olive trees. *Phytopathologia Mediterranea*, 53: 559–564.
- [628] Donadio, L.C. & Moreira, C.S. 1998. *Citrus variegated chlorosis*. Bebedoure, Brazil: FUNDECITRUS/FAPESP, 166 pages.
- [629] EPPO (European and Mediterranean Plant Protection Organization). 2010. ELISA tests for plant pathogenic bacteria. PM 7/101(1). *EPPO Bulletin*, 40: 369–372.
- [630] EPPO (European and Mediterranean Plant Protection Organization). 2015. First report of *Xylella fastidiosa* in France. *EPPO Reporting Service*, 8: 2015/144. Available at <http://archives.eppo.int/EPPOReporting/2015/Rse-1508.pdf> (last accessed 7 October 2016)
- [631] EPPO (European and Mediterranean Plant Protection Organization). 2016. *Xylella fastidiosa*. PM 7/24(2). *EPPO Bulletin*, 46: 463–500. Available at <http://onlinelibrary.wiley.com/doi/10.1111/epp.12327/epdf> (last accessed 7 October 2016)

- [632] **Firrao, G. & Bazzi, C.** 1994. Specific identification of *Xylella fastidiosa* using the polymerase chain reaction. *Phytopathologia Mediterranea*, 33: 90–92.
- [633] **Francis, M., Civerolo, E.L. & Bruening, G.** 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* cultivar SR1. *Plant Disease*, 92(1): 14–20.
- [634] **Francis, M., Lin, H., Cabrera-La Rosa, J., Doddapaneni, H. & Civerolo, E.L.** 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. *European Journal of Plant Pathology*, 115: 203–213.
- [635] **Galvez, L.C., Korus, K., Fernandez, J., Behn, J.L. & Banjara, N.** 2010. The threat of Pierce's disease to midwest wine and table grapes. *APSnet Features*. doi:10.1094/APSnetFeature-2010-1015.
- [636] **Gould, A.B. & Lashomb, J.H.** 2007. Bacterial leaf scorch (BLS) of shade trees. *The Plant Health Instructor*. doi:10.1094/PHI-I-2007-0403-07.
- [637] **Guan, W., Shao, J., Elbeaino, T., Davis, R.E., Zhao, T. & Huang, Q.** 2015. Specific detection and identification of American mulberry-infecting and Italian olive-associated strains of *Xylella fastidiosa* by polymerase chain reaction. *PLoS ONE*, 10(6): e0129330. doi:10.1371/journal.pone.0129330.
- [638] **Guan, W., Shao J., Singh, R., Davis, R.E., Zhao, T. & Huang, Q.** 2013. A TaqMan-based real time PCR assay for specific detection and quantification of *Xylella fastidiosa* strains causing bacterial leaf scorch in oleander. *Journal of Microbiological Methods*, 92: 108–112.
- [639] **Haelterman, R.M., Tolocka, P.A., Roca, M.E., Guzmán, F.A., Fernández, F.D., & Otero, M.L.** 2015. First presumptive diagnosis of *Xylella fastidiosa* causing olive scorch in Argentina. *Journal of Plant Pathology*, 97: 393.
- [640] **Harper, S.J., Ward, L.I. & Clover, G.R.G.** 2010, erratum 2013. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology*, 100: 1282–1288.
- [641] **Hartung, J.S., Beretta, J., Brlansky, R.H., Spisso, J. & Lee, R.F.** 1994. Citrus variegated chlorosis bacterium: Axenic culture, pathogenicity and serological relationships with other strains of *Xylella fastidiosa*. *Phytopathology*, 84, 591–597.
- [642] **He, C.X., Li, W.B., Ayres, A.J., Hartung, J.S., Miranda, V.S. & Teixeira, D.C.** 2000. Distribution of *Xylella fastidiosa* in citrus rootstocks and transmission of citrus variegated chlorosis between sweet orange plants through natural root grafts. *Plant Disease*, 84: 622–626.
- [643] **Hernandez-Martinez, R., Costa, H.S., Dumenyo, C.K. & Cooksey, D.A.** 2006. Differentiation of strains of *Xylella fastidiosa* infecting grape, almonds, and oleander using a multiprimer PCR assay. *Plant Disease*, 90: 1382–1388.
- [644] **Hill, B.L., & Purcell, A.H.** 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology*, 85: 209–212.
- [645] **Holland, R.M., Christiano, R.S.C., Gamliel-Atinsky, E. & Scherm, H.** 2014. Distribution of *Xylella fastidiosa* in blueberry stem and root sections in relation to disease severity in the field. *Plant Disease*, 98: 443–447.
- [646] **Hopkins, D.L.** 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. *Phytopathology*, 71: 415–418.
- [647] **Hopkins, D.L.** 2001. *Xylella fastidiosa*. In N.W. Schaad, J.B. Jones & W. Chun, eds. *Laboratory guide for identification of plant pathogenic bacteria*, 3rd edn, pp. 201–213. St. Paul, MN, APS Press.

- [648]Hopkins, D.L. & Purcell, A.H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease*, 86: 1056–1066.
- [649]Huang, Q. 2009. Specific detection and identification of *Xylella fastidiosa* strains causing oleander leaf scorch using polymerase chain reaction. *Current Microbiology*, 58(4): 393–398.
- [650]Huang, Q., Bentz, J., & Sherald, J.L. 2006. Fast, easy and efficient DNA extraction and one-step polymerase chain reaction for the detection of *Xylella fastidiosa* in potential insect vectors. *Journal of Plant Pathology*, 88: 77–81.
- [651]Jacques, M.A., Denancé, N., Legendre, B., Morel, E., Briand, M., Mississippi, S., Durand, K., Olivier, V., Portier, P., Poliakoff, F. & Crouzillat, D. 2016. New coffee plant-infecting *Xylella fastidiosa* variants derived via homologous recombination. *Applied and Environmental Microbiology*, 82: 1556–1568.
- [652]Janse, J.D. & Obradovic, A. 2010. *Xylella fastidiosa*: Its biology, diagnosis, control and risks. *Journal of Plant Pathology*, 92: 35–48.
- [653]Lee, R.F., Beretta, M.J.G., Derrick, K.S. & Hooker, M.E. 1992. Development of a serological assay for citrus variegated chlorosis – a new disease of citrus in Brazil. *Proceedings Florida State Horticultural Society*, 105: 32–35.
- [654]Leu, L.S. & Su, C.C. 1993. Isolation, cultivation, and pathogenicity of *Xylella fastidiosa*, the causal bacterium of pear leaf scorch in Taiwan. *Plant Disease*, 77: 642–646.
- [655]Li, W.B., Pria, W.D., Lacava, P.M., Qin, X. & Hartung, J.S. 2003. Presence of *Xylella fastidiosa* in sweet orange fruit and seeds and its transmission to seedlings. *Phytopathology*, 93: 953–958.
- [656]Li, W.B., Hartung, J. S., & Levy, L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. *Journal of microbiological methods*, 66(1), 104-115.
- [657]Li, W.B., Teixeira, D.C., Hartung, J.S., Huang, Q., Duan, Y., Zhou, L., Chen, J., Lin, H., Lopes, S., Ayres, A.J. & Levy, L. 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.
- [658]Loconsole, G., Potere, O., Boscia, D., Altamura, G., Djelouah, K., Elbeaino, T., Frasheri, D., Lorusso, D., Palmisano, F., Pollastro, P., Silletti, M.R., Trisciuzzi, N., Valentini, F., Savino, V. & Saponari, M. 2014. Detection of *Xylella fastidiosa* in olive trees by molecular and serological methods. *Journal of Plant Pathology*, 96: 7–14.
- [659]Loconsole, G., Saponari, M., Boscia, D., D'Attoma, G., Morelli, M., Martelli, G.P. & Almeida, R.P.P. 2016. Intercepted isolates of *Xylella fastidiosa* in Europe reveal novel genetic diversity. *European Journal of Plant Pathology*, 146: 85–94.
- [660]Mafra V., Kubo K.S., Alves-Ferreira M., Ribeiro-Alves M., Stuart R.M., Boava L.P., Rodrigues, C.M. & Machado, M.A. 2012. Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PLoS ONE* 7: e31263. doi:10.1371/journal.pone.0031263.
- [661]Marceletti, S. & Scortichini, M. 2016. Genome-wide comparison and taxonomic relatedness of multiple *Xylella fastidiosa* strains reveal the occurrence of three subspecies and a new *Xylella* species. *Archives of Microbiology*, 198: 803–812.
- [662]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.

- [663]Mircetich, S.M., Lowe, S.K., Moller, W.J. & Nyland, G. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. *Phytopathology*, 66: 17–24.
- [664]Mizell, R.F., Andersen, P.C., Tipping, C. & Brodbeck, B.V. 2015. *Xylella fastidiosa* diseases and their leafhopper vectors. University of Florida Institute of Food and Agricultural Services (UF/IFAS) Extension Series, ENY-683. Available at <http://edis.ifas.ufl.edu/in174> (last accessed 7 October 2016).
- [665]Monteiro, P.B., Renaudin, J., Jagoueix-Eveillard, S., Ayres, A.J., Garnier, M. & Bové, J.M. 2001. *Catharanthus roseus*, an experimental host plant for the citrus strain of *Xylella fastidiosa*. *Plant Disease*, 85(3): 246–251.
- [666]Montero-Astúa, M., Chacón-Díaz, C., Aguilar, E., Rodríguez, C.M., Garita, L., Villalobos, W., Moreira, L., Hartung, J.S. & Rivera, C. 2008. Isolation and molecular characterization of *Xylella fastidiosa* from coffee plants in Costa Rica. *Journal of Microbiology*, 46: 482–490.
- [667]Nigro, F., Boscia, D., Antelmi, I. & Ippolito, A. 2013. Fungal species associated with a severe decline of olive in southern Italy. *Journal of Plant Pathology*, 95: 668.
- [668]Nunney, L., Schuenzel, E.L., Scally, M., Bromley, R.E. & Stouthamer, R. 2014. Large-scale intersubspecific recombination in the plant-pathogenic bacterium *Xylella fastidiosa* is associated with the host shift to mulberry. *Applied and Environmental Microbiology*, 80: 3025–3033.
- [669]Nunney, L., Yuan, X., Bromley, R., Hartung, J., Montero-Astúa, M., Moreira, L., Ortiz, B. and Stouthamer, R., (2010). Population genomic analysis of a bacterial plant pathogen: novel insight into the origin of Pierce's disease of grapevine in the US. *PLoS One*, 5(11), p.e15488.
- [670]Ouyang, P., Arif, M., Fletcher, J., Melcher, U. & Ochoa Corona, F.M. 2013. Enhanced reliability and accuracy for field deployable bioforensic detection and discrimination of *Xylella fastidiosa* subsp. *pauca*, causal agent of citrus variegated chlorosis using Razor Ex technology and TaqMan quantitative PCR. *PLoS ONE*, 8(11): e81647. doi:10.1371/journal.pone.0081647.
- [671]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.
- [672]Purcell, A.H., Porcelli, F., Cornara, D., Bosco, D. & Picciau, L. 2014. *Characteristics and identification of xylem-sap feeders*. Workshop manual. 27 pp. Available at http://cartografia.sit.puglia.it/doc/Workshop_Manual_Insects.pdf (last accessed 10 October 2016)
- [673]Randall, J.J., Goldberg, N.P., Kemp, J.D., Radionenko, M., French, J.M., Olsen, M.W. & Hanson, S.F. 2009. Genetic analysis of a novel *Xylella fastidiosa* subspecies found in the southwestern United States. *Applied and Environmental Microbiology*, 75: 5631–5638.
- [674]Redak, R.A., Purcell, A.H., Lopes, J.R.S., Blua, M.J., Mizell, R.F. 3rd & Andersen, P.C. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annual Review of Entomology*, 49: 243–270.
- [675]Rodrigues, J.L.M., Silva-Stenico, M.E., Gomes, J.E., Lopes, J.R.S. & Tsai, S.M. 2003. Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and *gyrB* sequences. *Applied and Environmental Microbiology*, 69: 4249–4255.
- [676]Rossetti, V., Garnier, M., Bové, J.M., Beretta, M.J.G., Teixeira, A.R.R., Quaggio, J.A. & Negri, J.D. 1990. Occurrence of xylem-restricted bacteria in sweet orange trees affected by chlorotic variegation, a new citrus disease in Brazil. *Comptes Rendus de l'Academie des Sciences Series 3*, 310: 345–349.
- [677]Saponari, M., Boscia, D., Nigro, F. & Martelli, G.P. 2013. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (southern Italy). *Journal of Plant Pathology*, 95: 668.

- [678] **Scally, M., Schuenzel, E.L., Stouthamer, R. & Nunney, L.** 2005. Multilocus sequence type system for the plant pathogen *Xylella fastidiosa* and relative contributions of recombination and point mutation to clonal diversity. *Applied and Environmental Microbiology*, 71: 8491–8499.
- [679] **Schaad, N.W., Opgenorth, D., & Gaush, P.** 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. *Phytopathology*, 92: 721–728.
- [680] **Schaad, N.W., Postnikova, E., Lacy, G., Fatmi, M.B. & Chang, C.-J.** 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp. *piercei*, subsp. nov., *X. fastidiosa* subsp. *multiplex* subsp. nov., and *X. fastidiosa* subsp. *pauca* subsp. nov. *Systematic and Applied Microbiology*, 27: 290–300.
- [681] **Schuenzel, E.L., Scally, M., Stouthamer, R. & Nunney, L.** 2005. A multigene phylogenetic study of clonal diversity and divergence in North American strains of the plant pathogen *Xylella fastidiosa*. *Applied and Environmental Microbiology*, 71: 3832–3839.
- [682] **Sherald J.L. & Lei J.D.** 1991. Evaluation of a rapid ELISA test kit for detection of *Xylella fastidiosa* in landscaping trees. *Plant Disease*, 75: 200–203.
- [683] **Simpson, A.J.G. et al.** 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature*, 406: 151–157.
- [684] **Su, C.-C., Chang, J.C., Chang, C.-M., Shih H.-T., Tzeng K.-C., Jan F.-J., Kao C.-W. & Deng W.-L.** 2013. Pierce's disease of grapevines in Taiwan: Isolation, cultivation and pathogenicity of *Xylella fastidiosa*. *Journal of Phytopathology*, 161:389–396.
- [685] **Su, C.-C., Deng, W.-L., Jan, F.-J., Chang, C.-J., Huang, H., Shih, H.-T. & Chen, J.** 2016. *Xylella taiwanensis* sp. nov. cause of pear leaf scorch disease in Taiwan. *International Journal of Systematic and Evolutionary Microbiology*, 66: 4766–4771.
- [686] **Valentini, F. & Porcelli, F.** 2016. Sampling procedures. Presentation to the FAO-IPPC-CIHEAM International Workshop on *Xylella fastidiosa* & the Olive Quick Decline Syndrome, 19–22 April, 2016, Bari, Italy.
- [687] **Van Sluys, M.A. et al.** 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *Journal of Bacteriology*, 185: 1018–1026.
- [688] **Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J.** 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173: 697–703.
- [689] **Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N. & Stead, D.E.** 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology*, 66: 2853–2858.
- [690] **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.
- [691] **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.
- [692] **Yaseen, T., Drago, S., Valentini, F., Elbeaino, T., Stampone, G., Digiario, M. & D'Onghia, A.M.** 2015. On-site detection of *Xylella fastidiosa* in host plants and in “spy insects” using the real-time loop-mediated isothermal amplification method. *Phytopathologia Mediterranea*, 54: 488–496.

[693] Yuan, X., Morano, L., Bromley, R., Spring-Pearson, S., Stouthamer, R. & Nunney, L. 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. *Phytopathology*, 100: 601–611.