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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/24 (2)

Diagnostics
Diagnostic

PM 7/24 (2) *Xylella fastidiosa*

Specific scope

This Standard describes a diagnostic protocol for *Xylella fastidiosa*.[1]

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

Specific approval and amendment

First approved in 2004-09.
Revised in 2016-09.[2]

1. Introduction

Xylella fastidiosa causes many important plant diseases such as Pierce's disease of grapevine, phony peach disease, plum leaf scald and citrus variegated chlorosis disease as well as leaf scorch on almond and on shade trees in urban landscapes, e.g. *Ulmus* sp. (elm), *Quercus* sp. (oak), *Platanus sycamore* (American sycamore), *Morus* sp. (mulberry) and *Acer* sp. (maple). Based on current knowledge, *X. fastidiosa* occurs primarily on the American continent (Almeida & Nunney, 2015). A distant relative found in Taiwan on Nashi pears (Leu & Su, 1993) is likely to be a new species (see below). However, *X. fastidiosa* was also genuinely diagnosed on grapevine in Taiwan (Su *et al.*, 2014). The presence of *X. fastidiosa* on almond and grapevine in Iran (Amanifar *et al.*, 2014) was reported (based on isolation and pathogenicity tests, but so far strain(s) are not available). The reports from Turkey (Guldur *et al.*, 2005; EPPO, 2014), Lebanon (Temsah *et al.*, 2015; Habib *et al.*, 2016) and Kosovo (Berisha *et al.*, 1998; EPPO, 1998) are unconfirmed and are considered invalid. Since 2012, different European countries have reported interception of infected coffee plants from Latin America (Mexico, Ecuador, Costa Rica and Honduras) (Legendre *et al.*, 2014**;** Bergsma-Vlami *et al.*, 2015; Jacques *et al.*, 2016). The outbreak of *X. fastidiosa* in olive trees in Southern Italy (Saponari *et al.*, 2013; Martelli *et al.*, 2016) and the common presence of the bacterium in Mediterranean plant species, e.g. in *Nerium oleander* and *Polygala myrtifolia*, in the natural and urban landscape of Southern Italy, Corsica and along the Mediterranean coast in France (EPPO, 2015) constitutes an important change to its geographical distribution and also adds new host plants. As of 2016-02-09, the list of susceptible hosts includes 359 species from 75 botanical families (EFSA, 2016). The list of hosts in Europe is regularly updated with the results of surveys (EU, 2016).

Xylella fastidiosa is a member of the family *Xanthomonadaceae* of the Gammaproteobacteria. The genus *Xylella* contains only one species, *X. fastidiosa*, although the *Xylella* strain causing leaf scorch in Taiwanese pears may well be a second species in the genus (Su *et al.*, 2012; Marceletti & Scortichini, 2016). However, although the name *Xylella taiwanensis* has been proposed (Chen *et al.*, 2014) it has not been approved or published in the validation list of official names so far. There are three formally accepted subspecies of *X. fastidiosa*, i.e. subsp. *fastidiosa*, *pauca* and *multiplex* (Schaad *et al.*, 2004) based on DNA–DNA hybridization data, although only two, *fastidiosa* and *multiplex*, are so far considered valid names by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) (Bull *et al.*, 2012). Since that publiaction, several additional *X. fastidiosa* subspecies have been proposed based on multilocus sequence typing (MLST) analysis (Scally *et al.*, 2005; Yuan *et al.*, 2010), including subsp. *sandyi* (on *N. oleander*; Schuenzel *et al.*, 2005), subsp. *tashke* (on *Chitalpa tashkentensis*; Randall *et al.*, 2009) and subsp.

[1]The use of names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable.

[2]As more experience with the diagnosis of *Xylella fastidiosa* will be gathered in the coming months, the EPPO Secretariat intends to schedule a review of the Protocol at the next Panel on Diagnostics in Bacteriology in 2017-06.

morus (on mulberry; Nunney *et al.*, 2014). Recently, a revision of the *X. fastidiosa* subspecies has been proposed (Marceletti & Scortichini, 2016) based on genomic comparative analysis.

The bacterium colonizes two distinct habitats, i.e. the xylem network of plants and the foregut of insects belonging to the order Hemiptera, sub-order Auchenorrhyncha (Redak *et al.*, 2004), that feed on xylem fluid (Chatterjee *et al.*, 2008). Transmission of *X. fastidiosa* by insects does not require an incubation period in the vector and the bacteria are persistently transmitted (Almeida *et al.*, 2005). Both nymphs and adults can acquire the bacteria by feeding on the xylem fluid of an infected plant and transmit the pathogen to healthy plants immediately after acquisition. *Xylella* cells are typically organized as single layer biofilm in the foregut, cibarium and precibarium (Newman *et al.*, 2003; Backus & Morgan, 2011) and do not systemically colonize the insect body. Nymphs lose infectivity with every stage as the foregut is renewed with moulting. Newly emerged adults must feed on an infected plant to become infectious. The bacterium is not transmitted transovarially to the progeny of the vector (Freitag, 1951). However, once infected they can transmit the pathogen during their entire lifetime (Almeida *et al.*, 2005). Winged adults are the major means for dissemination.

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

2. Identity

Name: *Xylella fastidiosa* Wells *et al.* (1987)

Taxonomic position: Bacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae.

EPPO code: XYLEFA

Phytosanitary categorization: EPPO A1 list no. 166; EU Annex designation I/A1 as *Xylella fastidiosa*, IIAI as citrus variegated chlorosis and IVAI as peach phony rickettsia.

3. Detection

As stated in the Introduction over 300 plant species are host to *Xylella fastidiosa*. However, the bacterium does not appear to cause disease in many of these plant species. Colonization is frequently asymptomatic in many hosts for a long time after inoculation and does not necessarily result in disease development. There are also significant differences in susceptibility between hosts.

3.1. Disease symptoms

Symptoms depend on hosts and *X. fastidiosa* strain combinations. As the bacterium invades xylem vessels it blocks the transport of mineral nutrients and water. Generally, symptoms include leaf scorching, wilting of the foliage, defoliation, chlorosis or bronzing along the leaf margin and dwarfing. Bacterial infections can be so severe as to lead to the death of the infected plants. The bronzing may intensify before browning and drying (Janse & Obradovic, 2010). Symptoms usually appear on just a few branches but later spread to cover the entire plant. Depending on the plant species, the presence of yellow spots on leaves, chlorotic foliage often together with pronounced yellow discoloration between healthy and necrotic tissues, irregular lignification of bark, stunting, premature leaf drop, reduction of production and dimension of fruits, fruit distortion, crown dieback or a combination of symptoms may occur. Symptoms can be confused with those caused by other biotic or abiotic factors (other pathogens, environmental stresses, water deficiencies, salt, air pollutants, nutritional problems, sunburn etc.); illustrations of possible confusions can be seen at:

http://agriculture.gouv.fr/sites/minagri/files/xylella_fastidiosa_symptomes_et_risques_de_confusions_biotiques_et_abiotiques_dgal-1.pdf

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

3.1.1. Alfalfa dwarf

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

3.1.2. Almond leaf scorch

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

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

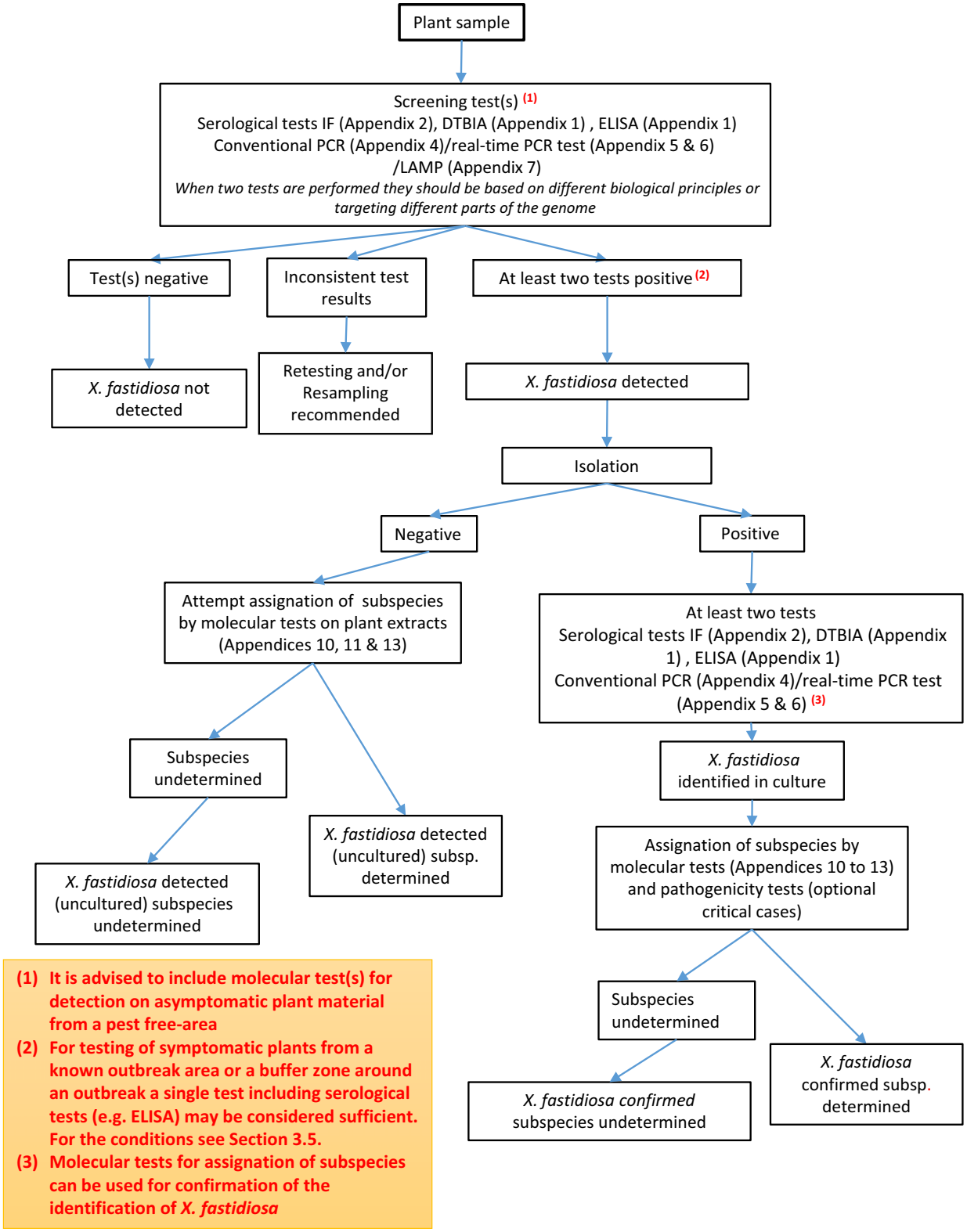

Fig 1 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* in plant material.

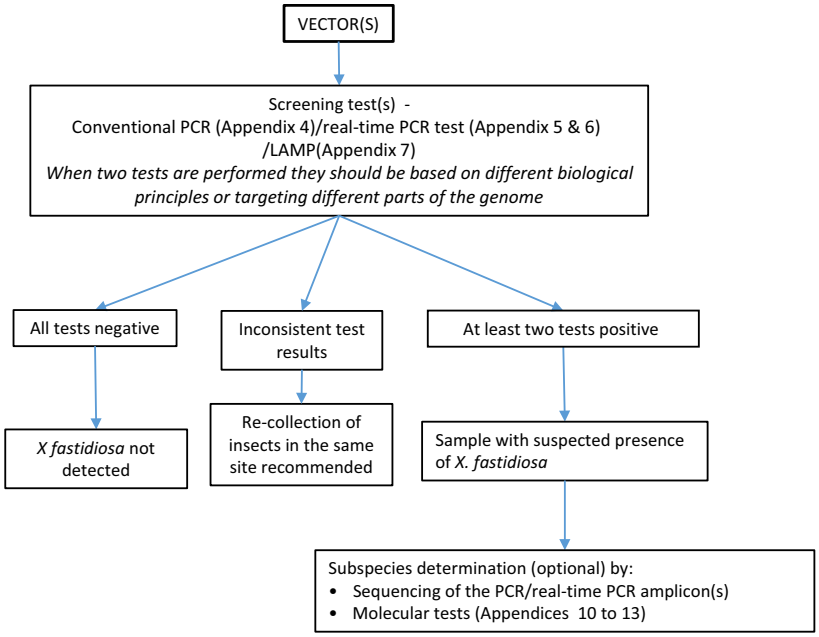

Fig 2 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* in vectors.

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

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

3.1.3. Bacterial leaf scorch of blueberry

The first symptom of bacterial leaf scorch of blueberry is a marginal leaf scorching (Fig. 4). The scorched leaf area may be bordered by a darker band (Brannen *et al.*, 2016). In the early stages of disease progression, symptoms may be localized, but over time symptoms can become uniformly distributed throughout the foliage. Newly developed shoots can be abnormally thin with a reduced number of flower buds. Leaf drop occurs and twigs and stems have a distinct 'skeletal' yellow appearance (Fig. 5). Following leaf drop the plant dies, typically during the second year after symptoms are observed (Chang *et al.*, 2009).

3.1.4. Bacterial leaf scorch of shade trees

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

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

may be killed by the disease. More information and pictures of symptoms are available in Gould & Lashomb (2007; available online).

3.1.5. Citrus variegated chlorosis

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

Affected trees show foliar interveinal chlorosis on the upper surface resembling zinc deficiency. Sectoring of symptoms in the canopy occurs on newly infected trees. However, citrus variegated chlorosis generally develops

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

Fig. 7 Small raised lesions appear on the underside of leaves. © USDA & University of Florida.

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

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

3.1.6. Coffee leaf scorch

Symptoms of coffee leaf scorch appear on new growth of field plants as large marginal and apical scorched areas on

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

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

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

3.1.7. Olive leaf scorching and quick decline

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

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

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

There are limited data on *X. fastidiosa* infecting olives, but evidence indicates that pathogen genotype defines pathogenicity. While *X. fastidiosa* is associated with but does not cause disease in olives in the USA (Krugner

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

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

et al., 2014), Koch's postulates have been fulfilled in Italy (Saponari *et al.*, 2016); pathogenicity data are not available from Brazil or Argentina. Nonetheless, a strong correlation between leaf scorching symptoms and presence of *X. fastidiosa* has been observed in three distant regions around the world (Southern Italy, Argentina and Brazil) (Coletta-Filho *et al.*, 2016).

3.1.8. Pierce's disease of grapes

On grapevine, the most characteristic symptom of primary infection is leaf scorch. An early sign of infection is a sudden drying of part of a green leaf, which then turns brown while adjacent tissues turn yellow or red (see Fig. 13). The leaf symptoms can be confused with fungal diseases, in particular with Rotbrenner, a fungal disease of grape vines caused by *Pseudopezicula tracheiphila* (Müll.-Thurg.) Korf & W.Y. Zhuang (1986)) (Fig. 14). The desiccation spreads over the whole leaf causing it to shrivel and drop, leaving only the petiole attached (Fig. 15). Diseased stems often mature irregularly, with patches of brown and green tissue. Chronically infected plants may have small, distorted leaves with interveinal chlorosis (Fig. 16) and shoots with shortened internodes. Fruit clusters shrivel. In later years, infected plants develop late and produce stunted chlorotic shoots. Symptoms involve a general loss of plant vigour followed by death of part of or the entire vine. Highly susceptible cultivars rarely survive for more than 2–3 years, although signs of recovery may be seen early in the second growing season. Young vines succumb more quickly than mature vines. More tolerant cultivars may survive chronic infection for more than 5 years.

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

Fig. 14 Symptoms caused by *Pseudopezicula tracheiphila*. Courtesy H. Reisenzein, AGES (AT).

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

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

3.1.9. Phony peach disease and plum leaf scald

On infected peach trees, young shoots are stunted and bear greener, denser foliage than healthy trees (Fig. 17). Lateral branches grow horizontally or droop, so that the tree seems uniform, compact and rounded. Leaves and flowers appear early, and remain on the tree longer than on healthy trees. Early in summer, because of shortened internodes, infected peach trees appear more compact, leafier and darker green

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

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

than normal trees. Affected trees yield increasingly fewer and smaller fruits until, after 3–5 years, they become economically worthless. Fruits may also be more strongly coloured and will often ripen a few days earlier than normal. Infected peach and plum trees bloom several days earlier than healthy trees and tend to hold their leaves later into the autumn. The leaves of infected peach never display the typical of leaf scorching seen on infected plum trees. Symptoms of plum leaf scald on leaves are a typical scorched and scalded appearance (Fig. 18). Plum leaf scald also increases the susceptibility of the tree to other problems. Phony peach disease and plum leaf scald can limit the life of peach and plum orchards (Mizell *et al.*, 2015).

3.1.10. Other hosts: leaf scorching symptoms seen in other hosts in Europe

For a general description of symptoms see Section 3.1 above. Besides olive, *X. fastidiosa* has been detected in different hosts under natural conditions in the current European outbreak areas. Most of these findings refer to symptomatic plants, which display typical leaf scorching symptoms. A list of hosts in which *X. fastidiosa* has been detected in Europe is available and regularly updated at: http://ec.europa.eu/food/plant/plant_health_biosecurity/legislation/emergency_measures/xylella-fastidiosa/susceptible_en.htm.

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

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

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

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

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

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

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

3.2. Sampling of plant material

3.2.1. Sampling period for symptomatic or asymptomatic plants

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

For outdoor plants in Europe this active growth period is usually from late spring to autumn.

Details based on specific observations during current outbreaks in Europe are presented below (EU, 2015).

(a) For *Polygala* spp., sampling can be performed from late spring to early autumn;
(b) For *O. europaea* and *N. oleander*, observations conducted in Italy (Apulia region) indicated that:
  - withering, desiccation and leaf scorching symptoms associated with *X. fastidiosa* infections are more strongly expressed in summer, although persistent during the entire year
  - in some cases, symptoms were also observed during winter at the start of the new vegetative growth.
(c) For deciduous plant species (e.g. *Prunus* spp.) in Italy (Apulia region) symptoms were consistently recorded, together with a detectable bacterium concentration, in leaves collected during summer. Asymptomatic leaves collected earlier in the vegetative period from the same trees tested negative.
(d) If necessary, dormant plants can be sampled by taking mature branches (e.g. woody cuttings), from which the xylem tissue is recovered and processed for detection of *X. fastidiosa*.

Experience in temperate areas in other parts of the world shows that in vines or deciduous trees, e.g. cherry and almond, that 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).

3.2.2. Sample collection

This section applies to sampling in places of production and in consignments. After taking samples they should be sent to the laboratory as soon as possible.

As *X. fastidiosa* is confined to the xylem tissue of its hosts, the petiole and midrib recovered from leaf samples are the best source for diagnosis as they contain a higher number of xylem vessels (Hopkins, 1981).

However, other sources of tissue include small twigs and roots of peach (Aldrich *et al.*, 1992), blueberry stem and roots (Holland *et al.*, 2014) and citrus fruit peduncles (Rossetti *et al.*, 1990).

Samples for the laboratory should be composed of branches/cuttings with attached leaves. The sample should include mature leaves. Young growing shoots should be avoided.

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

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

3.2.2.1. Symptomatic plants. The sample should consist of branches/cuttings representative of the symptoms seen on the plant(s) and containing at least 10 to 25 leaves depending on leaf size. Symptomatic plant material should

preferably be collected from a single plant; however, a pooled sample may also be collected from several plants showing similar symptoms.

3.2.2.2. Asymptomatic plants. For asymptomatic plants, the sample should be representative of the entire aerial part of the plant. Recent experimental data on the detection of *X. fastidiosa* in monumental and ancient olive trees showed that detection was more reliable when sampling the mid to upper part of the canopy. For testing individual asymptomatic plants, the number of branches to be collected is at least 4 to 10, depending on the host and plant size. There is limited experience of testing samples comprising leaves (including their petioles) collected from several asymptomatic plants. However, *X. fastidiosa* has been detected from samples of 100 to 200 leaves (including their petioles) collected from consignments of asymptomatic coffee plants (NRC, NL unpublished data)[1].

ISPM 31 (IPPC, 2008) provides useful information on the number of plants to be sampled.

3.3. Sampling of vectors

Field-collected insects can be analyzed to detect *X. fastidiosa* by polymerase chain reaction (PCR). The enzyme-linked immunosorbent assay (ELISA) test 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).

3.3.1. Sample collection

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. Identification keys with pictures are available online (Purcell *et al.* 2014).

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/acetone. Traps should be serviced on a weekly basis.

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

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

3.4. Sample preparation in the laboratory

3.4.1. Sample preparation for plant material

Samples should be processed as soon as possible after arrival.

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

For isolation, samples may be kept refrigerated for up to 3 days. For other, tests samples may be stored refrigerated for up to 1 week.

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

Dirty samples should be cleaned.

For isolation, samples should be surface disinfected (see Section 3.7).

3.4.1.1. Laboratory sample. From the sample received, indications on the minimum number of leaves (including their petioles) to be used and approximate weight of the laboratory sample are given in Table 1.

Tissue (preferably petioles and midribs or basal leaf portions) should be recovered from leaves and used directly for the preparation of the plant extract. The sample is processed according to the test to be used as described in this protocol.

3.4.2. Sample preparation for vectors

Since *X. fastidiosa* only colonizes the foregut and does not systemically spread into the body, only the head of the insect should be used for DNA extraction, thus avoiding the extraction of several contaminants that may inhibit the enzymatic reactions (Purcell *et al.*, 2014). Experience in Italy on *Philaenus spumarius* shows that up to 5 insects can be pooled to perform one test. Inhibitors may be present in the eyes and could affect PCR sensitivity Removing the eyes is recommended (B. Legendre pers. comm., 2016).

Before DNA extraction, it is imperative to remove the solvent (ethanol/acetone). To achieve this, the insects can be transferred for a few minutes to a dry filter paper and may be further dried in a SpeedVac centrifuge, to facilitate evaporation of the solvent. Total DNA can be extracted from single (or pooled) insect heads following different procedures (Appendix 3).

3.5. Screening tests

Unlike other EPPO protocols for bacteria, isolation is not recommended as a screening test because the bacterium is very difficult to isolate (see Figs 1 and 2). Samples should be considered as 'samples with *X. fastidiosa* detected' when at least two screening tests are positive based on different biological principles or targeting of different parts of the genome**.** Subspecies determination by molecular tests included in Section 4.2 and/or sequencing analysis should then be performed. Isolation should also be attempted. For

[1]The Panel on Diagnostics in Bacteriology is aware that this sampling recommendation is under revision.

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

Type of sample	Host plants/type of tissue	Minimum number of leaves per laboratory sample	Approximate weight of the laboratory sample
Samples from individual plants with leaves	Petioles and/or midribs or leaves of large size such as *Coffea* sp., *Ficus* sp., *Vitis* sp., *Nerium oleander*	5	0.5–1 g
	Petioles and/or midribs of leaves of small size such as *Polygala myrtifolia* and *Olea* sp.	25	0.5–1 g
	Plant species without petioles or with small petiole and midrib	25	0.5–1 g
Dormant plants or cuttings	Xylem tissue	N.A.	0.5–1 g
Composite sample from several coffee plants from a single lot with leaves (NRC, NL, procedure)	Samples of asymptomatic plants collected from, e.g., imported consignments or nursery monitoring	100–200	10–50 g

areas where the pest is known to be present or in buffer zones (see below) one positive test is sufficient to consider a sample as 'sample with suspected presence of *X. fastidiosa*'. In case of conflicting results between two tests, retesting and/or resampling is recommended.

- Symptomatic plant material
  Serological and molecular tests are both suitable for screening of symptomatic plant material.
- Asymptomatic plant material
  Testing asymptomatic plants in a pest-free area
  There is limited experimental data available on testing asymptomatic plant material. Consequently, the recommendations given in this Protocol are derived from data on testing symptomatic material and test performance studies. In most situations, the concentration of *X. fastidiosa* in asymptomatic plant material is likely to be lower than in symptomatic plant material (Purcell & Saunders, 1999; Almeida & Nunney, 2015). Consequently, it is advised to include molecular test(s) for detection on asymptomatic plant material.
  Testing asymptomatic plants in other areas
  Testing for asymptomatic plants in an outbreak area or a buffer zone around an outbreak often implies that a large number of tests need to be performed. In such a situation, and given that the concentration of the bacterium is expected to be higher than in an area thought to be pest free, a single test including serological tests (e.g. ELISA) may be performed. In serological tests in Italy in the outbreak area and the buffer zone around the outbreak area, 5% of the negative samples are also tested using a molecular test.

3.5.1. Serological tests

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

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

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

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

3.5.2. Molecular tests

Several molecular tests have been developed for *X. fastidiosa*. Only those that are commonly used in the EPPO region are described in full. Molecular tests can be performed on plants and insects. Validation data is available for testing of plants. These tests have been used for detection in insects but validation data is not available.

Although several PCR tests have been developed that effectively detect *X. fastidiosa* DNA in purified DNA extract, a recurrent problem with some matrices is the presence of inhibitors. These effects may be overcome by adequate DNA extraction protocols and dilutions of the extract.

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

The tests listed in this section allow the detection of *X. fastidiosa* regardless of the subspecies (tests specific for subspecies are presented in Section 4).

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

3.5.2.2. Real-time PCR. Three real-time PCR tests are recommended and have been validated:

- two tests based on Francis *et al.* (2006) are described in Appendix 5
- a test based on Harper *et al.* (2010) (and erratum 2013) is described in Appendix 6

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

A test based on primers developed by Harper et al. (2010, erratum 2013) using a commercial kit (Yaseen et al., 2015) is described in Appendix 7.

3.6. Additional tests

The bacterium can be detected in vessels in cross-sections of petioles by electron microscopy (Cariddi *et al.*, 2014).

3.7. Isolation

Xylella fastidiosa is very difficult to isolate and grow in axenic culture, even from symptomatic plants. The bacterium does not grow on most common culture media, and requires specific media. PD2 (Davis *et al.* 1980), BCYE (Wells *et al.*, 1981) or PWG (modified after Hill & Purcell, 1995) are widely used for the isolation from different host species. Media are described in Appendix 8.

The use of at least two different media is recommended, in particular when isolation is attempted for new hosts or in the case of a first detection.

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

Procedures for isolation from plant material are presented in Appendix 9.

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

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

- Colony morphology

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

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

  On PD2 and BCYE they are opaque and whitish (Figs 23 and 24 respectively). On BCYE they contrast with the black (charcoal) medium (Fig. 25).

  On modified PWG colonies are shiny and translucent. They take the colour of the medium (light caramel) (Figs 26 and 27).
- Cell morphology

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

  The isolation is negative if no bacterial colonies with growth characteristics and morphology similar to

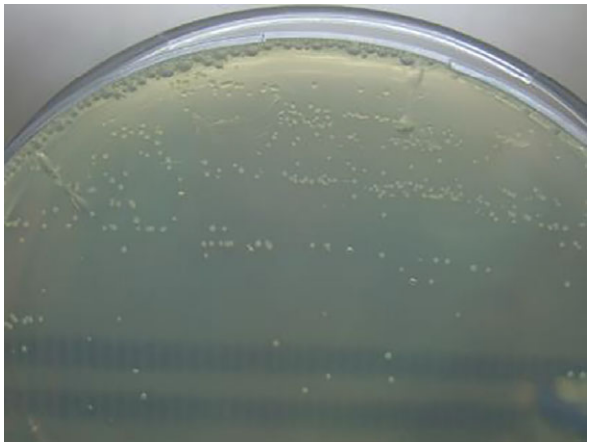

Fig. 23 Colonies of *Xylella fastidiosa* subsp. *fastidiosa* on PD2 (size < 2 mm after 3 weeks).

Fig. 24 Collection strain of *Xylella fastidiosa* subsp. *fastidiosa* ATCC 35879 on BCYE (size < 2 mm after 3 weeks).

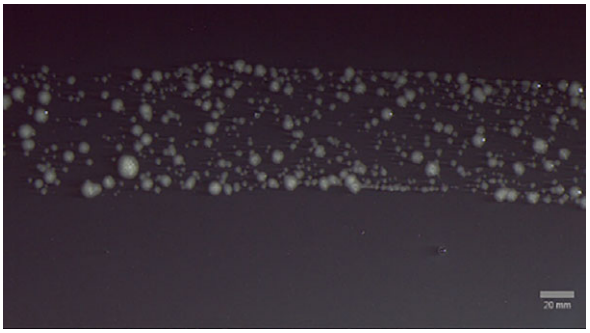

Fig 25 Colonies of *Xylella fastidiosa* subsp. *pauca* strain CoDiRO (ST53) on BCYE after 2 weeks. Courtesy M. Saponari, Institute for Sustainable Plant Protection (CNR). (Other pictures of colonies are available in the EPPO Global database.)

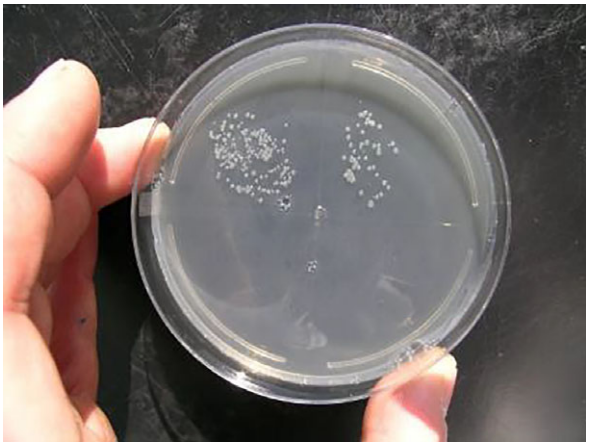

Fig. 26 *Xylella fastidiosa.* subsp. *fastidiosa* isolated from *Coffea canephora* on modified PWG (size < 2 mm after 3 weeks).

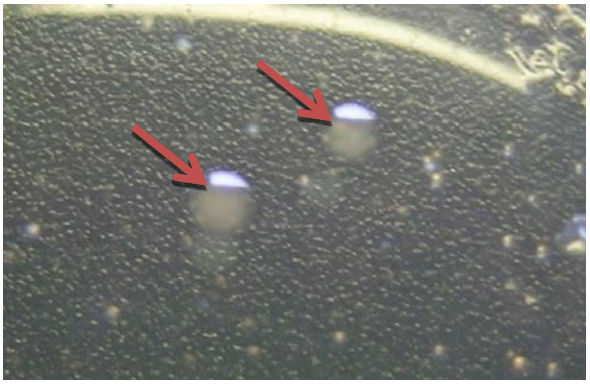

Fig 27 *Xylella fastidiosa* subsp. *pauca* isolated from *Coffea arabica* on modified PWG (size <2 mm after 3 weeks) (the background is a sheet of black paper below the plate).

X. fastidiosa are observed. Colonies are usually visible after 2–3 weeks but the plates should be observed for up 6 weeks.

The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed within the above-mentioned period on at least one medium. The reference culture should also have grown on the media used. The presumptive identification of *X. fastidiosa* colonies should be confirmed by serological or molecular tests (see Section 4).

4. Identification and subspecies determination

For this fastidious pathogen, subspecies determination on plant extracts is performed after positive screening test(s) using PCR-based molecular tests described in Appendices 10, 11, 12 and 13, respectively.

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

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

4.1.1. Serological tests

Serological tests can be used to identify a pure culture of *X. fastidiosa*; however, as no polyclonal antibodies are available the test cannot be used for the assignment of subspecies.

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

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

4.1.2. Molecular tests

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

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

4.1.2.2. Real-time PCR. Three real-time PCR tests are recommended and have been validated.

Two tests based on Francis *et al.* (2006) are described in Appendix 5.

The test based on Harper *et al.* (2010, erratum 2013) is described in Appendix 6.

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

Although different tests are available for subspecies assignment, MLST analysis is recommended for new findings. In other cases, subspecies assignment may be performed using subspecies-specific molecular markers (Pooler & Hartung,

1995; Hernandez-Martinez *et al.*, 2006); however, in the case of atypical/new patterns MLST should be performed.

The following tests are described:

- The MLST test based on Yuan *et al.* (2010) is described in Appendix 10 and allows an isolate to be assigned to the subspecies.
- The PCR test based on the primers described by Hernandez-Martinez *et al.* (2006) allows the subsp. *fastidiosa*, *multiplex* and *sandyi* to be assigned. This test can be performed either as a simplex test *in planta* or on isolates (Appendix 11) or a multiplex on isolates (Appendix 12).
- The PCR test based on Pooler & Hartung (1995) is described in Appendix 13. It allows assignment of an isolate to subsp. *pauca*.

4.3. Pathogenicity test

Verification of the pathogenicity of *X. fastidiosa* is sometimes difficult and can take several months. The pathogenicity test is described in Appendix 14.

4.4. Bioassay

The bioassay test from Francis *et al.* (2008) on *Nicotiana tabacum* (tobacco) is described in Appendix 15. Pathogenicity of strains can be evaluated with *N. tabacum*, but this has not been tested for all subspecies. Although virulence comparisons among isolates from different subspecies can be difficult due lack of efficient protocols for inoculation and the limited host ranges of isolates, citrus variegated chlorosis strains of *X. fastidiosa* are capable of colonizing and causing leaf scorch symptoms in *N. tabacum* (Lopes *et al.*, 2000; Alves *et al.*, 2003), and *X. fastidiosa* isolates from almond and grape showed differences in tobacco colonization and symptomatology (Francis *et al.*, 2008).

5. Reference material

Reference strains are available at:

CIRM-CFBP, Angers (FR)

BCCM/LMG Bacteria Collection, Ghent (BE)

NCPPB, Fera, York (GB)

Q-bank (http://www.q-bank.eu/) includes sequences of *Mut*S for properly documented species and strains present in collections.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis.*

7. Performance criteria

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

8. Further information

Further information on this organism can be obtained from:

Anses-LSV, Unit of bacteriology, virology and GMO, 7 rue Jean Dixméras, 49044 Angers Cedex 01 (FR). Contact: B. Legendre (bruno.legendre@anses.fr) or V. Olivier (valerie.olivier@anses.fr).

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

9. Feedback on this Diagnostic Protocol

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

10. Protocol revision

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

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BR). The Panel on Diagnostics in Bacteriology reviewed this revision in 2016-06.

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Appendix 1 – ELISA

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

Tissue sources for ELISA tests can be leaves (including petioles), twigs or canes.

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

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

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

1. Double antibody sandwich (DAS)-ELISA test

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

- The ELISA kits from Agritest and Loewe have been validated for olives, oleander, almond, citrus, oak, grape and other species (i.e. weeds) (Loconsole *et al.*, 2014).

Analytical sensitivity

In a test performance study performed at the Institute for Sustainable Plant Protection (Bari, IT) in 2015 the analytical sensitivity of the Agritest and Loewe kits, using dilutions ranging from 10^7 to 10 cfu mL^{-1}, prepared by spiking inactivated bacterial culture in olive, was around 10^4 cfu mL^{-1}.

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

Analytical specificity

Data from Loewe

No cross-reaction noted with:

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

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

Agritest: data provided by Plant Pathology Research Centre (CREA-PAV, Rome, IT)

Analytical specificity evaluated on 34 non-target bacterial strains.

No cross-reaction noted found with the following plant pathogens:

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

Diagnostic sensitivity
100% (in comparison with naturally infected samples).

Diagnostic specificity
100% (in comparison with naturally infected samples).

- *ELISA kit from Agdia*

Analytical sensitivity not available yet (evaluation in progress).

Analytical specificity: cross-reaction noted with:

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

No cross-reaction noted with:

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

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

2. Direct tissue blot immunoassay (DTBIA)

DTBIA for the detection of *X. fastidiosa* in olive plant material for large-scale screening of symptomatic trees (Djelouah *et al.*, 2014) has been developed. Fresh cross-sections of young twigs are printed onto nitrocellulose membranes and the membrane incubated with the specific antiserum prior to development. This method has the advantages of being easy to perform and cost-effective in terms of reagents and labour; the membranes can be printed directly in the field preventing movement of infected plant materials to other areas.

Performance criteria available
In a test performance study performed at the Institute for Sustainable Plant Protection (Bari, IT), DTBIA was evaluated for the identification of *X. fastidiosa* strain CoDiRO in naturally infected olives (12 samples; 4 laboratories)

The DTBIA results were scored as the number of imprints showing specific purple coloration within the spotted sections.

Two different kits (Agritest and Enbiotech) were compared, which consisted of different detecting antisera. In the case of the protocol provided by Agritest, the imprints were made by squeezing the cuttings prior to spotting the fresh cut sections on the membrane.

Following both procedures, the olive samples were correctly categorized as positive and negative in the four laboratories. However, reactions seen with the Agritest kit were consistently stronger and easy to assess and interpret, even without observation of the imprinted membrane under the microscope.

Appendix 2 – Immunofluorescence (IF) test

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009)

The IF test is usually performed on plant tissue that is mechanically homogenized in extraction buffer (e.g. 50 mM phosphate buffer) or demineralized water.

A commercial polyclonal antibody is available from Loewe.

Analytical specificity on pure cultures (data is provided by the supplier; Loewe) concentrations of up to 10^4 cfu mL^{-1} tested on pure cell cultures).

Inclusivity: 100%

Number of *X. fastidiosa* strains tested: 5 (*X. fastidiosa*, *X. fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *fastidiosa*; CoDiRO, Lecce, IT).

Exclusivity: 100%

Number of non-target strains: 9 (*Agrobacterium vitis*, *Clavibacter michiganensis* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus*, *Dickeya chrysanthemi*, *Pseudomonas syringae* pv. *syringae*, *Rhodococcus fascians*, *Xylophilus ampelinus*, *Xanthomonas vesicatoria*, *Xanthomonas campestris* pv. *campestris*).

No cross-reaction observed.

A preliminary test performance study on diagnostic sensitivity was performed during a workshop in Germany involving 13 laboratories using naturally infected coffee plant samples.

Diagnostic sensitivity 100% of agreement at 10^4 cells per mL.

Repeatability: 100%

Appendix 3 – DNA extraction

Extraction of DNA for molecular analyses can be achieved using standard commercial kits (e.g. Bextine & Child, 2007; Huang, 2009), and CTAB buffer (Hendson *et al.*, 2001; Rodrigues *et al.*, 2003; de Souza *et al.*,

2003). The following commercial kits are widely used and validated in several European Union (EU) laboratories to process samples from different plant species: DNeasy Plant Mini Kit-based extraction (Qiagen), Modified DNeasy® mericon™ Food Standard Protocol (Qiagen), QuickPick™ SML Plant DNA Kit-based extraction (Bio-Nobile). Validation data are available in the EPPO Database on Diagnostic Expertise.

1. DNA extraction for plant material

CTAB-based extraction

0.5–1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized) should be weighed, put into the extraction bags or into suitable tubes with 5 mL of CTAB buffer and homogenized using a homogenizer (e.g. Homex, Polytron, etc.).

1 mL of extract should be transferred into a 1.5-mL micro-centrifuge tube and the sample should be heated at 65°C for 30 min and then centrifuged at 16 000 *g* for 5 min. 1 mL of the supernatant from centrifugation should be transferred to a new 2-mL micro-centrifuge tube, with care being taken not to transfer any of the plant tissue debris. 1 mL of chloroform:isoamyl alcohol (24:1) should be added and the sample should be mixed well by shaking. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant should be transferred to a 1.5-mL micro-centrifuge tube and 490 µL (approximately 0.7 volumes) of cold 2-propanol should be added. After mixing by inverting twice, the tube should be incubated at −20°C for 20 min. Centrifugation of the samples at 16 000 *g* for 20 min will allow recovery of a pellet that should be washed with 1 mL of 70% ethanol. An additional centrifugation at 16 000 *g* for 10 min and decantation in 70% ethanol should be performed. Sample should be air or vacuum-dried. The pellet should be resuspended in 100–150 µL of TE buffer or RNase- and DNase-free water.

Commercial kits

- DNeasy Plant Mini Kit-based extraction (Qiagen)
  An aliquot of 200 mg of fresh small pieces of midribs and petioles is put into extraction bags with the addition of lysis buffer and homogenized using available equipment (Polytron, Homex, etc.). Lysis and purification are carried-out following the manufacturer's instructions.
- Modified DNeasy® Mericontm Food Standard Protocol (Qiagen)
  This kit, designed for the extraction of total DNA from a large-scale sample of raw or processed food material, has been successfully adapted to recover high-quality DNA from a wide range of plant species. For this purpose, plant samples should consist of 0.5–1 g of fresh small pieces of midribs, petioles, basal leaf part or twigs (1/4 of the indicated amount, if lyophilized). The recovered tissue should be transferred into the extraction bags or suitable tubes, 5 mL of Food Lysis Buffer added, and homogenized using a homogenizer (e.g. Homex, Polytron, etc.); 1 mL of sap should be transferred into a 1.5-mL micro-centrifuge tube and incubated for 30 min at 60°C. The sample is then processed following the manufacturer's instructions. The protocol can be performed manually or automated using a dedicated workstation.
- QuickPickTM SML Plant DNA Kit-based extraction (Bio-Nobile)
  0.5–1 g of fresh small pieces of midribs, petioles, basal leaf part or twigs is crushed in sterile water (5 mL g^{-1}), then left to soak for at least 15 min, under gentle shaking. 250 µL of the plant extract is centrifuged for 20 min at 20 000 *g*. The pellet is suspended in 75 µL of lysis buffer with 5 µL of proteinase K and the manufacturer's instructions followed. The extraction can be either manual or automated.

Extraction efficiency depends on the matrix and this is reported in the section on performance criteria of the rele vant tests.

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

2. DNA extraction for vectors

CTAB-based extraction

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

A single insect head or a pool of 5 heads, preferably with the eyes removed, should be homogenized in a 2-mL tube with 1–2 tungsten carbide beads (for a maximum of 15–20 s at a frequency of 24 cycles per s, in Mill300 Qiagen mixer/Tissue Lyser II Qiagen or similar equipment). 500 µL of CTAB buffer should be added and the tube should be mixed well by shaking or vortexing. The sample should be heated at 65°C for 30 min. 500 µL of chloroform:isoamyl alcohol 24:1 should be added and sample should be mixed again by shaking or vortexing. After centrifugation at 16 000 *g* for 10 min, 400 µL of the supernatant should be transferred to a 1.5-mL micro-centrifuge tube and 280 µL (approximately 0.7 volumes) of cold 2-propanol should be added. After mixing by inverting twice, the tube should be incubated at −20°C for 20 min. The centrifugation of the samples at 16 000 *g* for 20 min will allow a pellet to be recovered; this should then be washed with 1 mL of 70% ethanol. An additional centrifugation at 16 000 *g* for 10 min followed by decantation into 70% ethanol should be performed. The sample should be air or vacuum dried. The pellet should be resuspended in 30–80 µL of TE buffer or RNase- and DNase-free water,

depending on the amount of starting material (single or pooled insect heads).

Commercial kits

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

Validation of the QuickPick™ is included in the EPPO Database on Diagnostic Expertise.

For the LAMP test (Appendix 7), insects are used directly without DNA extraction.

Appendix 4 – Conventional PCR (Minsavage *et al.*, 1994)

1. General information

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

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

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

1.4 Amplicon size: 733 bp.

1.5 The forward primer RST31 sequence is 5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3′; the reverse primer RST33 sequence is 5′-CACCATTCGTATCCCGGTG-3′.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 Matrices: plants, insects or pure culture suspension.

2.1.2 See Appendix 3 for extraction procedures from plants and insects.

2.1.3 For pure cultures, a single colony of fresh pure culture is suspended in approximately 1 mL of molecular-grade water; lysis should be performed at 100°C for 5 min.

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

2.2. Conventional PCR

2.2.1. Master mix

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

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	18.6	N.A.
Taq DNA polymerase buffer (Invitrogen)	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (RST31)	20 µM	0.375	0.3 µM
Reverse primer (RST33)	20 µM	0.375	0.3 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.15	0.03 U μL^{-1}
Subtotal		23	
Genomic DNA from plant tissue extract or bacterial suspension		2	
Total		25	

*Molecular-grade water should preferably be used or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix, or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least by botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *X. fastidiosa*,

isolated from a suspension with approximately 10^7 cfu mL^{-1}.

3.2. Interpretation of results

Verification of the controls:
- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of the expected size.

When these conditions are met:
- A test will be considered positive if amplicons of 733 bp are produced
- A test will be considered negative if it produces no band or band(s) of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

From Anses (FR) using the DNeasy® Plant mini kit (Qiagen):

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

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

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

4.4 Data on reproducibility
Not available.

4.5 Diagnostic sensitivity data
Vitis vinifera: 81%
Prunus persicae: 81%
Citrus sinensis: 82%
Coffea arabica: 81%
Coffea canephora: 74%
Compared with spiked matrices with bacterial concentrations from 101 to 106 bacteria per mL: 21 samples per matrix, 63 DNA extractions per matrix, 126 amplifications per matrix. (On orange tree 18 samples per matrix, 54 DNA extractions per matrix, 108 amplifications per matrix.)

4.6 Diagnostic specificity data
Citrus sinensis: 100%
Coffea arabica: 100%
Coffea canephora: 100%

4.7 Other information
In 2014, a test performance study was performed on a new set of spiked samples.

Performance criteria
Analytical sensitivity (with a probability of detection of 100% on coffee and orange only):
Coffea spp: ≈10^4 bacteria per mL (100%: 4 labs/4)
Olea europaea: ≈10^6 bacteria per mL (3 labs/4)
Vitis vinifera: ≈10^6 bacteria per mL (2 labs/4)
Citrus sinensis: ≈10^2 bacteria per mL (100%: 4 labs/4)
Prunus persica: ≈10^4 bacteria per mL (3 labs/4)
Diagnostic sensitivity (based on results on spiked samples to the following concentrations):
Coffea spp: 70% (10^2–10^4 bacteria per mL)
Olea europaea: 30% (10^4–10^6 bacteria per mL)
Vitis vinifera: 40% (10^4–10^6 bacteria per mL)
Citrus sinensis: 80% (10^1–10^3 bacteria per mL)
Prunus persica: 60% (10^2–10^4 bacteria per mL)
Note: these results obtained by several laboratories are different from those obtained in the intra-laboratory evaluation, mainly on grapevine (variability linked to a matrix effect?)
TPS (test performance study) performed with extraction kit from Qiagen (DNeasy Plant Mini Kit)
Diagnostic specificity: 100%
Reproducibility: 84%
Repeatability: 95% (from 88–100% according to the 4 laboratories)
4 samples per matrix
2 extractions per sample
2 amplifications per DNA extract

Additional validation data

Performance criteria obtained by other laboratories with Minsavage *et al.* (1994) with slightly different master mixes are available and can be downloaded from the EPPO Database on Diagnostic Expertise (http://dc.eppo.int/validationlist.php).

A validation study with non-European *X. fastidiosa* strains (Harper *et al.*, 2010) showed that the Rst31/33 primer failed to detect the following American strains from grapes and oaks: *X. fastidiosa*, *Vitis vitifolia*, US (PD0001); *X. fastidiosa*, *V. vitifolia*, US (R. Almeida); *X. fastidiosa*, *V. rotundifolia*, US (C. Chang); *X. fastidiosa*, *Quercus laevis*, US (OAK0023); *X. fastidiosa*, *Quercus rubra*, US (OAK0024); *X. fastidiosa*, *Quercus rubra*, US (C. Chang).

Appendix 5 – Real-time PCR tests (based on Francis *et al.*, 2006)

(A) SYBR green version

1. General information

1.1 This PCR is suitable for the detection and identification of *Xylella fastidiosa*.
1.2 The test is based on Francis *et al.* (2006).
1.3 The target sequence is a conserved hypothetical protein HL gene.
1.4 Amplicon size: 221 bp.
1.5 Forward primer HL5 sequence: 5′-AAGGCAATAA-ACGCGCACTA-3′; reverse primer HL6 sequence: 5′-GGTTTTGCTGACTGGCAACA-3′.
1.6 The real-time PCR systems used to generate the validation data presented below were: Applied Biosystems® 7500 Fast, ThermoFisher Scientific or CFX 9600, Bio-Rad.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 Matrices: plant, insects or pure bacterial suspensions.
2.1.2 See Appendix 3 for extraction procedures from plants and insects.
2.1.3 For pure bacterial suspensions, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.
2.1.4 Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for later use.

2.2. Real-time real-time PCR

2.2.1. Master mix

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

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR conditions

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Alternative internal positive controls (IPCs) can include:

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

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

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

When these conditions are met:

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

4. Performance criteria available

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

DNA extraction: CTAB

4.1 Analytical sensitivity data

Olea europaea plant extracts spiked with ten fold dilution of *X. fastidiosa* subsp. *pauca* CoDiRo strain suspensions: ≈ 10 cfu mL^{-1}.

4.2 Analytical specificity data

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

Exclusivity: 100%. Evaluated on 34 non-target bacterial strains: 3 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *fragariae*, 1 *X. arboricola* pv. *corylina*, 1 *X. arboricola* pv. *celebensis*, 1 *Xanthomonas axonopodis* pv. *citri*, 1 *Xanthomonas campestris* pv. *campestris*, 1 *X. campestris* pv. *populi*, 2 *Xanthomonas hortorum* pv. *pelargonii*,3 *Pseudomonas savastanoi* pv. *savastanoi*, 1 *Pseudomonas marginalis*, 4 *Pseudomonas syringae* pv. *syringae*, 4 *Brenneria* (ssp. *rubrifaciens*, *quercina*, *salicis*, *populi)*, 2 *Pantoea stewartii*, 1 *Pantoea agglomerans*, 1 *Erwinia amylovora*, 3 *Agrobacterium tumefaciens*, *2 Rhizobium vitis.Xanthomonas arboricola* pv. *celebensis* and *Brenneria populi* gave an amplification curve corresponding to melt peak values of 87°C and 87.5°C, respectively. *Pantoea agglomerans*, *Brenneria quercina*, *Pseudomonas marginalis* and *Xanthomonas hortorum* pv. *pelargoni* gave an amplification curve with an inconsistent melting peak.

4.3 Data on repeatability

Olea europaea: 100%

4.4 Data on reproducibility

Olea europaea: extraction: 100%

4.5 Data on diagnostic sensitivity

Olea europaea: 100%

4.6 Data on accuracy

Olea europaea: 96%

(B) Taqman version

1. General information

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

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

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

1.4 Amplicon size: 221 bp.

1.5 Forward primer HL5 sequence: 5′-AAGGCAA-TAAACGCGCACTA-3′; reverse primer HL6 sequence: 5′-GGTTTTGCTGACTGGCAACA-3′; the probe sequence is: 5′/FAM/-TGGCAGGCAG-CAACGATACGGCT-/BHQ1/3′.

1.6 Validation data below has been generated using the real-time PCR system: ViiA™ 7 Real-Time PCR System, Thermo Fisher Scientific.

1.7 Automatic baseline and manual threshold of 0.1.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: plant insects or pure bacterial suspensions

2.1.2 For extraction procedures from plants see Appendix 3

2.1.3 For pure bacterial suspension, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR grade water; lysis should be performed at 100°C for 5 min.

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	1	N.A.
Real-time PCR buffer (TaqMan® Universal PCR Master Mix, Thermo Fisher Scientific, 2×)	2×	5	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Forward primer (*HL5*)	10 µM	0.9	0.9 µM
Reverse primer (*HL6*)	10 µM	0.9	0.9 µM
Probe 1 (probe)	10 µM	0.2	0.2 µM
Subtotal		8	
Bacterial suspension or DNA extract		2	
Total		10	

*Molecular-grade water should preferably be used or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions

Pre-incubation (UNG step) at 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 45 cycles of (95°C for 15 s and 60°C for 60 s).Heating and cooling ramp speed: standard temperature ramping mode, corresponding to ±1.6°C on 7900HT Fast Real-Time PCR System (Applied Biosystems) and ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific).

3. Essential procedural information

3.1. Controls

For positive controls, inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

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

Alternative internal positive controls (IPC) can include:

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

Other possible controls:

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

3.2. Interpretation of results

Verification of the controls:

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

When these conditions are met:

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

4. Performance criteria available

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

4.1 Analytical sensitivity data

Determined on *X. fastidiosa* DNA dilutions. The lowest concentration tested in which all replicates were positive was found to be 2.6, 3.2 and 3.5 [log (target copies of DNA per mL), determined with digital PCR using the same primers and probe as in real-time PCR, and corresponding to concentration of cells per mL], *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* and *X. fastidiosa* subsp. *pauca* CoDiRO strain, respectively. *Plant material (spiked)*: 94% determined in plant material prepared as for symptomatic testing (31/33) spiked

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

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

4.2 Analytical specificity data

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

100% inclusivity. Four *X. fastidiosa* tested: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* and *X. fastidiosa* subsp. *pauca* CoDiRO strain.

100% exclusivity: *Xylophilus ampelinus* and five unidentified bacteria isolated from olive plants; DNA extracted from healthy olive, oleander, rosemary and lavender.

4.3 Data on repeatability

No data available.

4.4 Data on reproducibility

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

Additional validation data

Validation studies with non-European *X. fastidiosa* strains (Harper *et al.*, 2010 and Li *et al.*, 2013) showed that the HL5/6 primer failed to detect some American strains from *Morus alba* (mulberry, US MUL), *Acer negundo* (box elder, US BE1), *Quercus rubra* (red oak, US OAK0024) and *Liquidambar styraciflua* (sweetgum, US) and one Brazilian strain from *Citrus sinensis* (sweet orange, Brazil 20-1381).

Appendix 6 – Real-time PCR (Harper *et al.*, 2010; erratum 2013)

1. General information

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

1.2 The test is based on Harper *et al.* (2010, erratum 2013).

1.3 The target sequence is located at the level of the gene coding for the 16S rRNA processing rimM protein. Forward primer XF-F sequence: 5′-CACGGCTGGTAACGGAAGA-3′; reverse primer XF-R sequence: 5′-GGGTTGCGTGGTGAAATCAAG-3′; probe XF-P sequence: 5′-6-FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1-3′.

1.4 Real-time PCR system used to generate the validation data below: Applied Biosystems® 7500 Fast, ThermoFisher Scientific (Anses) or CFX 96 Bio-Rad (Anses, ISPP & CREA).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 Matrices: plant, insects or pure cultures.

2.1.2 See Appendix 3 for extraction procedures from plants and insects.

2.1.3 For pure cultures, 2 µL of bacterial suspension should be used; lysis should be performed at 100°C for 5 min.

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

2.2. Real-time PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	6.48	N.A.
Taqman Fast Universal Master Mix (Applied Biosystems)	2 x	10	1x
Forward primer (*XF-F*)	10 µM	0.6	0.3 µM
Reverse primer (*XF-R*)	10 µM	0.6	0.3 µM
Probe 1 (*XF-P*)	10 µM	0.2	0.1 µM
BSA	50 µg µL^{-1}	0.12	0.3 µg µL^{-1}
Subtotal		18	
Bacterial suspension or DNA extract		2	
Total		20	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions

Pre-incubation at 50°C for 2 min followed by 95°C for 10 min, followed by 40 cycles of (94°C for 10 s and 62°C for 40 s). Heating ramp speed: 5°C s^{-1}.

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of sterile extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid

extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: the molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: add nucleic acid of the *X. fastidiosa* reference strain, e.g. to a PCR reaction.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

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

4. Performance criteria available

(A) Anses (Angers, FR)

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

A4.1 Analytical sensitivity data:
Vitis vinifera: machine-assisted extraction $\approx 10^3$ bacteria per mL; manual extraction $\approx 10^3$ bacteria per mL
Citrus spp: machine-assisted extraction $\approx 10^2$ bacteria per mL; manual extraction $\approx 10^2$ bacteria per mL
Olea europaea: machine-assisted extraction $\approx 10^5$ bacteria per mL; manual extraction $\approx 10^5$ bacteria per mL
The above concentrations gave a probability of detection of 100%.

A4.2 Analytical specificity data:
Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section Validation (http://dc.eppo.int/validationlist.phphttp://dc.eppo.int/validationlist.php).
Inclusivity 100% evaluated using 19 targets strains belonging to *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *sandyi*, *X. fastidiosa* subsp. *multiplex*.
Exclusivity 100% evaluated on 29 non-target strains : 16 *Xanthomonas* spp., 1 *Xylophilus ampelinus*, 1 '*Candidatus*' Liberibacter asiaticus, 1 'Candidatus' Liberibacter africanus, 6 saprophytic bacteria isolated from *Coffea* spp. and 4 saprophytic bacteria on *Citrus sinensis*. No cross-reactions were observed.

A4.3 Data on repeatability:
Vitis vinifera: machine-assisted extraction 96%; manual extraction 100%
Citrus spp.: machine-assisted extraction 100%; manual extraction 100%
Olea europaea: machine-assisted extraction 100%; manual extraction 88%

A4.4 Data on reproducibility
All matrices: machine-assisted extraction 98%; manual extraction 90%

A4.5 Data on diagnostic sensitivity
Vitis vinifera: machine-assisted extraction 94%; manual extraction 100%
Citrus spp.: machine-assisted extraction 100%; manual extraction 100%
Olea europaea: machine-assisted extraction 67%; manual extraction 50%

A4.6 Data on accuracy
Vitis vinifera: machine-assisted extraction 96%; manual extraction 100%
Citrus spp.: machine-assisted extraction 100%; manual extraction 100%
Olea europaea: machine-assisted extraction 75%; manual extraction 63%

(B) Institute for Sustainable Plant Protection (Bari, IT)

DNA extraction: DNeasy Mericon Food Kit (Qiagen)

B4.1. Analytical sensitivity data
Up to 10^2 cfu mL^{-1} (corresponding to 7 cfu per reaction) using dilutions ranging from 10^7 to 10 cfu mL^{-1}.

B4.2. Analytical specificity data
Not available

B4.3. Data on repeatability
100%

B4.4. Data on reproducibility
100%

B4.5. Diagnostic sensitivity data
100%: 108 samples gave a positive result out of 108 expected

B4.6. Diagnostic specificity data
100%: 90 samples gave a negative result out of 90 expected

(C) Plant Pathology Research Centre (CREA-PAV, Rome, IT) in collaboration with Institute for Sustainable Plant Protection (CNR-IPSP, Bari, IT)

DNA extraction: CTAB

C4.1. Analytical sensitivity data
Olive: ≈ 10^2 bacteria per mL
C4.2. Analytical specificity data
Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section Validation (http://dc.eppo.int/validation-list.php).
Exclusivity 100% evaluated on 34 non-target bacterial strains: 3 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *fragariae*, 1 *X. arboricola* pv. *corylina*, 1 *X. arboricola* pv. *celebensis*, 1 *Xanthomonas axonopodis* pv. *citri*, 1 *Xanthomonas campestris* pv. *campestris*, 1 *X. campestris* pv. *populi*, 2 *Xanthomonas hortorum* pv. *pelargonii*,3 *Pseudomonas savastanoi* pv. *savastanoi*, 1 *Pseudomonas marginalis*, 4 *Pseudomonas syringae* pv. *syringae*, 4 *Brenneria* (ssp. *rubrifaciens*, *quercina*, *salicis*, *populi*), 2 *Pantoea stewartii*, 1 *Pantoea agglomerans*, 1 *Erwinia amylovora*, 3 *Agrobacterium tumefaciens*, *2 Rhizobium vitis.*
C4.3. Data on diagnostic sensitivity
Olea europaea: 91%
C4.4. Data on diagnostic specificity
Olea europaea: 100%
C4.5. Data on repeatability
Olea europaea: 100%
C4.6. Data on reproducibility
Olea europaea: 90%
C4.7. Data on accuracy
Olea europaea: 93%

Appendix 7 – Real-time LAMP test

1. General information

1.1 This test is suitable for the detection of *Xylella fastidiosa* in host plants and insects.
1.2 The test is based on primers developed by Harper *et al.* (2010; erratum 2013) and was developed by Yaseen *et al.* (2015).
1.3 The target sequence is located at the 16S rRNA processing gene *rimM* of *X. fastidiosa*.
1.4 The following primers are used: external XF-F3 primer sequence 5′-CCGTTGGAAAACAGATGGGA-3′; external XF-B3 primer sequence 5′-GAGACTGGCAAGCGTTTGA-3′; internal XF-FIP primer sequence 5′-ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC-3′; internal XF-BIP primer sequence 5′-GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA-3′; loop XF-LF primer sequence 5′-TGCAAGTACACACCCTTGAAG-3′; loop XF-LB primer sequence 5′-TTCCGTACCACAGATCGCT-3′

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 Matrices: plants or insects (Yaseen *et al.*, 2015).
2.1.2 Plant tissues (thin slices of 1-year-old twigs, 1–2 mm thick) or single captured insects (adults not homogenized) are immersed in 1 mL of extraction buffer (1% Triton X-100, 20 mM Tris-HCl, 20 mM EDTA) and denatured at 95°C for 10 min.
2.1.3 Alternatively, use the CTAB-based total nucleic acid extraction procedure for plants or insects (see Appendix 3).
2.1.4 Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for later use.
2.2 LAMP
2.2.1 Ready to use kits are commercially available to perform the test on a specific device or using a standard real-time thermal cycler (e.g. Enbiotech, Qualiplante).
2.2.2 PCR conditions: follow the manufacturer's instructions.

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix, or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed using isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results

Verification of the controls:
- NIC and NAC should produce no fluorescence.
- PIC and PAC: for real-time measurement a positive reaction is defined by time of positivity (minutes) and/or TM (°C ± known variation) as given by the manufacturer.

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

4. Performance criteria available from Plant Pathology Research Institute (CRA, Rome, IT) and Institute for Sustainable Plant Protection, CNR (Bari, IT)

4.1 Analytical sensitivity data
Qualiplante S.A.S. kit: 10^2–10^3 cfu mL^{-1}; Enbiotech s.r.l. kit: 10^3 cfu mL^{-1}.

4.2 Analytical specificity data
Strain numbers are available on the validation sheet in the EPPO Database on Diagnostic Expertise section Validation (http://dc.eppo.int/validationlist.php).
Exclusivity evaluated by LAMP-PCR (Enbiotech s.r.l. kit) tested on the following bacterial strains: 3 *Xanthomonas arboricola* pv. *pruni*, 1 *X. arboricola* pv. *juglandis*, 2 *X. arboricola* pv. *fragariae*, 1 *X. arboricola* pv. *corylina*, 1 *X. arboricola* pv. *celebensis*, 1 *Xanthomonas campestris* pv. *campestris*, 1 *X. campestris* pv. *populi*, 2 *Xanthomonas hortorum* pv. *pelargonii*, 3 *Pseudomonas savastanoi* pv. *savastanoi*, 1 *Pseudomonas marginalis*, 4 *Pseudomonas syringae* pv. *syringae*, 4 *Brenneria* (ssp. *rubrifaciens*, *quercina*, *salicis*, *populi*), 2 *Pantoea stewartii*, 1 *Pantoea agglomerans*, 1 *Erwinia amylovora*, 3 *Agrobacterium tumefaciens*, 2 *Rhizobium vitis*. No cross-reactions were observed.

4.3 Data on repeatability
Not available.

4.4 Data on reproducibility
Qualiplante S.A.S. kit: 90%; Enbiotech s.r.l. kit: 100%

4.5 Diagnostic sensitivity
Qualiplante S.A.S. kit: 84%; Enbiotech s.r.l. kit: 83%

4.6 Diagnostic specificity
Qualiplante S.A.S. kit: 92%; Enbiotech s.r.l. kit: 88%

4.7 Accuracy: 86%

Appendix 8 – Buffers and media

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

(A) Buffers

Sterile succinate-citrate-phosphate buffer

Disodium succinate ($Na_2C_4H_4O_4$)	1.0 g
Trisodium citrate ($C_6H_5Na_3O_7$)	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Distilled water to	1 L

Adjust pH to 7.0 before autoclaving.

PBS (1×)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4•12 H_2O	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L

Adjust pH to 7.2 before autoclaving.

Phosphate buffer (0.01M PB)

Na_2HPO_4 (anhydrous)	4.26 g
KH_2PO_4	2.72
Distilled water to	1 L

Adjust pH to 7.0 before autoclaving.

CTAB buffer*

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

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

TE buffer (100 mL)

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

(B) Media

Ingredients should be dissolved in the order given.

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

Soy peptone (Difco, 0436-01)	2.0 g
Bacto tryptone (Oxoid, LP0042	4.0 g
Disodium succinate (Sigma, S-2378)	1.0 g
Trisodium citrate (Sigma, S-4641)	1.0 g

(continued)

K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH) (Sigma, H-5533)	10.0 mL
Microbiological grade agar (Oxoid, LP0028)	15.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Sterile distilled or deionized water to	1.0 L
Adjust pH to 6.9	
BSA fraction V (20% w/v)* (Sigma Aldrich, A7030)	10.0 mL

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

• BCYE medium modified

Component (supplier/order no.)	Quantity
Demineralized water	940 mL
Aces Buffer (Sigma/A-3594)	10 g
KOH solution 1M	40 mL*
Yeast extract (Difco/212750)	10 g
Activated charcoal (Sigma/C-9157)	2 g
Agar no. 1 (Oxoid/LP011)	17 g

*Adjust the pH to 6.9 before adding the agar. This is done by adding approximately 40 mL KOH 1M until the appropriate pH value is reached.
Adjust the total volume to 980 mL with the demineralized water.
Autoclave and cool down afterwards to 45–50°C.
Then add the following components by filter sterilization:

Component (supplier/order no.)	Quantity
Cysteine hydrochloride (Sigma/C-7880)	5 mL
Ferric pyrophosphate (Sigma/P-6526)	15 mL

Agitate for at least 1 min.
The final pH is approximately 6.9.

Stock solutions (filter sterile)

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

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

• PWG medium modified [Anses based on Hill & Purcell (1995) and information provided at the COST Workshop – Bari 2010 (Rodrigo Almeida pers. comm.)]

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

Use a 2 L bottle and autoclave at approximately 121°C for 20 min.
Ingredients except BSA and L-glutamine are added mixed and dissolved in the order given.
After autoclaving, allow the temperature to cool to 50°C and under a horizontal air flow add filtered sterile BSA dissolved in 50 mL of deionized water and L-glutamine dissolved in 100 mL of water at about 50°C.

Stock solution of red phenol

Red phenol (0.2% aqueous solution)	50 mg
Sterile distilled or deionized water	25 mL

Store for a maximum of 1 month at 5 ± 4°C.

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

Stock solution of Hemin chloride

Hemin chloride (0.1% solution NaOH 0.05 N)	50 mg
Solution NaOH 0.05 N	50 mL

Store for a maximum of 1 month at 5 ± 4°C.

Appendix 9 – Isolation procedures

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

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

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

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

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

After surface sterilization, tissues are cut into pieces, placed in a mortar or in a container/test tube with sterile succinate-citrate-phosphate buffer or PBS (see Appendix 8) at a ratio of 1:10 (w:v). Tissues are then ground with a homogenizer (Polytron, Homex, etc.). An aliquot of 100 µL of sap is added to 900 µL of sterile succinate-citrate-phosphate buffer or PBS, and used to prepare a serial 10-fold

dilution (up 10^{-5}). Aliquots of 100 µL of 10^{-2}, 10^{-3}, 10^{-4} dilutions are then plated on the specific media, incubated at approximately 28°C, and monitored for colony development over 6 weeks. Plates should be sealed or kept in plastic bags to prevent desiccation.

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

2. Isolation from several leaves option 2 : 10–50 g of tissue collected from 100–200 leaves

For each sample, at least 10–50 g of tissue (petioles and midribs or basal leaf portions) is used.

Plant material is disinfected by soaking in a bleach solution (0.5% for 5 min or 2% for 2 min), then rinsed three times with sterile water. The plant material is then dried in tissue paper and briefly disinfected with 70% alcohol. Then the material is dried in a flow cabinet. After disinfection the plant material is crushed in a stomacher bag. 40 mL of buffer (PBS 0.01 M, see Appendix 8) is added and agitated for approximately 30 min at room temperature. The required volume of the extract for screening and for isolation is directly used from the extract obtained after the agitation step. The remaining extract volume is subsequently concentrated (centrifugation for 20 min at 10 000 *g* and 4°C) and resuspended in 1.5 mL PB 0.01 M. This concentrated extract is also used for screening and for isolation. In both cases, non-concentrated and concentrated extract, isolation is performed by preparation of serial dilutions (non-diluted; 1:10; 1:100) and plating on the specific medium. Incubation should be done at approximately 28°C, and plates monitored for colony development up to 6 weeks. Plates should be sealed or kept in plastic bags to prevent desiccation.

3. Isolation from individual leaves

After disinfection of the leaf with 70% (v/v) ethanol, a petiole or midrib approximately 1 cm long is collected with a sterile scalpel. Symptomatic leaves should be used preferably if available. The midrib or petiole is briefly soaked in ethanol at 96% (v/v) and flamed very quickly to achieve surface disinfection without causing a significant temperature rise in the tissues which could kill the bacteria. The sample is immediately placed in a sterile Petri dish with 1–2 mL of sterile saline solution or sterile demineralized water, comminuted and left to soak for at least 15 min, under gentle shaking. 100 µL of the macerate is plated without dilutions. Plates should be sealed or kept in plastic bags to prevent desiccation.

Appendix 10 – Multilocus sequence typing (MLST) (Yuan *et al.*, 2010)

1. General information

1.1 This test is suitable for the assignment of an isolate to one of the known subspecies from DNA from a pure bacterial culture. It has also been used with DNA from plant extracts (Loconsole *et al.*, 2016) although it is recognized that the quantity and quality of target DNA may not always be suitable for obtaining all amplicons.

1.2 The test is based on Yuan *et al.* (2010).

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

1.4 Amplicon size: 708 bp for l*euA*, 533 bp for *petC*, 730 bp for *malF*, 600 bp for *cysG*, 379 bp for *holC*, 557 bp for *nuoL*, 654 bp for *gltT*.

1.5 The sequences for the primers are as follows: forward primer leuA-for 5′-GGTGCACGCCAAATCGAATG-3′; reverse primer leuA-rev 5′-GTATCGTTGTGGCGTACACTG-3′; forward primer petC-for 5′-GCTGCCATTCGTTGAAGTACCT-3′; reverse primer petC-rev 5′-GCACGTCCTCCCAATAAGCCT-3′; forward primer malF-for 5′-TTGCTGGTCCTGCGGTGTTG-3′; reverse primer malF-rev 5′-GACAGCAGAAGCACGTCCCAGAT-3′; forward primer cysG-for 5′-GCCGAAGCAGTGCTGGAAG-3′; reverse primer cysG-rev 5′-GCCATTTTCGATCAGTGCAAAAG-3′; forward primer holC-for 5′-ATGGCACGCGCCGACTTCT-3′; reverse primer holC-rev 5′-ATGTCGTGTTTGTTCATGTGCAGG-3′; forward primer nuoL-for 5′-TAGCGACTTACGGTTACTGGGC-3′; reverse primer nuoL-rev 5′-ACCACCGATCCACAACGCAT-3′; forward primer gltT-for 5′-TCATGATCCAAATCACTCGCTT-3′; reverse primer gltT-rev 5′-ACTGGACGCTGCCTCGTAAACC-3′.

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

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 Nucleic acid source: bacterial culture or plant extract.

2.1.2 For pure cultures, a single colony of a fresh pure culture is resuspended in 0.9 mL of

PCR-grade water; lysis should be performed at 100°C for 5 min.

2.1.3. See Appendix 3 for extraction procedures from plants.

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

2.2. PCR for MLST

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	36.2	N.A.
PCR buffer (Invitrogen)	10×	5	1×
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	20 mM	0.5	0.2 mM
Forward primers (leuA-for, petC-for, malF-for, cysG-for, holC-for, nuoL-for, gltT-for)	20 µM	0.75	0.3 µM
Reverse primers (leuA-rev, petC-rev, malF-rev, cysG-rev, holC-rev, nuoL-rev, gltT-rev)	20 µM	0.75	0.3 µM
DNA Polymerase Platinum (Invitrogen)	5 U μL^{-1}	0.3	0.03 U μL^{-1}
Subtotal		45	
Genomic DNA		5	
Total		50	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR conditions

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reaction with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2. Interpretation of results

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

Verification of the controls for each PCR:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the relevant size.

When these conditions are met:

- Sequencing is performed when expected amplicons are produced (see point 1.4).
- Subspecies assignment is not possible if no band, a different number of bands, or band(s) of a different size are produced.
- The test should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

No validation data is available.

Appendix 11 – Conventional simplex PCR (Hernandez-Martinez *et al.*, 2006)

1. General information

1.1 This conventional PCR is suitable for assignation of subspecies *in planta* and assignation of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* and *sandyi* isolates. A multiplex PCR for isolates is described in Appendix 12.

1.2 The test is based on the following publication: Hernandez-Martinez *et al.* (2006).

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

1.4 Amplicon size in base pairs: 638 bp with subsp. *sandyi*, *multiplex*; 521 bp with subsp. *multiplex*; 412 bp with subsp. *fastidiosa*, *multiplex*.

1.5 Oligonucleotides for subsp. *sandyi*, *multiplex*: forward primer XF1968-L 5′-GGAGGTTTACCGAAGACAGAT-3′; reverse primer XF1968-R 5′-ATCCACAGTAAAACCACATGC-3′.

1.6 Oligonucleotides for subsp. *multiplex*: forward primer ALM1 5′-CTGCAGAAATTGGAAACTTCAG-3′; reverse primer ALM2 5′-GCCACACGTGATCTATGAA-3′.

1.7 Oligonucleotides for subsp. *fastidiosa*, *multiplex*: forward primer XF2542-L 5′-TTGATCGAGCTGATGATCG-3′; reverse primer XF2542-R 5′-CAGTACAGCCTGCTGGAGTTA-3.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 The test can be performed on DNA extracts or on pure culture suspension.

2.1.2. See Appendix 3 for extraction procedures from plants.

2.1.3 For pure cultures, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.

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

2.2. Conventional PCR

2.2.1. Master mix

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

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2. Interpretation of results

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

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the relevant size.

When these conditions are met:

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

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

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

4.1 Analytical sensitivity data

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

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

4.2 Analytical specificity data

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

4.3 Data on repeatability

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

4.4 Data on reproducibility

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

Appendix 12 – Conventional multiplex PCR (Hernandez-Martinez *et al.*, 2006)

1. General information

1.1 This conventional PCR technique is mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*.

1.2 The test is based on the following publication: Hernandez-Martinez *et al.* (2006).

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

1.4 Amplicon size: 638 bp with subsp. *sandyi*, *multiplex*, 521 bp with subsp. *multiplex* and 412 bp with subsp. *fastidiosa*, *multiplex*.

1.5 Oligonucleotides for subsp. *multiplex*, *sandyi*: forward primer XF1968-L 5′-GGAGGTTTACCGAAGACAGAT-3′; reverse primer XF1968-R 5′-ATCCACAGTAAAACCACATGC-3′.

1.6 Oligonucleotides for subsp. *multiplex*: forward primer ALM1 5′-CTGCAGAAATTGGAAACTTCAG-3′; reverse primer ALM2 5′-GCCACACGTGATCTATGAA-3′.

1.7 Oligonucleotides for subsp. *fastidiosa*, *multiplex*: forward primer XF2542-L 5′-TTGATCGAGCTGATGATCG-3′; reverse primer XF2542-R 5′-CAGTACAGCCTGCTGGAGTTA-3.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 For pure cultures, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.

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

2.2. Multiplex PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	15.25	N.A.
PCR buffer (Promega)	10×	2.5	1×
$MgCl_2$	50 mM	1.25	2.5 mM
dNTPs	20 mM	1	0.8 mM
Forward primers (XF1968-L, ALM1, XF2542-L)	20 µM	1.25	1 µM
Reverse primers (XF1968-R, ALM2, XF2542-R)	20 µM	1.25	1 µM
Promega Taq DNA polymerase	5 U μL^{-1}	0.5	0.1 U μL^{-1}
Subtotal		23	
Genomic DNA extract		2	
Total		25	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free water.

2.2.2 PCR conditions

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* can be used instead of living cultures. For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix, or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC (and if relevant IC) should produce amplicons of the relevant size.

When these conditions are met:

- A test will be considered positive if amplicons of 638 bp (subsp. *sandyi*, *multiplex*), 521 bp (subsp. *multiplex*) or 412 bp (subsp. *fastidiosa*, *multiplex*) are produced. Some strains of subsp. *multiplex* have two bands (638 bp and 531 bp – Type ST6) and others three bands (638 bp, 531 bp and 412 bp – Type ST7) (see Fig. 28).
- Subspecies assignment is not possible if no band, a different number of bands, or band(s) of a different size are produced.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

From the publication DNA extraction available with the Qiagen DNA tissue kit (Qiagen Inc., Valencia, CA).

4.1 Analytical sensitivity data

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

4.2 Analytical specificity data

Inclusivity 100% evaluated on 15 strains of

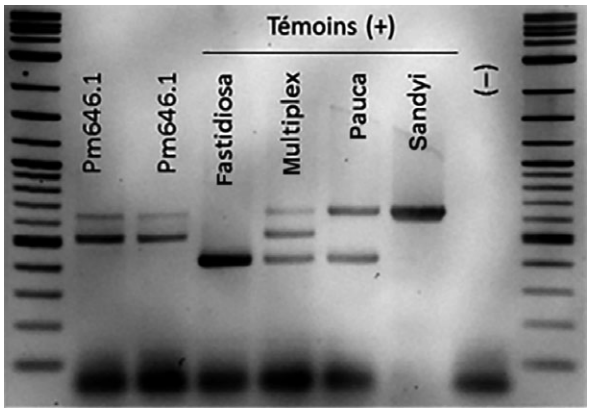

Fig. 28 Samples of *Polygala myrtifolia* and positive controls (subspecies of strains).

X. fastidiosa subsp. *fastidiosa*, 12 strains of *X. fastidiosa* subsp. *sandyi* and 25 strains of *X. fastidiosa* subsp. *multiplex*.

4.3 Data on repeatability
No data available.

4.4 Data on reproducibility
No data available.

4.5 Data on diagnostic sensitivity
Oleander: 100%
Grape: 100%

Appendix 13 – Conventional PCR (Pooler & Hartung, 1995)

1. General information

1.1 This conventional PCR is suitable for the detection and identification of *Xylella fastidiosa* and assignation of subsp. *pauca* in planta or for an isolate.

1.2 The test is based on Pooler & Hartung (1995).

1.3 The primers target a gene coding for a hypothetical protein (BLASTing; CVC strain *X. fastidiosa* 9a5c).

1.4 Amplicon size: 500 bp.

1.5 Forward primer CVC-1: 5′-AGATGAAAACAATCATGCAAA-3′; reverse primer 272-2-Int: 5′-GCCGCTTCGGAGAGCATTCCT-3′.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1 Tissue source: plant or pure culture suspension.

2.1.2 See Appendix 3 for extraction procedures from plants.

2.1.3 For pure cultures, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.

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

2.2. Conventional PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	18.3	N.A.
	10×	2.5	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Taq DNA polymerase buffer (Invitrogen)			
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (CVC-1)	20 µM	0.5	0.4 µM
Reverse primer (272-2-Int)	20 µM	0.5	0.4 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.2	0.04 U μL^{-1}
Subtotal		23	
Genomic DNA from plant extract (final concentration and its 10- and 100-fold dilutions) or bacterial suspension		2	
Total		25	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

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

3. Essential procedural information

3.1. Controls

For positive controls inactivated cultures or lysates of *X. fastidiosa* subsp. *pauca* can be used instead of living cultures. For a reliable test result to be obtained the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). For a series of analyses including samples from different plant species, whenever possible one PIC should be included per plant species to be analysed, or at least per botanical genus.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of

the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results

Verification of the controls:
- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the expected size.

When these conditions are met:
- A test will be considered positive if amplicons of 500 bp are produced.
- A test will be considered negative if it produces no band or band(s) of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

In France 2 different strains, isolated from coffee and identified as subsp. *pauca* by MLST (Yuan *et al.*, 2010) have been tested positive with the Pooler & Hartung (1995) method. It has not been possible to test other strains, in particular from Brazil, as these are not available in reference collections.

Appendix 14 – Pathogenicity test

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

1. Test plants

Pathogenicity tests use host plants grown in pots. The plants should not be herbaceous and xylem tissue should be well differentiated. Optimal stages for inoculation are illustrated in Fig. 29. When known, the most susceptible cultivars should be selected. Examples for some hosts are given below.

Olea europaea: successful inoculation has been obtained with Cellina di Nardò, Frantoio, Leccino, Coratina (M. Saponari, in prep. 2016).

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

Citrus sinensis: Pera, Hamlin, Natal and Valencia.

Coffea: Coffea arabica, *Coffea canephora* (no data is available to recommend specific cultivars).

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

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

Olive seedlings | Grape self-rooted cuttings | Seedlings of *Prunus* spp

Fig. 29 Illustration of size of plants used for the inoculations. Courtesy M. Saponari, CNR-Institute for Sustainable Plant Protection (IT).

2. Inoculation

Inoculation techniques should ensure infiltration directly into the xylem vessels in order for symptoms to develop. The most widely used method for plant inoculation is by needle puncture in the stem at the insertion of the petiole (Fig. 30).

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

Low passage (2–3) cultures of the bacterium grown for 8–10 days on the most suitable medium (see Appendix 8) at 28–30°C should be used for inoculation. Bacteria are removed from solid media and resuspended in PBS, or succinate-citrate buffer (see Appendix 8). The bacterial suspension should contain a high bacterial concentration (approximately 10^9 cfu mL^{-1}).

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

Another conventional procedure consists of the inoculation of the plants using a syringe (i.e. a 1-mL tubercolin syringe) with droplets of inoculum containing *X. fastidiosa* at approximately 10^8 cfu mL^{-1}.

An alternative method for inoculating citrus is to raise a flap of bark tissue by cutting tangentially upward with a razor blade, and apply 10–30 µL of suspension (10^8 cfu mL^{-1}) under the flap; a piece of excised bark tissue should be placed in an Eppendorf tube containing 500 µL of bacterial suspension for 2 h then the tissue piece should be replaced, and wrapped with grafting tape.

Fig. 30 Needle inoculation. Courtesy M. Saponari, CNR-Institute for Sustainable Plant Protection (IT).

If plants have multiple stems (i.e. *Polygala myrtifolia* or blueberry) inoculations should be performed on at least 2 stems.

To increase the effectiveness of the inoculations, the plants can be subjected to a second round of inoculation 3–8 weeks after the first inoculations.

3. Symptom monitoring

Symptoms usually appear (see Section 3.1) 60–80 days after inoculation in grapes and 8–10 months in citrus. It took 6 years in Brazil to complete the pathogenicity tests (and fulfil Koch's postulates) for the *X. fastidiosa* strains infecting coffee. In Italy, symptoms on the most susceptible cultivars of olive started to appear after 13 months.

The test plants showing symptoms should be tested as recommended in Section 3.5 and isolation should be attempted, although it may not be successful.

As it is not easy to obtain symptoms, the testing of asymptomatic test plants is also recommended.

Appendix 15 – Bioassay on tobacco plants (Francis *et al.*, 2008)

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

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

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

Around 1 month after transplant, tobacco plants are prepared for inoculation by cutting the top of the stem and removing the lower juvenile leaves so that only three healthy adult leaves in the lower portion of the plant remain (numbered 1–3).

Bacterial inoculum is prepared from *X. fastidiosa* cultured on solid media at 28°C for about 1 week. Bacteria from two plates are scraped off and resuspended in 1.5 mL succinate-citrate phosphate buffer (Appendix 8).

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

Plants continue growing from the site where the stem was cut. Leaves are classified according to their appearance

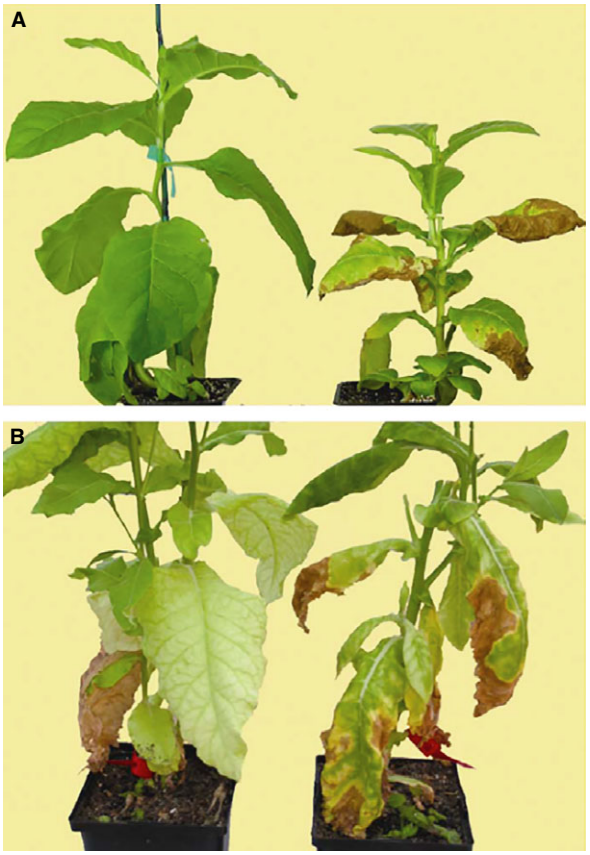

Fig 31 Symptoms on *Nicotiana tabacum* cv. SR1 after inoculation with *Xylella fastidiosa* Pierce's disease Temecula-1 strain. Symptoms were fully developed 6 weeks after inoculation. (A) The control plant mock inoculated with water (left) and plant inoculated with *X. fastidiosa* Temecula-1 (right). (B) Advanced symptoms at flowering time (2–3 months after inoculation). The water mock-inoculated control plant is showing normal leaf senescence (left) and the *X. fastidiosa* inoculated plant is showing marginal leaf scorching and a chlorotic halo around the edge of the scorch symptoms (right). Reproduced from Francis *et al.* (2008).

as control (healthy) or senescent (showing browning symptoms) from buffer-inoculated control plants and asymptomatic (healthy) or symptomatic (marginal leaf scorch) from *X. fastidiosa*-inoculated plants.

Symptoms start to develop 10–14 days after inoculation (leaf scorch symptoms). Francis *et al.* (2008) reports that tobacco inoculated with stains associated with almond leaf scorch and Pierce's disease showed typical symptoms resembling those of grapes and almond infected with *X. fastidiosa* (Fig. 31).