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Diagnostic Procedures to Detect *Xylella fastidiosa* in Nursery Stocks and Consignments of Plants for Planting

Giuliana Loconsole [1,*], Stefania Zicca [1], Lorenzo Manco [2], Oumaima El Hatib [3], Giuseppe Altamura [1], Oriana Potere [2], Vito Elicio [4], Franco Valentini [3], Donato Boscia [1] and Maria Saponari [1]

[1] Consiglio Nazionale delle Ricerche-Istituto per la Protezione Sostenibile delle Piante (CNR-IPSP), Sede Secondaria di Bari, 70126 Bari, Italy; stefania.zicca@ipsp.cnr.it (S.Z.); giuseppe.altamura@ipsp.cnr.it (G.A.); donato.boscia@ipsp.cnr.it (D.B.); maria.saponari@ipsp.cnr.it (M.S.)
[2] Dipartimento di Scienze della Pianta, del Suolo e degli Alimenti (DiSSPA), Università degli Studi di Bari Aldo Moro, 70126 Bari, Italy; lorenzomanco@hotmail.it (L.M.); oriana.potere@uniba.it (O.P.)
[3] Istituto Agronomico Mediterraneo di Bari (IAMB), 70010 Valenzano, Italy; oumaima.elhtb@gmail.com (O.E.H.); valentini@iamb.it (F.V.)
[4] Agritest Srl, Tecnopolis Casamassima, 70010 Valenzano, Italy; v.elicio@agritest.it
* Correspondence: giuliana.loconsole@ipsp.cnr.it

Abstract: Preventive measures for infectious diseases caused by the harmful plant pathogenic bacterium *Xylella fastidiosa* include inspections and diagnostic tests on imported consignments of plants and in nurseries. Currently, mandatory checks on plant propagating materials are enforced in Europe (EU regulation 2021/1201) for the most susceptible species found in the European outbreaks, and prior to move propagating materials of the "specified plants" from nurseries located in the so-called "demarcated areas". These requirements imply sampling and laboratory manipulation of a large number of samples, nevertheless plants to be sampled are often small size potted plants. While statistically based methods for inspections and sampling are available, namely the International Standards for Phytosanitary Measures n. 31, validated laboratory procedures to test large volumes of plant materials are lacking. In this work, we optimized two distinct protocols to detect *X. fastidiosa* in pooled plant materials collected from lots of plants for planting. The first protocol was designed to test in pool few samples (up to 8), the second to process through a single diagnostic test plant material from a high number of samples (up to 225). Accuracy of the newly developed protocols was assessed by pooling at different ratio tissues collected from healthy and infected *Polygala myrtifolia*, *Nerium oleander*, *Olea europaea*, *Lavandula stoechas* and *Prunus avium*. Moreover, tests included pools of plantlets of *Brassicaceae* and *Solanaceae* artificially inoculated with stem portions of infected periwinkle. Using both protocols, high diagnostic sensitivity values were generated using serological and molecular tests, with qPCR consistently yielding the highest performance values, regardless the host species tested.

Keywords: inspection; sampling; consignments; nursery stocks; composite samples; laboratory tests

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1. Introduction

Xylella fastidiosa is a Gram-negative bacterium belonging to Xanthomonadaceae family (Wells et al. 1987); however, several distinctive molecular and pathogenic traits separate this bacterium from many common phytopathogenic bacteria of this family. It is spread through infected plant propagating materials (i.e., mainly responsible for the long-distance spread) and by xylem-feeding insects (i.e., responsible for local spread), and infects a wide range of plant species. Based on the literature search [1], the bacterium has been detected in more than 500 plant species, mainly perennial species, with most of the infected species not showing remarkable alterations, even if destructive diseases are known to occur in important crops such as grapevine [2], citrus [3], stone fruits [4,5] and olives [6], as well as in numerous ornamental and forest species [7]. The wide host-range of this bacterium

is related to its wide genetic diversity. At least six different subspecies of *X. fastidiosa* have been proposed [8], and based on the multilocus sequence typing (MLST) analyses, 87 different sequence types (ST) have been so far described worldwide [9]. Several analyses indicated that the subspecies evolved in geographical isolation [10], and experimental research has shown host pathogen specificity within subspecies, although the determinants of the host specificity remain unknown [11].

International movements of infected plants for commercial or landscape planting are most likely the main pathway that contributed to the spread and establishment of *X. fastidiosa* outside of the Americas [12], where the pathogen was thought to be confined until its emergence in Europe during the last decade. Since the second half of the 18th century, its potential destructive impact on important woody crops (i.e., Pierce's disease in grapes, leaf scorch in almond and citrus variegated chlorosis in citrus) has been well-documented in the Americas, supporting the adoption in Europe of preventive measures, including its classification as "harmful quarantine pathogen", and more recently as one of the European priority pests (Regulation EU 2019/1702).

However, given the biological complexity of this pathogen (wide number host species to inspect and latent infections in several species), these measures failed to effectively protect the European territories, with several independent introductions occurring in the past years [12], followed by the establishment of field infections where favorable conditions occurred. As a result, the pathogen is currently threatening olives, almond and several other species in the different outbreaks discovered mainly in southern Europe countries. More specifically, the bacterium has been detected in 2013 initially in southern Italy associated to a deadly severe disease affecting olives [6,13], then in 2015, infections were detected on ornamentals and on several Mediterranean shrubs in natural habitats in Corsica and southern mainland France [14]; in 2016, the bacterium was detected in the Balearic Islands and mainland Spain [15]; in late 2018, two outbreaks were found, respectively, in Tuscany region (Italy) [16] and in Portugal (Europhyt, Outbreak No. 753); while the latest reports are from France in the region Occitanie where an outbreak was detected on lavender plants in 2020 (EPPO Reporting Service 2020/197) and from Portugal on rosemary plants in 2021 (JN/Agências, August 2021). With regard to these European outbreaks and the numerous interceptions of infected coffee plants, a total of almost 80 different host species have been found infected by a large panel of genetically distinct strains, representing the main subspecies and clustering in 9 different ST: ST1 (*fastidiosa*), ST6, ST7, ST79, ST81, ST87 (*multiplex*), ST53, ST80 (*pauca*) and ST76 (*sandyi*) [17]. Due to these outbreaks and the numerous interceptions in imported consignments [18], strict emergency measures have been enforced in Europe to avoid further introductions in the *Xylella*-free areas and cross-contaminations of strains among the currently known European outbreaks areas. Inspection of many plant propagating materials before marketing is one of the main requirements of the European regulations. Plants for planting, other than seeds, of *Coffea* spp., *Lavandula dentata* L., *Nerium oleander* L., *Olea europaea* L., *Polygala myrtifolia* L. and *Prunus dulcis* (Mill.) found to be susceptible to multiple bacterial strains in different European outbreaks, shall only be introduced/moved within Europe if tested for the presence of *X. fastidiosa*, using a sampling scheme in accordance with the international standard ISPM 31, to identify with at least 80% of confidence a level of presence of infected plants of 1% (EU Regulation 2020/1201).

These mandatory checks imply large scale sampling and testing programs in nurseries and in imported/marketed consignments, and pose major challenges for the affordability and reliability of the tests. To this end, composite sampling may represent an acceptable practice to satisfy sample size requirements, while keeping the number of diagnostic tests affordable. Given the lack of experience and the limited experimental data on the detection of the bacterium in pooled samples [19,20], in this study efforts were made for developing and optimizing diagnostic procedures to detect *X. fastidiosa* in composite samples of different host species. Experiments aimed at defining the amount of tissue to be sampled from a single plant of the lots, and the maximum number of plants to

be pooled when performing a single test. Protocols were developed using artificially or naturally infected plants sampled in the outbreak area of Apulia (southern Italy) where isolates harboring the genotype ST53 are spreading and causing infections in more than 35 plant species including olives, the most affected and susceptible species to this bacterial genotype.

Validation of the developed procedures was then performed using composite samples made by a small or large number of individual plant samples.

2. Materials and Methods

2.1. Plant Sources and Tissue Used for the Diagnostic Tests

Naturally infected olives (*Olea europaea* L.), oleander (*Nerium oleander* L.), myrtle-leaf milkwort (*Polygala myrtifolia* L.), lavender (*Lavandula stoechas*) and cherry (*Prunus avium*) were sampled in the contaminated area of Apulia (southern Italy). All infected sources were infected by *X. fastidiosa* subsp. *pauca*, strain ST53. Other than these perennial host species, diagnostic procedures were also tested on herbaceous plants, using plantlets of infected periwinkle, needle-inoculated with the abovementioned bacterial genotype, (*Catharantus roseus)* to artificially contaminate pools of young plantlets of *Solanum lycopersicum* and *Brassica oleracea*.

For each species, composite samples were prepared by pooling at different ratio portions recovered from asymptomatic or symptomatic *Xylella*-infected source plants and from *Xylella*-free sources.

Plant tissues used in our tests consisted of leaf tissues (either petioles or midribs or leaf basal part) for oleander and olives, leaf basal part and stem portions (1.5–2.0 cm long) for myrtle-leaf milkwort, scraped xylem tissue from hardwood cutting for cherry, and stem portions for lavender (2.5–3.0 cm long), *S. lycopersicum* and *B. oleracea* and periwinkle.

2.2. Detectability and Distribution of the Bacterium under Natural Infection Conditions

To gather information of the minimum amount of plant tissues to be collected during the inspections of lots of plants for planting, naturally infected plants (selected based on previous molecular diagnostic tests [21,22]) were selected in the infected area of Apulia, and diagnostic tests performed on single leaves or single stem portions. More specifically, 15 different infected olive trees (including both symptomatic and asymptomatic trees) and 1–2 infected symptomatic plants for the other species were selected and sampled. Single leaves/stem portions harvested from these infected sources were processed using the modified "Dneasy Mericon food kit standard protocol" (Qiagen) [PM 7/24 (4)] [21] and the recovered total DNA extracts tested by qPCR [22]. From each infected source the number of individual samples, corresponding to single leaves or single stem portion (hereafter referred as sub-samples), that were collected and tested ranged from 12 in olives to 22 in myrtle-leaf milkwort plants.

2.3. Detection of X. fastidiosa by Pooling a Small Number of Individual Samples

To adapt the conventional diagnostic protocols., i.e., the same used for individual samples, for testing composite samples containing a small number of individual samples (from 5 to 8), we used a variable number of leaves, stem portions and wood chips of olives, oleander, myrtle-leaf milkwort, cherry and lavender.

Tests were performed on artificially contaminated composite samples, also simulating the situation in which only one asymptomatic plant in the pool was infected (the worst case scenario). Thus, the experimental samples were obtained by mixing plant materials with no symptoms from one infected source and one non-infected source. An increasing number of petioles (from 1 to 4) and leaf basal parts (1 to 3) recovered from an infected olive and oleander, respectively, and 2, 4 or 6 leaf basal parts recovered from infected myrtle-leaf milkwort plants were mixed at different ratio with a variable and increasing number of the same plant material recovered, respectively, from non-infected sources of olive, oleander and myrtle-leaf milkwort. Due to the seasonality of the detectability of the

bacterium in the leaves of cherry trees compared to xylem tissues, the samples used in the experiments consisted of 2 and 4 wood chips recovered from mature cuttings from an infected tree, mixed with a variable and increasing number of woody chips (8 and 16) from a non-infected tree.

In the case of olive, tests included also the use of semi-hardwood cuttings. More specifically, pieces of 1–1.5 cm long (from 1 to 4), recovered from different cuttings harvested from an asymptomatic infected source, were mixed with a variable number of pieces from a non-infected source, simulating the worst situation in which only one cutting per tree was infected.

For each mixture, at least 3 replicates were processed in 2 independent assays and tested as individual samples by using one or more procedures described in the diagnostic standard EPPO PM 7/24 (4) [21], namely ELISA, Real time Lamp, total DNA extraction using the modified "Dneasy Mericon food kit protocol" followed by qPCR [22] on thermal cycler CFX96 (Biorad). Accuracy (diagnostic sensitivity and specificity) was calculated according to the criteria defined in the EPPO, PM 7/76 (5) [23].

2.4. Detection of X. fastidiosa by Pooling a Large Number of Individual Samples

2.4.1. Sample Preparation

Plant materials recovered from a high number of individual samples (from 100 to 225 plants), corresponding to 20 g (for the perennial species) and 40 g (for the herbaceous species), were pooled and singularly tested. As described in Section 2.3, artificially contaminated samples were prepared by mixing asymptomatic plant materials (portions from 2 to 4) recovered from infected sources at different ratio with a variable number of portions recovered from non-infected sources. In the case of the *S. lycopersicum* and *B. olearacea*, both not susceptible to the Apulian bacterial genotype, the infected source was the infected periwinkle.

The pooled tissues excised from the individual samples were either soaked or macerated in extraction bags in phosphate buffered saline (PBS) 1x−2% Natrium metabisulfite (w:v 1:1/1:1.5 for the herbaceous species and 1:3 for the perennial species).

After an incubation at room temperature (RT) for 30 min, the plant homogenates were recovered from the extraction bags and centrifuged for 5 min at 3000× *g*; then, the resultant supernatants were centrifuged at 10,000× *g* for 20 min, and the pellets resuspended in the appropriate extraction buffer according to the diagnostic procedure to be used. For each mixture, at least 3 replicates were prepared and processed in 2 independent tests.

2.4.2. Enzyme-Linked Immunosorbent Assay (ELISA) and Real Time Loop-Mediated Isothermal Amplification (LAMP)

For ELISA and real time LAMP assays, the pellets were resuspended in 1 mL of PBS 1x. ELISA was performed as described in the EPPO Standard PM 7/101 (1) [24], using the kit provided by Agritest [16], whereas Real time Lamp assays were performed using the ready-to-use "Xylella screen glow" kit (Enbiotech) [21], with 5 µL of the resuspension added to 200 µL of extraction buffer provided by kit. Samples were incubated at 65 °C for 10 min, and an aliquot of 3 µL was added to the Lamp Mix; amplification was performed following the manufacturer's instructions using the Icgene-Enbiotech device.

2.4.3. Extraction of Total DNA and qPCR Assays

For qPCR tests, DNA extraction was performed by comparing three different methods: the standard CTAB protocol [20], the Dneasy Mericon food kit protocol (Qiagen) modified [21], and the Maxwell® RSC PureFood GMO and Authentication Kit" (Promega) following the manufacturer's instructions. For all three protocols, the pellets recovered as described in Section 2.4.1 were resuspended in 1 mL of the corresponding extraction buffer and then an aliquot processed following the standard CTAB protocol or the manufacturer's instructions for the commercial kits. Three replicates for each artificially contaminated composite sample were processed.

Diagnostic sensitivity and specificity was calculated based on the qPCR results [22,23] gathered from the reactions set up with the DNA extracts purified using the three selected protocols.

3. Results

3.1. Distribution of Xylella fastidiosa in Naturally Infected Sources

The number of qPCR-positive sub-samples (single leaves or stem portions) detected within each tested infected plant, and the corresponding quantification cycles (Cq), were variable and depending on the host species (Table 1).

Table 1. *Xylella fastidiosa* qPCR-positive sub-samples recovered from each infected plants. Values of the quantification cycles (Cq) generated in the positive samples are indicated. In bold are indicated the symptomatic olive trees.

Source Infected Plant	Tissue Sampled	N. of qPCR-Positive/ N. of Total Sub-Samples Individually Tested	%Positive Sub-Samples/Plant	Range of Cq Values (min-max)
Olive	Leaf petioles/midribs			
OL-108		12/12	100	18.44–20.75
OL-109		12/12	100	18.44–20.75
OL-13		12/12	100	21.01–34.52
OL-MF		12/12	100	15.31–28.54
OL-A1		23/24	96	20.74–33.91
OL-A2		12/12	100	19.13–29.03
OL-B1		12/12	100	15.20–24.23
OL-96		5/12	42	23.37–34.25
OL-123		6/12	50	23.84–34.50
OL-142		7/12	58	22.14–34.40
OL-37		8/12	67	24.25–34.50
OL-19		8/12	67	27.06–34.45
OL-34		7/12	58	22.31–34.57
OL-38		5/12	42	23.15–34.18
OL-151		4/12	33	21.29–34.70
Oleander	Leaf basal part			
No-C		9/12	75	21.38–26.88
No-B		6/12	50	19.45–23.40
Myrtle-leaf milkwort	1.5–2.0 cm of stem			
Poly-1		22/22	100	15.90–26.12
Lavender	1.5–2.0 cm of stem			
Lav-1		15/15	100	23.88–32.20
Cherry	Wood chips recovered from portion of 2 cm excised from mature cuttings			
Ch-1		19/20	95	19.23–32.96
Ch-2		20/20	100	19.43–30.63

For olive, high rates of qPCR-positive leaves (>96%) were obtained from the sub-samples harvested from the seven symptomatic trees sampled in the heavily infected area, where most of the infected olives show typical symptoms of desiccation. The Cq values produced by these positive sub-samples were consistently below 32; with only few leaves yielding Cq values higher than 32.

When leaf petioles where collected from five of the asymptomatic olive trees the range of qPCR-positive leaf petioles dropped to 50–70%, with Cq values generated in the majority of the sub-samples (approx. 60%) comprised between 33.00 and 34.50; however, few sub-samples generated Cq below 25. In the remaining three asymptomatic olive trees (OL-38,

OL-96, OL-123), the rate of qPCR-positive leaves was below or equal to 50%; however, almost all Cq values recorded from these trees were lower than 30.

From the myrtle-leaf milkwort and lavender infected plants, 100% of qPCR-positive sub-samples was obtained, with Cq values comprised in the optimal range. Similar results were gathered from the two cherry trees sampled, yielding 95–100% of qPCR-positive wood chips.

With regard to the two infected oleander plants, the rate of qPCR-positive sub-samples per plant was 50 and 75%, respectively, with Cq values in the optimal range.

The data gathered by these tests, clearly support the notion that in infected plants the bacterium is not uniformly distributed, with some leaves and shoots escaping the bacterial colonization or harboring the bacterium at a concentration below the limit of detection of the qPCR assay, currently considered the most sensitive method. As shown by our data, under some specific conditions, the percentage of sub-samples testing positive can be as low as 33%. This implies that multiple leaves or shoots need to be sampled from each individual plant in order to reduce the risk of false negative results.

3.2. Detection of Xylella fastidiosa in Composite Samples by Pooling Few Samples

In line with the results reported in the previous paragraph, consistent bacterial detection occurred when the composite samples were prepared by using more than one single portion from a known infected plant.

Table 2 shows the values of the diagnostic sensitivity recorded when testing composite samples prepared using a variable number of plant portions from the infected sources and from the non-infected sources. As shown, for olive, the highest values of diagnostic sensitivity (89% and 100%) were obtained when at least four leaves from an infected tree were used in composite samples pooled with 20 and 40 leaves. In olives, when pieces of cuttings were used, the bacterium was detectable by qPCR even in the combination including one single piece from the infected sources out of 28, yielding Cq values ranging from 28.02 to 31.45.

Table 2. Assessment of the qPCR diagnostic sensitivity when testing composite samples prepared by mixing a variable number of portions from infected and non-infected sources. The pools that generated 100% of diagnostic sensitivity with at least one diagnostic test are highlighted in grey.

Host Species (Tissue)	**Composite Samples**			**Diagnostic Sensitivity**		
	N. of Portions from the Infected Source	N. of Portions from the Non-infected Source	Weight (grams)	qPCR (Harper et al. 2010)	Real Time Lamp	ELISA
Olive (petioles/ midribs)	2	8	0.2	83%	67%	67%
		18	0.5	92%	83%	83%
		28	0.8	78%	78%	67%
	4	16	0.6	100%	100%	100%
		36	1.0	89%	100%	89%
Olive (1.0–1.5 cm pieces of cutting)	1, 2, 3, 4	27, 26, 25, 24	1.2	100%	-	-
Oleander (leaf basal part)	2	16	1.7	100%	100%	89%
	3	24	2.2	89%	100%	89%
Myrtle-leaf milkwort (leaf basal part)	4	32	0.5	83%	83%	83%
	6	48	1	100%	100%	100%
Cherry (woody chips)	2	8	1.0	100%	83%	100%
	4	16	2.0	100%	83%	100%

For oleander, two leaves from an infected plant mixed with 16 leaves recovered from the non-infected source allowed us to reach the highest values for diagnostic sensitivity, whereas six leaves from infected myrtle-leaf milkwort were needed to reach a diagnostic

sensitivity of 100% for all diagnostic techniques used. For cherry, 100% of diagnostic sensitivity was obtained either when two or four sub-samples from the infected sources were used in mixture with eight or 16 sub-samples from the non-infected sources, respectively.

Regardless of the host species, all composite samples that do not containing infected materials were properly identified, with diagnostic specificity of 100% recorded by ELISA and molecular tests.

Based on these results, for large scale sampling and testing programs in nurseries and in imported/marketed consignments, the minimum number of leaves/plant to be recovered when pooling multiple samples and the maximum number of plants to be pooled were estimated (Table 3). In those cases in which large trees need to be sampled, xylem tissues recovered from mature cuttings can be preferred to leaves [19,20]. From large trees, generally multiple shoots are sampled (at least 4), as such, from each sample to be pooled, at least four pieces or cuttings or woody chips are used. Under our experimental conditions, this allowed us to pool up to seven olives or five cherry trees (Table 3).

Table 3. Estimation of: (i) the number of plant portions to be collected from each plant species; (ii) the number of plants that can be pooled for testing composite samples when using the same diagnostic protocols adopted for testing individual samples.

Host Species (Tissues)	Minimum n. of Portions to Be Collected for Each Plant/Tree	Maximum n. of Portions Cab Be Pooled	N. Plants/Trees That Can Be Pooled
Olive (petioles)	4	20	5
Olive (pieces from cuttings)	4	28	7
Oleander (leaf basal part)	2	16	8
Myrtle-leaf milkwort (leaf basal part)	6	48	8
Cherry (woody chips)	4	20	5

3.3. Detection of Xylella fastidiosa in Composite Samples Prepared by Pooling a High Number of Samples

Detection failed in almost all composite samples processed by soaking the diced tissues in PBS 1X for 30 min. On the contrary, homogenization in extraction bags using the semi-automated homogenizer (Homex, Bioreba) yielded positive detections in almost all expected positive samples.

False negative results were obtained with composite samples of myrtle-leaf milkwort, even if six leaves from the infected sources were used, as previously determined (see Section 3.2). As such, we repeated the experiment with this species using small pieces (1.5–2 cm long) of shoots (from semi-hardwood cuttings) instead of leaves.

The combinations of composite samples that yielded the best values of diagnostic sensitivity are summarized in Table 4.

Regarding olive and oleander, a diagnostic sensitivity of 100% was obtained for all three detection methods by mixing the leaf basal part of four and two leaves recovered from the infected source with those recovered from 800–900 and 200 leaves harvested from the *Xylella*-free plants, respectively.

Concerning myrtle-leaf milkwort and lavender, *X. fastidiosa* was successfully detected in composite samples prepared by mixing two pieces of shoots from the infected sources with 250 or 180–200 pieces recovered from non-infected sources, respectively.

A diagnostic sensitivity of 100% was obtained for the three detection methods, even if for real time LAMP, inhibition occurred in lavender when the resuspended pellets were used directly to set up the reactions; dilution of the resuspended pellets at a ratio of 1:3 v:v improved the bacterial detectability and the diagnostic sensitivity.

Table 4. Number of plants that were be pooled when preparing large composite samples of olive, oleander, myrtle-leaf milkwort, lavender, cherry and herbaceous species. Values of diagnostic sensitivity generated by qPCR using different DNA extraction methods, ELISA and Real time Lamp assay are also reported.

Species	Maximum Size of the Composite Sample	Diagnostic Sensitivity (%)				
		qPCR			Real Time LAMP	ELISA
		CTAB	Dneasy Mericon Food Kit (Qiagen)	Maxwell® RSC PureFood GMO and Authentication Kit (Promega)		
Olea europaea L.	20 g of leaf tissue corresponding to approx. 800–900 * leaf basal parts. Given that from each plant 4 leaves are used, the maximum number of plants pooled corresponds to 200–225.	100	100	100	100	100
Nerium oleander	20 g of leaf tissue corresponding to approx. 200 leaf petioles. Given that from each plant 2 leaves are used the maximum number of plants pooled corresponds to 100.	100	100	100	100	100
Polygala myrtifolia	20 g of stems corresponding to approx. 250 shoot pieces of 1.5–2.0 cm. Given that from each plant 2 shoot pieces are used, the maximum number of plants pooled correspond to 125.	100	100	100	100	100
Lavandula stoechas	20 g of stems corresponding to approx. 180–200 shoot pieces of 2.5–3.0 cm. Given that from each plant 2 shoot pieces are used, the maximum number of plants pooled correspond to 90–100.	100	100	100	100 **	100
Prunus avium	20 g of xylem tissue corresponding to approx. 200 wood chips. Given that from each plant 2 wood chips are used, the maximum number of plants pooled correspond 100.	100	100	100	0	0
Herbaceous host(*Solanum lycopersicum*)	40 g of stems corresponding to approx. 200 stems. Given that from each plant 1 stem portion is used, the maximum number of plants pooled correspond to 200.	100	100	100	85.6	57.1
Herbaceous host (*Brassica oleracea*)	40 g of stems corresponding to approx. 200 stems. Given that from each plant 1 stem portion is used, the maximum number of plants pooled correspond to 200.	100	100	100	37.5	37.5

* The range is calculated based on minimum and maximum weight of the plant portions (petioles/midribs or stem pieces). ** 4 out of 8 replicates generated negative results in a first assay; when diluted 1:3 in PBS, positive results were obtained.

For cherry, 100% diagnostic sensitivity was obtained only by qPCR tests on replicates obtained when at least 0.1 g of xylem tissue from the infected sources were recovered and mixed in a total of 20 g of xylem tissue from Xylella-free plants. Real time LAMP and ELISA tests failed to detect the bacterium in composite samples of cherry.

A diagnostic sensitivity of 100% was obtained by qPCR on composite herbaceous samples prepared by mixing one stem portion from an infected periwinkle with 200 stem portions of tomato or cabbage plantlets. Whereas both real time Lamp and ELISA tests produced lower values for diagnostic sensitivity.

The failure of ELISA and real time LAMP assays to correctly detect the bacterium in composite samples of cherry and herbaceous plants, may be due to the presence of contaminants in the pellet recovered after the centrifugation, which affected the diagnostic results. Thus, qPCR should be preferably used to test the bacterium in large composite samples for these species.

With regard to the DNA extraction methods compared, except that for the composite samples of lavender, CTAB protocol generated the quantification cycles (Cq) values lower or comparable to those obtained on the DNA purified using both commercial kits (Table 5).

Table 5. Range of the quantification cycles (Cq) values generated by qPCR reactions using different DNA extraction methods for large composite samples (according to Table 4). The lowest and the highest value obtained for the 6 replicates tested for each plant species are indicated.

Host Species	Range of Cq Values for Positive Sample (min-max)		
	CTAB	Dneasy Mericon Food Kit (Qiagen)	Maxwell® RSC PureFood GMO and Authentication Kit (Promega)
Olive	26.20–31.04	30.10–31.10	29.40–31.18
Oleander	24.03–28.38	26.21–27.71	24.38–26.09
Myrtle-leaf milkwort	25.55–27.03	27.81–30.17	28.51–29.03
Lavender	32.04–32.56	29.96–30.14	28.40–30.89
Cherry	28.17–30.84	31.03–32.94	31.11–33.08
Herbaceous host (*Solanum lycopersicum*)	27.43–28.42	26.66–30.23	28.85–30.02
Herbaceous host (*Brassica oleracea*)	26.25–31.69	28.40–31.41	27.13–30.02

Optical density (OD) values recorded in the ELISA assays (Table 6) clearly differentiated the positives from the negative controls, except that in the case of cherry for which high level of background were recorded (Table 6).

Table 6. Results of the ELISA tests for composite samples. The lowest and the highest OD values obtained for each plant species are indicated in parenthesis.

Host Species	N. of Positive Replicates/ Total Expected Positive ($OD_{405\ nm}$ Values at 120 min)	$OD_{405\ nm}$ Values at 120 min Recorded in the Xylella-Negative Control
Olive	6/6 (0.212–0.394)	0.021
Oleander	8/8 (0.493–1.248)	0.010
Myrtle-leaf milkwort	6/6 (0.256–0.913)	0.022
Lavender	8/8 (0.307–1.004)	0.063
Cherry	0/6 -	0.300
Herbaceous host (*Solanum lycopersicum*)	4/8 (0.174–0.308)	0.039
Herbaceous host (*Brassica oleracea*)	3/8 (0.217–0.590)	0.070

All composite samples prepared with non-infected plants did not produce any positive reaction with the three diagnostic methods tested, yielding values of 100% of diagnostic specificity, with the exception of ELISA tests performed on cherries that were affected by the occurrence of background reactions.

4. Discussion

The use of composite samples can substantially reduce analytical costs for large scale pathogen diagnosis, because the number of required analysis is reduced by pooling several samples into one that can be tested as individual sample. Composite sampling refers to mix physically individual samples to form a composite sample, and a single analysis is then performed. An accurate selection of the plant materials to be sampled and processed as composite samples may allow to retrieve the same information that could be gathered from multiple analyses on individual plants. The main limitation/drawback when testing composite samples is related to the lower diagnostic sensitivity, as individual contaminated/infected samples are diluted with "clean" samples. However, when a large number of samples is selected to satisfy sample size requirements, the number of analytical measurements should be kept affordable.

In this work, we standardized and optimized the preparation of composite samples to detect *X. fastidiosa*, by simulating the worst case scenario:, i.e., only one asymptomatic infected plant in the pooled samples. Optimization was performed at two scales, for small scale composite samples (from 5 to 8) and for large scale composite samples (from 100 to 225 pooled samples).

When pooling plant samples, the key aspect is to reduce as much as possible the plant tissues, so that the resultant plant material volumes can be easily processed. However, when selecting plant tissues for testing, a limitation can be represented by the erratic distribution of the target pathogen. The occurrence of *X. fastidiosa* in infected sources is generally not uniformly distributed, especially during the early stage of the infections (latent period), with some leaves and shoots escaping the bacterial colonization or harboring the bacterium at concentration below the limit of detection of the qPCR assay, currently considered the most sensitive method.

As shown by our data, under some specific conditions, the percentage of sub-samples testing positive can be as low as 33%. This implies that multiple leaves or shoots need to be sampled from each individual plant in order to reduce the risk of false negative results. Through our work, we experimentally assessed the minimum number of plant portions to be collected from the single unit of a lot of plants, to ensure high diagnostic sensitivity of the tests on the recovered composite samples.

For olives, we proved that during the early infection stage less than 40% of the trees may be colonized or harbor a detectable level of the pathogen. Under our experimental conditions, we found that at least four leaves per tree should be used in the composite samples, in order to generate 100% of diagnostic accuracy. Based on this finding, we have then defined the maximum number of samples that can be pooled, when using the same detection pipeline used to process individual samples or when using the procedure optimized in this work.

In oleander, tests on single sub-samples (single leaves) from infected plants showed 50% of qPCR positives. As such, inconsistent results were obtained when only one leaf petiole was used to prepare the composite samples, whereas using 2–3 leaf petioles tests generated 100% of diagnostic accuracy. Given the large size and weight of the oleander leaf petioles, a maximum of eight oleander plants can be pooled and tested using the standard diagnostic pipeline, and up to 100 plants can be pooled for the large scale composite samples.

In the remaining naturally infected host species, the bacterium was consistently detected in almost all tested sub-samples. In this case, it should be remarked that the only available plants were those with well-established bacterial infections. For all these species, when at least two shoots were sampled to recover the tissues for preparing the

composite samples, diagnostic sensitivity of 100% was obtained. The number of plants that were efficiently pooled at large scale corresponded to 125 for myrtle-leaf milkwort and 100 for lavender and cherry, while eight plants could be pooled for myrtle-leaf milkwort and lavender and five for cherry, when small scale composite samples need to be tested.

Experiments performed using herbaceous plants showed that efficient bacterial detection occurred in composite samples prepared by pooling as much as 200 individual samples (approx. 40 g of tissues).

With regard to the diagnostic test, qPCR assay gave the highest performance values for all plant species, regardless the procedure used for the purification of the total plant DNA. ELISA and real time LAMP performed equally to qPCR when used on composite samples of olive, oleander and myrtle-leaf milkwort, while gave lower values of diagnostic sensitivity on the remaining plant matrices tested.

Our experiments confirmed the importance to subject to an accurate homogenization step the plant tissues prior to perform the serological or the molecular tests. Bacterial detection failed in composite samples prepared by dicing and soaking the tissues in the extraction buffer without prior homogenization.

Inspections and controls for *X. fastidiosa*, a priority pest (2019/1702) for Europe, are now mandatory on imported consignments and in place of productions for the most susceptible host plants listed in the EU Regulation 2020/1201, as well as on the numerous "specified plants" propagated in nurseries located in the infected, containment and buffer zones.

Guidelines on inspections are available from different international standards (i.e., EPPO PM 3/81 Inspection of consignments for *X. fastidiosa*; EPPO PM 3/82 Inspection of places of production for *X. fastidiosa*, and ISPM 31), which provide useful information on the numbers of units to be sampled, and to determine the sample sizes in lots of plants for planting. To complement such guidelines, in this study we standardized the procedures for pooling materials, at small and large scale, collected from lots of plants, by defining conditions for testing composite samples. These conditions included: (i) the type of tissue to be sampled and processed according to the plant species; (ii) the minimum number of plant portions to be collected from the single unit of the lot; (iii) the maximum number of plants that can be pooled and processed as single composite sample; (iv) the approximate weight of the laboratory composite samples to be subjected to the diagnostic test; (v) the diagnostic test that provided the highest-performance values.

Overall, the data and the procedures herein developed provide useful guidance when large number of samples (often from small size plants, i.e., from consignments or from nurseries) need to be subjected to laboratory tests, satisfying sample size requirements and keeping the diagnostic tests technically and economically affordable. These indications were promptly included in the revision of the EPPO 7/24 (4) diagnostic standard.

Indeed, some of the procedures have been transferred to the Italian Plant Protection Organization, adopting the composite samples for the ongoing 2021 monitoring campaign in the demarcated areas.

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