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Optimisation of sampling and testing for asymptomatic olive trees infected by *Xylella fastidiosa* in Apulia region, Italy

Anna Maria D'ONGHIA*, Franco SANTORO, Serena Anna MINUTILLO, Dajana FRASHERI, Marilita GALLO, Stefania GUALANO, Giuseppe CAVALLO, Franco VALENTINI

Centre International de Hautes Etudes Agronomiques Méditerranéennes Bari (CIHEAM Bari), Via Ceglie, 9 - 70010 Valenzano (BA), Italy
*Corresponding author. E-mail: donghia@iamb.it

Summary. Early detection of *Xylella fastidiosa* outbreaks in Apulian olive groves is crucial, especially in buffer zones and pathogen-free areas where olive trees are asymptomatic. Three studies were conducted. Two were on the spatial and temporal progression of *X. fastidiosa* infections in tree canopies of asymptomatic or mildly symptomatic olive trees of tolerant ('Leccino') and susceptible ('Cellina di Nardò' and 'Ogliarola salentina') cultivars. Despite different trends in pathogen infection rates and concentrations between 'Leccino' and susceptible olive cultivars over the study period, results showed that sampling was most effective in the mid-upper part of tree canopies throughout the year, excluding the warmest and coldest periods. Stem xylem tissues were the most appropriate for detecting the pathogen compared to lower parts of mature leaves with petioles, using serological and molecular assays. Based on these results, a third study was conducted to compare molecular and serological tests (qPCR, real-time LAMP, DAS-ELISA, DTBIA) for detection of *X. fastidiosa* in the mid-upper part of asymptomatic branches of infected 'Leccino' trees that were sampled in an appropriate collection time, using stem xylem tissue as the most appropriate matrix for testing. The molecular methods showed the greatest sensitivity, with no undetermined results, while among the serological assays, DTBIA was more sensitive than DAS-ELISA. An improved protocol for monitoring asymptomatic olive trees is recommended.

Keywords. Plant matrices, diagnosis, qPCR, real-time LAMP, DAS-ELISA, DTBIA.

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ORCID:
AMD'O: 0000-0002-1817-4637
FS: 0000-0003-0793-866X
SAM: 0000-0002-6403-0685
DF: 0000-0001-6423-2394
MG: 0000-0003-4981-485X
SG: 0000-0002-2221-6529
GC: 0000-0001-9192-9955
FV: 0000-0001-5724-8291

INTRODUCTION

Xylella fastidiosa (Wells *et al.*, 1987), a xylem-limited Gram negative bacterium, causes a number of serious plant diseases, including Pierce's disease of grapevine and citrus variegated chlorosis (CVC), and this pathogen can infect more than 655 plant species among crops, ornamentals, wild vegetation and forestry (EFSA *et al.*, 2022). The bacterium is classified into six genetically different subspecies: *fastidiosa*, *multiplex*, *pauca*, *sandyi*, *tashke*,

and *morus,* each with its own host range (Schaad *et al*., 2004; Randall *et al*., 2009; Janse and Obradovic, 2010; Nunney *et al*., 2014).

Xylella fastidiosa was first recorded in Europe and Mediterranean region in olive trees in the Apulia region of Southern Italy (Saponari *et al*., 2013). A novel strain of *X. fastidiosa* subsp. *pauca*, De Donno strain ST53 (Elbeaino *et al*., 2014; Loconsole *et al*., 2016) causes the dramatic 'Olive Quick Decline Syndrome (OQDS)'. This strain, which primarily infects olive trees (*Olea europaea* L.), is transmitted by three spittlebugs, among which *Philaenus spumarius* is the most efficient (Ben Moussa *et al*., 2016; Cavalieri *et al*., 2019). *Xylella fastidiosa* causes withering, desiccation, leaf scorch and some dieback in olive trees, symptoms that can extend over entire canopies and lead to death of susceptible cultivars, within a few years from the onset of the symptoms (Martelli *et al*., 2016). With no pathogen control methods being available, strategies for managing *X. fastidiosa* infections currently rely on vector control and the use of *X. fastidiosa*-resistant cultivars (Morelli *et al*., 2021). Apulian native olive cultivars, such as 'Cellina di Nardò' and 'Ogliarola salentina', are highly susceptible and succumb to the disease, while 'Leccino' and 'Favolosa FS-17' show some resistance to the infections (Boscia *et al*., 2017).

Apulia is the most important olive oil producing region in Italy (ISTAT, 2021) and is almost totally covered with olive trees, so spread of the bacterium by vectors has been rapid and destructive. The pathogen is a serious threat to the olive industry and to the landscape of this region, and the heritage value of centuries-old olive trees is also important (Strona *et al*., 2017; Schneider *et al*., 2020).

Early detection of *X. fastidiosa* in olive trees through monitoring programmes is important for assessing new outbreaks, especially when there are no or very mild symptoms, particularly in early stages of infections and because some trees are resistant/tolerant to the pathogen. Following the EPPO diagnostic protocol PM 7/24 (4) *Xylella fastidiosa* (2019) for survey of the bacterium in olive trees, samples should be collected close to the symptomatic portions of branches or should be representative of whole tree canopies for asymptomatic trees. Sampling of olive could also be carried out throughout year, regardless of temperature and physiological phases, because symptoms associated with *X. fastidiosa* infections are persistent, although more strongly expressed in summer.

Several official diagnostic assays are available for the detection of *X. fastidiosa* in olive trees (Harper *et al*., 2010; Djelouah *et al*., 2014; Loconsole *et al*., 2014; Yaseen *et al*., 2015), using leaf petioles and midribs from mature leaves, which are the most suitable source for diagnosis due to the high number of xylem vessels they contain (Hopkins, 1981). Serological tests are usually performed in large-scale monitoring of *X. fastidiosa* in infected and containment areas, where the concentration of the bacterium is expected to be high. Instead, molecular tests are recommended for *X. fastidiosa*-free areas and buffer zones. However, apart from diagnostic sensitivity and specificity, these tests differ in terms of required processing time, necessary technical skills, and specialised instrumentation, which impact the efficiency of *X. fastidiosa* monitoring programmes, especially from an economic viewpoint.

Concentration of the bacterium and its distribution in olive trees are not uniform and depend on pathogen strains, olive cultivars and environmental factors (primarily air temperature and soil water content). As well, EPPO sampling procedures for official monitoring of *X. fastidiosa* in pathogen-free areas are not fully supported by specific research results. For these reasons, the present study aimed to maximize probability of detecting *X. fastidiosa* ST53 in canopies of asymptomatic or mildly symptomatic olive cultivars in the Apulian *X. fastidiosa*-demarcated area. It was therefore necessary to develop effective sampling and testing procedures for detecting the pathogen in olive trees on a large scale.

To this aim, two studies were carried out on the temporal, spatial and quantitative evolution of the *X. fastidiosa* population at olive canopy level of the tolerant 'Leccino' cultivar in comparison with susceptible olive cultivars, using different matrices and diagnostic methods. Results achieved led to a third study for the detection of *X. fastidiosa* in infected 'Leccino' olive trees that were asymptomatic or showed mild infection symptoms through the comparative evaluation of four diagnostic assays, including: Double antibody sandwich - enzyme-linked immunosorbent assay (DAS-ELISA), Direct tissue blot immunoassay (DTBIA), real time Loop-mediated isothermal amplification (real time LAMP), and quantitative polymerase chain reaction (qPCR). For field data acquisition, the XylApp software (Santoro *et al*., 2017) was used, and samples were analysed in the official plant quarantine laboratory of CIHEAM Bari (Italy).

MATERIALS AND METHODS

Spatial, temporal and quantitative evolution of the infections

A preliminary study was conducted in 2017 on 80 'Leccino' (*X. fastidiosa*-tolerant) and 46 'Cellina di

Nardò' (susceptible) olive cultivars in a 50-year-old olive grove in the *X. fastidiosa* infected zone (Lecce province, Apulia). In March, the status of infection in the grove was assessed by testing all the olive trees using DAS-ELISA. For each tree, four semi-hardwood twigs with mature leaves (15–20 cm in size) were collected from the mid-upper and on four sides of the tree canopy (EPPO, 2019). A total of five *X. fastidiosa*-positive trees for each cultivar were selected, based on absence or low levels of symptoms (Boscia *et al.*, 2017). Asymptomatic branches were chosen for sampling. This study aimed to evaluate three levels of canopy and twig portions for preliminary screening, before performing a more in-depth sampling in the second study. The canopy of these trees was divided into three height levels: low (L_1), medium (L_2), high (L_3). Four twigs were randomly collected at each level and each twig was divided into three portions: basal, middle, apical. A total of 240 twigs per cultivar were collected in May and July and twig sections were tested for *X. fastidiosa* using DTBIA, following the protocol of Djelouah *et al.* (2014). DTBIA-negative samples were retested using real-time LAMP following the protocol of Yaseen *et al.* (2015).

Preliminary results from the first study led to a more extensive investigation in the period from December 2017 (T_0) to March 2019 (T_4), focusing on two canopy levels using semi-hardwood twigs. Due to the high *X. fastidiosa* infection pressure in the grove used in the first study, the second study was carried out in two olive groves (approx. 50 years old) located in the Northern part of the *X. fastidiosa* infected zone bordering the containment area. In this area, one olive grove was found with the tolerant 'Leccino' (189 trees), the target cultivar, but not with the susceptible 'Cellina di Nardò', which was replaced with the susceptible 'Ogliarola salentina' (109 trees). The total infection rates in these two groves were first assessed by DAS-ELISA at T_0 in December 2017 (EPPO, 2019). Based on these results and absence of symptoms or presence of mild symptoms, ten infected trees per cultivar were chosen, and asymptomatic branches were periodically tested at two canopy levels (low and high) at four collection times: June 13, 2018 (T_1), September 6, 2018 (T_2), December 18, 2018 (T_3), March 6, 2019 (T_4).

A total of eight semi-hardwood twigs with mature leaves per tree (four from each canopy level) were tested for *X. fastidiosa* at each collection time, using DAS-ELISA, DTBIA and qPCR (Harper *et al.*, 2010), and xylem tissues either from twigs or from mature leaves (lower portion of the leaf with petiole). Each twig with mature leaves was considered as a sample. Throughout the whole sampling period, a total of 640 samples from both cultivars were analysed by serological assays (80 samples per cultivar at each collection time) and half of the samples were randomly selected for qPCR tests. As shown in Figure 1, the same portion of each host matrix was first analysed by DTBIA (two prints per sample) and afterwards by DAS-ELISA (0.5 g per sample) and qPCR (0.3 g per sample).

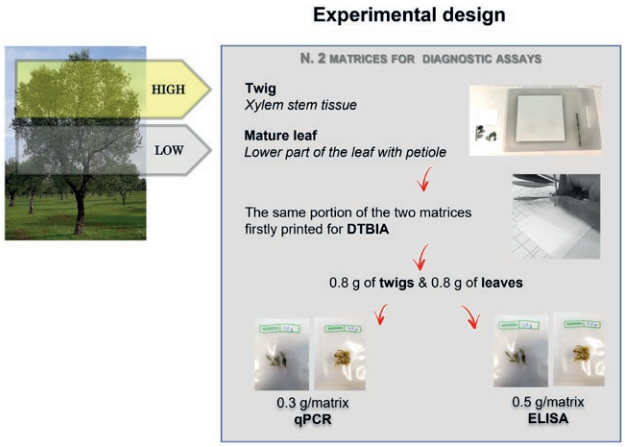

Figure 1. The experimental design used for sampling olive trees. This shows the two canopy levels that were sampled (four semi-hardwood twigs with mature leaves were collected from each level), and the analyses for detection of *Xylella fastidiosa* using two matrices: the stem xylem tissue and the lower part of the mature leaves with their petioles. Each host matrix was first printed on nitrocellulose membrane for DTBIA and was then used for qPCR and DAS-ELISA.

Diagnostic assays

DAS-ELISA was applied using a commercial kit (Agritest S.r.l.) following the manufacturer's instructions. The reaction was determined to be positive if, at regular intervals within 120 min, the absorbance value (at 405 nm) with a microplate reader was three times greater than the mean absorbance of negative control samples. Samples were considered undetermined when the absorbance value was at least two times greater than the mean absorbance of negative control samples. For "undetermined" samples, testing was repeated using the same matrix sample.

DTBIA was carried out using the protocol of Djelouah *et al.* (2014). Twig sections (2–5 mm diam.) and mature leaf petioles were printed on nitrocellulose membranes. Each type of sample was printed twice. Blotted membranes were immersed in 1% bovine serum albumin at room temperature, and then with antigen-specific alkaline phosphatase-labelled antibodies to *X. fastidiosa* (Agritest S.r.l.) at a dilution of 1:500. They were then

dipped in a substrate solution containing one tablet of Sigma Fast TM BCIP-NBT at room temperature until purple-violet colour appeared in the positive controls. The reaction was stopped by washing with tap water. The sample was considered positive when a clear purple violet colour reaction, in one or more prints, was visible with a 10× magnification stereoscope. For a mild positive reaction, results were considered undetermined, and the test was repeated with the same matrix sample.

qPCR was applied after using the CTAB-based protocol for total DNA extraction from 0.3 g of xylem tissue per host matrix. For absolute pathogen quantification, inactivated bacterial cells of *X. fastidiosa* subsp. *pauca* ST53 (supplied by CNR Bari, Italy) were used for generating the standard calibration curves. Inactivated bacterial suspension with an initial OD_{600} of 0.5, corresponding to ca. 10^8 CFU mL^{-1}, was spiked into crude extract of healthy olive xylem tissues to obtain serial ten-fold dilutions ranging from 10^7 to 10^5 CFU mL^{-1}. Bacterium quantifications from samples were inferred by the standard calibration curve, using Cqs from qPCR. Reactions were performed on 50 ng μL^{-1} total DNA. In all analyses, appropriate negative controls containing no template DNA were used. Amplifications and data analyses were carried out using CFX96™ Real-Time PCR Detection Systems (BIO-RAD). Samples were considered positive for Cq values not exceeding 32 and undetermined for 33 to 34 Cq. In cases of no amplification curves or Cq values exceeding 35, samples were considered negative (molecular detection of *X. fastidiosa* by real-time tests https:// upload.eppo.int/download/298ocd8b7f525).

Real-time LAMP was carried out using a commercial kit (Enbiotech S.r.l.), following the manufacturer's instructions, and based on primers developed by Harper *et al.* (2010; erratum 2013) and modified by Yaseen *et al.* (2015). An aliquot (5 µL) of crude sap, prepared for DAS-ELISA using xylem tissues from each matrix (mature leaf with petioles and twig samples), was processed in two steps (DNA extraction and real-time LAMP). Amplifications and data analyses were carried out using CFX96™ Real-Time PCR Detection Systems (BIO-RAD). Samples were considered positive in presence of amplification curves within 30 min from the beginning of each reaction.

Comparative evaluation of serological and molecular tests

Based on results obtained in the two previous studies, a comparative evaluation of serological (DAS-ELISA, DTBIA) and molecular (qPCR, real-time LAMP) tests for the detection of *X. fastidiosa* was carried out on 75 asymptomatic/mildly symptomatic infected trees of the tolerant 'Leccino' cultivar. Four semi-hardwood twigs from four sides at the high canopy level of each tree were collected and tested in October 2019. A portion from each twig was printed twice on nitrocellulose membranes for the DTBIA tests. Xylem tissue from the same twig portion (approx. 1 g) was divided into two equal parts: one was homogenized to obtain plant sap for DAS-ELISA and real time LAMP tests, and the other part was used for qPCR. Positive controls from Agritest S.r.l. and Enbiotech S.r.l. commercial kits were used for serological assays and real-time LAMP, respectively, whereas inactivated bacterial cells of *X. fastidiosa* subsp. *pauca* ST53 (supplied by CNR Bari, Italy) were used for qPCR. Negative controls were obtained from healthy olive plants maintained in the insect-proof greenhouse of CIHEAM Bari (Italy). Serological and molecular tests for the detection of *X. fastidiosa* were performed following the EPPO diagnostic protocol PM 7/24 (4) *Xylella fastidiosa* (EPPO, 2019).

Statistical analyses

Descriptive and parametric statistical analyses were carried out for the two studies on xylem tissues from twigs and mature leaves (lower part with petioles) of *X. fastidiosa*-infected olive trees. Incidence of infection, with respect to the type of olive cultivars (susceptible 'Cellina di Nardò' and 'Ogliarola Salentina' and tolerant 'Leccino'), was estimated using the relative frequency of selected trees *versus* the total number of trees tested by serological and molecular methods. Pathogen concentrations were expressed as a base-10 logarithmic scale (Log CFU mL^{-1}).

To investigate differences and infection evolution rates, and pathogen concentrations with respect to different conditions, six categorical variables were defined: varietal susceptibility ('Cellina di Nardò' *vs* 'Ogliarola salentina', 'Leccino'), canopy level (low, medium, high), twig portion (basal, medium, apical), plant matrix (xylem tissue from the lower part of mature leaves with petioles and from twigs), sampling time (T_1, T_2, T_3, T_4), and diagnostic method (DTBIA, DAS-ELISA, qPCR). Parametric univariate tests ("one way" and "factorial" ANOVAs) were applied to test for separability and interactions between levels of defined categorical variables. The applicability condition of the univariate models was verified by normality tests on the data (Shapiro-Wilks), admitting a slight deviation from the ideal condition, and Levene's test to verify the homogeneity of variances. The comparison between serological (DAS-ELISA, DTBIA) and molecular (qPCR, real-time LAMP) tests, with respect to the condition of infected plants of

the tolerant 'Leccino' with no or mild symptoms, was assessed through evaluation of the relative percentages of positive, false negative and undetermined samples detected by the diagnostic techniques. Microsoft Excel® was used for preparation of some datasets. All statistical analyses with derived graphs were carried out using Statistica 7 software (StatSoft Inc.).

RESULTS

Spatial, temporal and quantitative evolution of the infection

Results from the preliminary study in 2017 showed that the average infection rates from the three canopy levels of the five olive trees per cultivar (240 twigs per cultivar) were the least in the tolerant 'Leccino' (23.1%) compared to that of the susceptible 'Cellina di Nardò' (89.3%). In the susceptible cultivar, most of the tested twigs were *X. fastidiosa*-infected, whereas in 'Leccino' the number of infected twigs was much less. In addition, no DTBIA-negative sample was assessed as positive by real-time LAMP.

For host canopy levels, a statistically significant difference in infection rate was detected only in the low canopy level (L_1) compared to L_2 and L_3 of 'Leccino' (one way ANOVA), whereas no significant difference in infection rate among the three canopy levels was shown for the susceptible cultivar (Figure 2, A and B).

Effects of the distribution of infection along three longitudinal portions of olive tree twigs (apical, middle, basal) were analysed by a factorial ANOVA analysis, with respect to the two different cultivars (Figure 3). The results showed no significant differences in the infection rate between the three portions of the two cultivars, although the greatest values were found in the apical twigs of 'Leccino'.

The second study (December 2017 to March 2019) on ten asymptomatic/mildly symptomatic trees/cultivar analysed effects of two canopy sampling levels using different diagnostic methods and two host matrices (factorial ANOVA). The total number of positive samples over the full sampling period was greater for the upper part of tree canopies of the susceptible cultivar 'Ogliarola salentina', regardless of the plant matrix or diagnostic test used (Figure 4, A and B). The same result, but less evident, was obtained for the 'Leccino' cultivar. The statistical model revealed a greater percentage of positives in the xylem tissues of twigs (approx. 5 - 10%) compared to leaf tissues, regardless of the diagnostic test used, variety susceptibility, and host canopy level (Figure 4 B). These results were more marked from qPCR, which had greater diagnostic sensitivity than the serological tests.

In the susceptible 'Ogliarola salentina', infection rates for twig samples detected with qPCR ranged from 45% in the low canopies to 75% in the high canopies. This effect was much less marked in tolerant 'Leccino' trees where low canopy samples gave 40% infection and high canopy samples gave 48% infection. DTBIA and DAS-ELISA gave similar results for both cultivars using twig tissues (Figure 4 B). However, DTBIA was more sensitive for detecting infections in leaf tissues in 'Ogliarola salentina' (Figure 4 A).

For the evolution of infection incidence (positive samples for each host canopy level), statistically signifi-

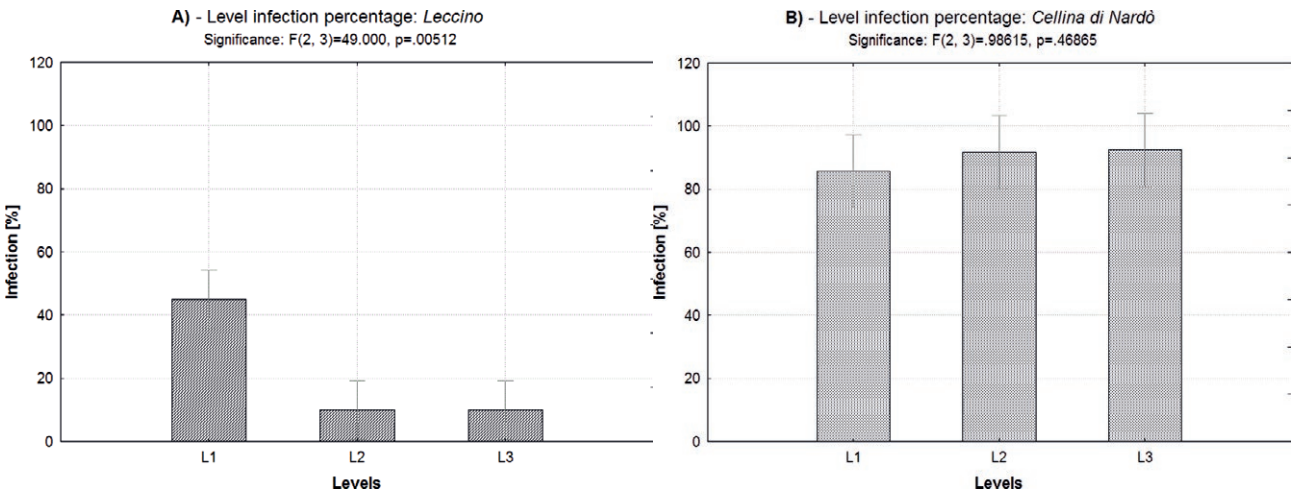

Figure 2. Mean infection rates in 'Leccino' (A) and 'Cellina di Nardò' (B) olive cultivars, as indicated from testing 240 twig samples per cultivar at the three canopy levels: low (L_1), medium (L_2), high (L_3).

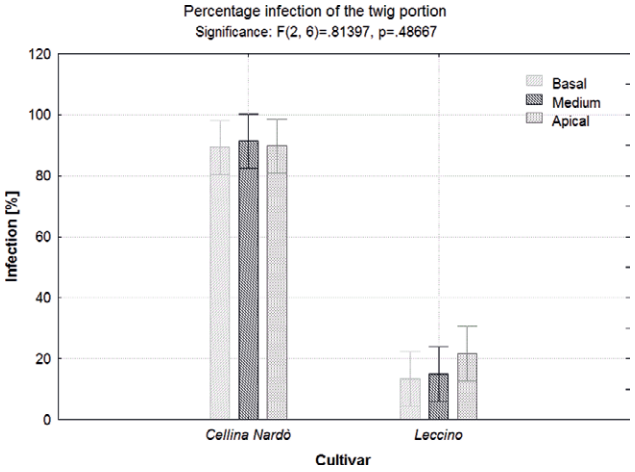

Figure 3. Mean infection rates for different olive twig portions (basal, middle, apical) for the *Xylella fastidiosa* tolerant 'Leccino' and the susceptible 'Cellina di Nardò' cultivars.

cant differences were recorded within the sampling period (F = 84.16, *P* = 0.00000) and according to the cultivar factor (F = 15.13, *P* = 0.0000); no differences for host canopy level, plant matrix, and diagnostic method were detected (Figures 5 and 6). 'Ogliarola salentina' showed a progressive increase over time in infection rates at both host canopy levels for both types of plant matrices (Figures 5B and 6B). This trend was more evident for twigs from the high canopy level, reaching values close to 100% infection at the last collection month. In contrast, percentage of infection for 'Leccino' fluctuated regardless of host canopy level or matrix type, with ranging from approx. 30 to 50% for qPCR-positive samples (Figures 5A and 6A). In this cultivar, the infection rates increased from September to December and then decreased from December to March. In contrast, decreased infection was observed in the period June to September. In general, a slight increase in incidence of infection was detected in the high host canopy level, using twig tissue samples.

For the different diagnostic methods, in general qPCR gave the greatest numbers of positive results regardless of olive variety, host canopy level and type of plant matrix (Figures 5 and 6). However, the greatest diagnostic sensitivity of qPCR was more evident in 'Leccino' when twigs were used for testing (Figure 6A). The two serological methods gave similar results with twigs from both olive varieties (Figure 6). In contrast differences were recorded when leaves were used (Figure 5). In this case, DTBIA was more sensitive than DAS-ELISA, showing the greatest diagnostic sensitivity in the high canopy level of 'Ogliarola salentina' (Figure 5B).

For evolution of overall mean *X. fastidiosa* concentration (Log[CFU mL^{-1}]) in both cultivars, at each collection time during the sampling period (T_1, T_2, T_3, T_4), the statistical analyses confirmed significant differences between the four assessed concentrations (F = 24.2, *P* = 0.00002, with Shapiro-Wilk: W = 0.98, *P* < 0.001, and Levene test: F = 2.94, *P* < 0.001), highlighting September 2018 as the month with the lowest mean value (4.426 Log[CFU mL^{-1}] or approx. 26,000 CFU mL^{-1}), and March 2019 with the greatest mean value (5.166 Log[CFU mL^{-1}] or approx. 140,000 CFU mL^{-1}) (Figure 7).

The overall average concentration of *X. fastidiosa* detected in 'Leccino' (approx. 50,000 CFU mL^{-1}) was less than that measured in the susceptible 'Ogliarola salentina' (approx. 100,000 CFU mL^{-1}) (F = 18.915, *P* =

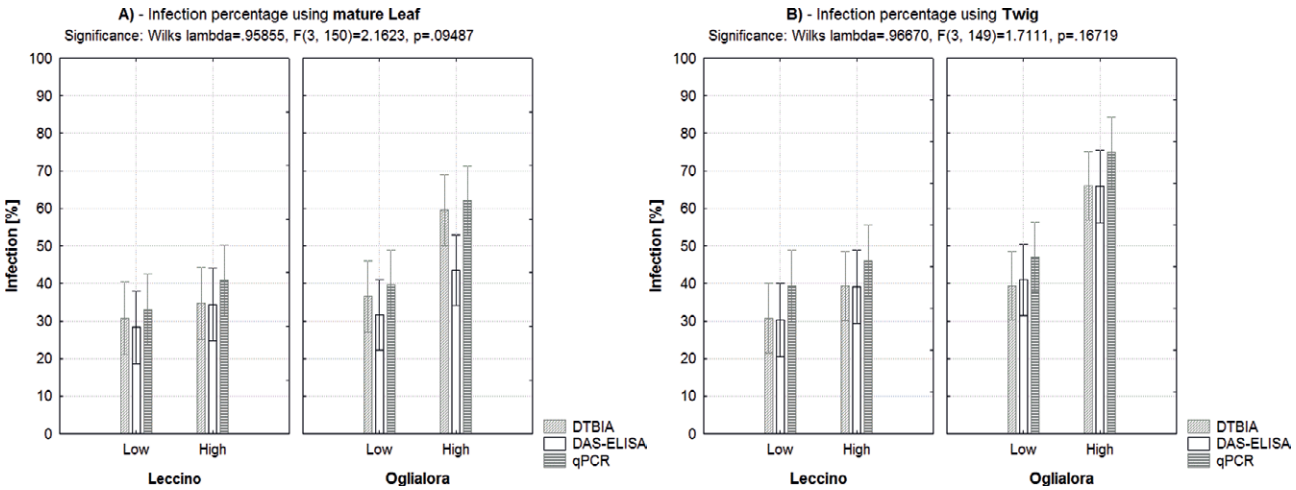

Figure 4. Mean *Xylella fastidiosa* infection rates in two olive cultivars ('Ogliarola salentina' and 'Leccino') assessed using xylem tissue from leaves (A) and twigs (B), from two host canopy levels (high, low) and using three diagnostic methods (DAS-ELISA, DTBIA, qPCR).

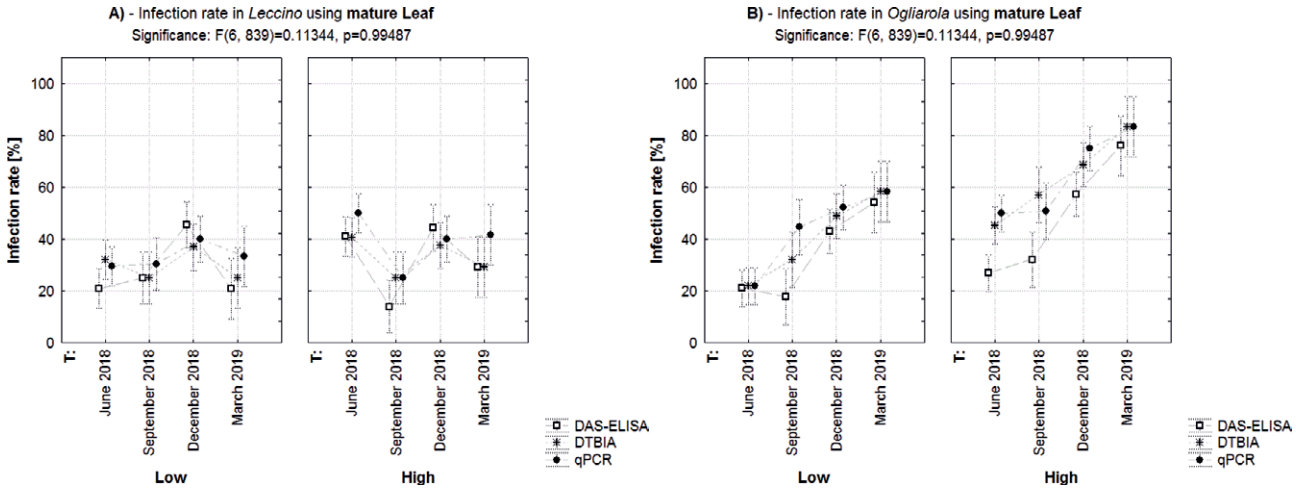

Figure 5. Mean *Xylella fastidiosa* infection rates in 'Leccino' (A) and 'Ogliarola salentina' (B) olive trees assessed using the host leaves, and three diagnostic assays at four different sampling times (T).

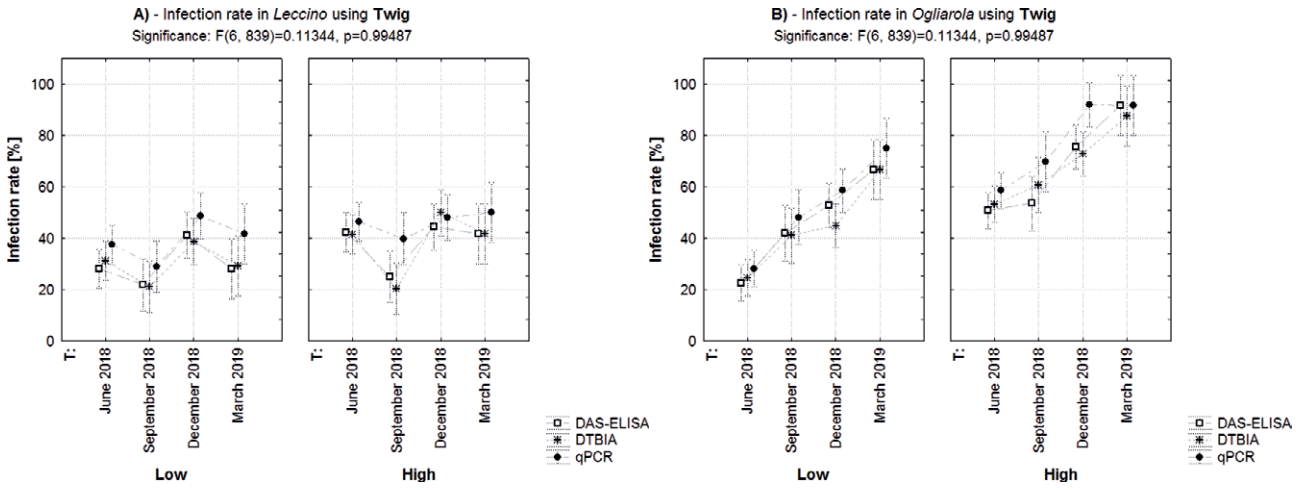

Figure 6. Mean infection rates in olive trees of 'Leccino' (A) and 'Ogliarola salentina' (B) assessed using the host twigs and three different diagnostic assays at four different sampling times (T).

0.00002), and the overall mean amount of bacterial cells extracted from mature leaves (approx. 53,000 CFU mL^{-1}) was less than that extracted from twigs (approx. 100,000 CFU mL^{-1}) ($F = 15.1$, $P = 0.00011$).

Figure 8 shows the time course of *X. fastidiosa* concentrations expressed in logarithmic units for the two olive cultivars in the two assessed host tissue types.

Only in 'Leccino', the evolution of *X. fastidiosa* concentration in the June-December period was similar to that of the infection rate in leaf tissues, i.e., this decreased from June to September, followed by an increase until December. In contrast, a different trend in this cultivar was observed for twigs, which showed decreases in bacterial population size from June to December, followed by increases from December to March. Unlike the infection rate, the pathogen concentration trend was different in 'Ogliarola salentina', which showed a decrease in the summer period using both matrices, followed by increases in the remaining months. In both olive varieties, however, there was a steady increase in pathogen concentrations from December to March, regardless of the tissues assayed. In general, twigs yielded greater pathogen concentrations than leaves (Figure 8).

Comparative evaluation of serological and molecular tests

The comparisons of the different diagnostic tests for the detection of *X. fastidiosa* on asymptomatic branch-

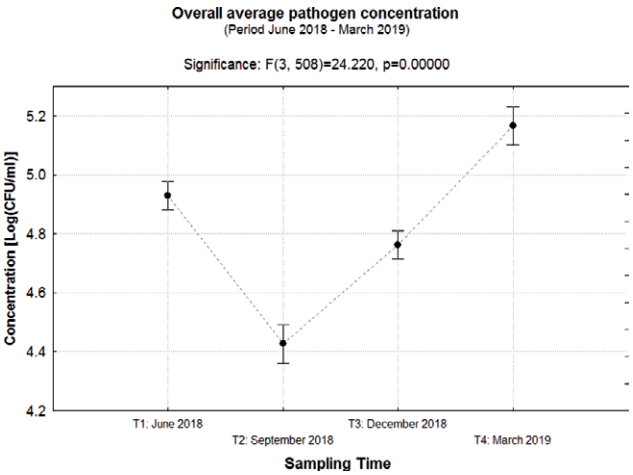

Figure 7. General trend of mean *Xylella fastidiosa* concentrations in two olive cultivars at four sampling times (T1 to T4) during 2018/19.

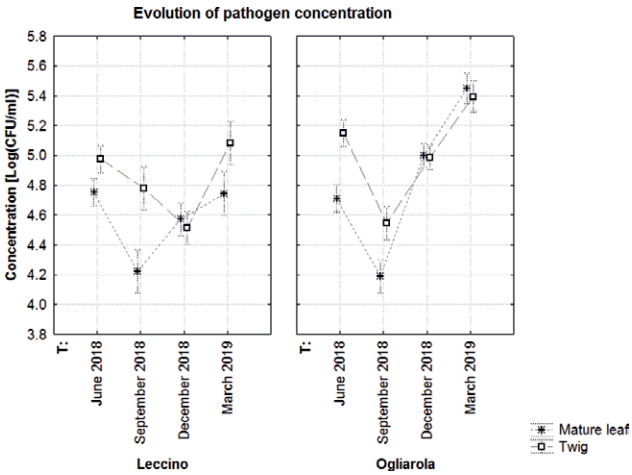

Figure 8. Mean *Xylella fastidiosa* concentration in different tissues (lower parts of mature leaves with petioles and twigs) of two olive cultivars at different sampling times (T).

es of 'Leccino' trees was based on the most effective sampling procedure and most suitable collection time obtained in the previous studies. The proportions of positives, false-negatives and undetermined samples detected with the four diagnostic tests showed that qPCR was the most sensitive, followed by real-time LAMP, DTBIA and DAS-ELISA. DTBIA detected 90.7% of the infected trees, with 5.3% of false-negatives and 4.0% of undetermined samples. ELISA was less sensitive for infected trees (86.7%), with a greater proportion of false-negatives (12%) and fewer undetermined samples (1.3%). For the molecular tests, qPCR (98.7%; 1.3% false-negatives) performed slightly better than real time LAMP (97.3%; 2.7% false-negatives) and without undetermined results. The only false-negative sample detected using qPCR was positive by real-time LAMP and DTBIA. Both serological techniques gave greater proportions of false-negatives and undetermined results when compared with the molecular tests, which showed few false-negatives. All undetermined samples in the serological tests gave positive results in the second round of testing, thus increasing the performance of DTBIA by approx. 3% compared to ELISA.

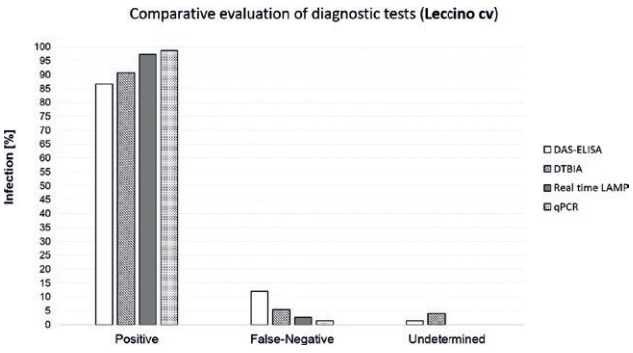

Figure 9. Infection levels indicated in different *Xylella fastidiosa* diagnostic tests (DTBIA, DAS-ELISA, qPCR, real-time LAMP), expressed as percentages of positive, false-negative and undetermined samples.

DISCUSSION

This research has provided relevant technical results for optimizing sampling and analyses of olive trees for *X. fastidiosa* in buffer zones and pathogen-free areas. In these areas, trees infected by this pathogen could be asymptomatic or mildly symptomatic, depending on cultivar susceptibility or low bacterial content in new infections. Therefore, studies on the spatial and quantitative distribution of the bacterium in the olive canopies, and the seasonal dynamics of the pathogen in naturally infected olive trees have focused on the tolerant 'Leccino' cultivar compared with susceptible olive cultivars.

The difference in the host canopy distribution of the pathogen between tolerant and susceptible olive cultivars was evident in most of the situations analysed. Comparing results of the first two studies, it appears that in addition to the increase in the number of samples (from five to ten trees per cultivar) and collection times (from two to four), and the robust statistical analysis conducted in the second study, the different infection pressure at the two study sites may also have influenced the results obtained for both cultivars. However, sampling of the high host canopy level in the second study yielded the

best results for both cultivars. These trends were much less evident in 'Leccino'. For this cultivar, the resistance mechanism likely reduces the pathogen multiplication rate and spread in host plants (Giampetruzzi *et al*., 2016).

For host xylem tissue samples, a difference was also noted between the two cultivars, i.e., xylem tissue from twigs gave better results than for leaf samples in the susceptible 'Ogliarola salentina'. This trend was less evident in 'Leccino'. In contrast, use of xylem tissue from twigs was the most effective for the quantitative detection of the pathogen in both varieties, to a greater extent in the case of 'Leccino'.

These results confirm the differences between the two varieties, both for symptom expression and for spatial, temporal, and quantitative distribution of *X. fastidiosa* infections.

A fluctuating trend in infection rates was observed in 'Leccino', where the infection apparently remained within a range, regardless of canopy level, tissue type, and diagnostic method. Pathogen detection decreased from June to September and increased from September to December, followed by a slight decrease in the winter period. In contrast, 'Ogliarola salentina' had a continuous increase in infection rate, reaching almost 100% in different canopy parts at the end of the sampling period.

The quantitative evolution of *X. fastidiosa* concentrations also differed between the two cultivars, for twig tissues in the June-December period. There was a decrease in bacterial concentrations in 'Leccino', with Ct values greater in twigs than in leaves. The increase in pathogen concentration in the winter months occurred irrespective of cultivars, tissue types or canopy levels. Effects of air and xylem temperatures estimated by regression models have indicated buffer effects of trunk tissues, especially for maximum temperatures occurring during summer (Román-Écija *et al*., 2022). These effects could explain the different results obtained with xylem tissues from twigs compared to those from leaves.

De Pascali *et al*. (2019) indicated that *X. fastidiosa* resistance in 'Leccino' could be linked to low resistance to water stress which enhances the defence mechanisms, as compared to 'Ogliarola salentina'. In addition, effects of temperature on *in vitro* cell cultures of the pathogen and on xylem vessel temperature dynamics in olive grove showed that extreme low or high temperatures differentially influence growth and survival of *X. fastidiosa* strains (Román-Écija *et al*. (2022). Temperatures between 4 and 10°C did not affect cell survival, while incubation at 36 and 40°C for 7 d killed the bacterial cells. However, the widest optimum growth temperature range was estimated for *X. fastidiosa* subsp. *fastidiosa* (19 to 33°C) and for subsp. *multiplex* (20 to 31°C), while *X. fastidiosa* subsp. *pauca* strains had lower optimal ranges (19 to 27°C).

The olive groves studied here, like most of those in southern Apulia, were not irrigated, and the trees in this region are usually under water stress during the summer months. This could explain the decrease of *X. fastidiosa* infection rates and pathogen populations in 'Leccino' from June to September. Also during the study period between 2018 and 2019, the temperature trends in the vicinity of the two olive groves (measured approx. 10 km away) showed that the summer was hot, with average temperatures often exceeding 36°C for whole weeks (July and August were the warmest months), followed by a mild autumn with an average minimum temperature rarely below 10°C (November was the coldest month), and a mild winter with average minimum temperature of 5 to 6°C (January, February and March were the coldest months). Low daily temperature values (1.5–3.5°C) occurred occasionally between January and February, and never exceeded more than 1 d duration (data provided by the weather station of the Regional Agency for Environmental Prevention and Protection, ARPA: http://www.webgis.arpa.puglia.it/meteo/index.php). As *X. fastidiosa* subsp. *pauca* strains have low optimal temperature ranges (19 to 27°C), the pathogen decline in both cultivars in late summer (September) was probably influenced by the persistent high temperatures during midsummer. Mild autumn and winter temperatures did not seem to inhibit the bacterium.

Results of diagnostic tests used during the second study were confirmed in the comparative study that was conducted only on asymptomatic/mildly symptomatic infected 'Leccino' trees in a suitable sampling month (October), by testing xylem stem tissues from asymptomatic branches in the mid-upper parts of the tree canopies.

Choice of the most suitable tests for *X. fastidiosa* monitoring programmes depends on several factors, but most importantly, their sensitivity. Although qPCR was the most sensitive test, in large-scale monitoring programmes, samples are usually processed with serological tests, that are less expensive and do not require sophisticated equipment. Real-time LAMP (97.3% detection) was slightly less sensitive than qPCR (98.7%), but has several advantages, as it is user-friendly and less time-consuming. It can be performed either with a real-time instrument for processing many samples, or with hand-held devices for fewer samples, allowing pathogen detection on site (Yaseen *et al*., 2015). Among serological tests, DTBIA also offers several advantages, but it requires well-trained personnel to read mild positive reactions with microscope. This is a limitation to the

use of DTBIA in most diagnostic laboratories, which often prefer to use DAS-ELISA for large-scale monitoring of *X. fastidiosa* (Djelouah *et al*., 2014). Considering the high sensitivity of both molecular assays compared to serological tests, qPCR and real-time LAMP are the preferred tests to detect low pathogen concentration in asymptomatic olive trees.

Results obtained on the 'Leccino' cultivar provide technical indications for optimizing sampling and testing procedures to increase probability of detecting new *X. fastidiosa* infections in asymptomatic olive trees, especially when buffer zones and pathogen-free areas are monitored. As recommended by EPPO and substantiated by this work, the mid-upper parts of olive canopies are the most efficient for sampling asymptomatic trees. Different from the EPPO procedure is the sampling period and the olive xylem tissues to be used in diagnostic assays. For sampling period, this should consider the environmental temperature range rather than the seasonal period, as recommended by EPPO (late spring to autumn). Thus, sampling can be conducted year-round, avoiding the warmest periods (temperatures above 36°C for weeks), and the coldest periods (temperatures below 10°C for weeks). In addition, xylem tissues of semi-hardwood twigs should be preferred over the basal parts of mature leaves with petioles for detection of the pathogen using serological and molecular assays. For the diagnostic tests recommended by EPPO, DTBIA should be the preferred test for monitoring in *X. fastidiosa*-infected and containment areas, while qPCR or real-time LAMP are appropriate for buffer zones and pathogen-free areas. However, the advantages of real-time LAMP over qPCR make this test more suitable for large-scale *X. fastidiosa* monitoring programmes.

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