An Evaluation of Monitoring Surveys of the Quarantine Bacterium *Xylella fastidiosa* Performed in Containment and Buffer Areas of Apulia, Southern Italy

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Abstract

Introduction: *Xylella fastidiosa* is a quarantine phytopathogen for the European Plant Protection Organization and currently infects olive trees in the Apulia region (southern Italy). Upon the Implementing Decision of the European Union 2016/764 of May 12, 2016, extensive monitoring surveys were performed on approximately 190 000 ha to ascertain the possible occurrence of *X. fastidiosa*.

Objectives: The primary objectives of the analysis were to start to collect epidemiological data on *X. fastidiosa* occurrence in areas far from the initial outbreaks and discuss the results of the pathogen detection.

Methods: A total of 220 279 olive trees were inspected. Basic information on farm and trees management was obtained. A total of 13 706 olive trees were analyzed through serological and molecular techniques to verify the possible occurrence of the bacterium.

Results: The cultivars “Nociara,” “Cima di Melfi,” and “Cellina di Nardò” showed the highest occurrence of decline symptoms. Tree age appears to be related to the incidence of decline symptoms. Olive trees growing in well-managed soils showed fewer symptoms than trees cultivated in farms where such agronomic techniques are not regularly performed. *X. fastidiosa* was detected in 2078 samples taken from symptomatic trees and 1653 samples obtained from asymptomatic trees. In 3300 samples taken from symptomatic trees, the bacterium was not detected.

Conclusions: Implementation and utilization of reliable in situ detection techniques could increase the number of sampled trees in each plot, thus allowing a more extensive and robust assessment of *X. fastidiosa*-infected plants in areas where the pathogen inoculums are still low.

Keywords

biocontainment, inspection, pathogen, biorisk management, infectious agent

Introduction

Xylella fastidiosa is a Gram-negative, slow-growing, xylem-limited bacterium that spreads to cultivated crops and wild plants through the feeding activities of insect vectors. It is considered among the most dangerous phytopathogens and is capable of killing many woody and herbaceous crops. Until recently, *X. fastidiosa* was restricted to the American continents, and consequently, the European Plant Protection Organization (EPPO) originally placed it in the A1 list of quarantine bacteria not present in Europe. However, starting in October 2013, many occurrences of this pathogen were reported for some European countries (ie, Italy, France, Spain, Germany, Switzerland, Portugal) and Iran.

X. fastidiosa includes some subspecies showing distinct molecular and pathogenic features. In the United States, *X. fastidiosa* subsp *pauc*, the causal agent of “citrus variegated chlorosis” in South America, is a regulated biological agent, and it is included in the Agricultural Bioterrorism Protection

Act. In Italy, *X. fastidiosa* subsp *pauc* is associated with the “olive quick decline syndrome” (OQDS), a severe disease widely occurring in Salento (Apulia region, southern Italy). The main symptoms include leaf, twig, and branch dieback followed by collapse of the crown and plant death. Most likely, the pathogen was introduced in Apulia through ornamental coffee plants originally grown in Central America and imported to Italy for commercial trade.^{1,2}

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Due to its quarantine status and according to EPPO phytosanitary legislation, measures to avoid its further spread in the region were adopted soon after the pathogen discovery in the Apulia region. Among these, there was the Implementing Decision EU 2016/764 published on May 12, 2016, that delineated the so-called containment and buffer areas bordering the northern limit of the infected area. The latter is the area where the initial outbreaks of the disease were observed and where the infection has widely spread. In this area, due to the occurrence of the vector(s) and the very high and uniform density of olive orchards, the eradication of the pathogen has not been feasible.³ According to the European quarantine legislation, within the 20 km-wide containment area, each olive tree found to be infected upon the performing of techniques developed to specifically detect *X. fastidiosa* must be uprooted. Within the 10 km-wide buffer area, more severe rules are applied, and within a radius of 100 m that starts from the infected tree, all olive trees must be uprooted. The monitoring surveys of these areas are performed by inspectors of the Apulia region, who follow ad hoc rules for sampling.

The collection of field data related to OQDS and *X. fastidiosa* is also particularly important to assess some epidemiological aspects of the disease. Indeed, despite a relevant number of studies that have been performed to elucidate some features linked to the bacterium such as its taxonomy, origin, host range, vectors, and detection, the epidemiological aspects of OQDS and *X. fastidiosa* subsp. *pauca* in Apulia are still largely unknown. For a more comprehensive understanding of olive tree decline, it is also important to analyze the information obtained from the areas where the infection is at an initial stage or absent and the symptoms of the decline are not widespread or very severe. In addition, no specific investigation on a large area has been performed aiming at ascertaining the occurrence of decline symptoms among the many olive cultivars currently grown in Apulia or verifying whether the positive or negative detection of *X. fastidiosa* is correlated with the presence of the symptoms.

To this aim, we analyzed and discuss the official data regarding 220 279 olive trees obtained by monitoring the containment and buffer areas of Taranto and Brindisi provinces carried out between September 2017 and March 2018 by inspectors of the Agenzia Regionale per le attività Irrigue e Forestali (ARIF) of the Apulia Region. These surveys followed the Implementing Decision EU 2016/764 published on May 12, 2016, and the Ministerial Decree (December 7, 2016) of the Italian Ministry of Agriculture, Food and Forestry, “Misure di emergenza per la prevenzione, il controllo e l’eradicazione di *Xylella fastidiosa* Wells et al. nel territorio della Repubblica italiana.” Previously, such an area was not intensively assessed for the presence of OQDS symptoms and the occurrence of the bacterium.

Materials and Methods

Recording of Field Data and Sampling Procedures

Between September 2017 and March 2018, the ARIF inspectors surveyed olive orchards and surrounding areas included

within both the 20 km-wide containment area and the 10 km-wide buffer area of Brindisi and Taranto provinces, as delineated by the aforementioned Ministerial Decree. The 2 areas of approximately 190 000 ha in total were divided into subareas of 250 ha, and each subarea was in turn subdivided into plots of 1 ha. Each plot was monitored to reveal the possible occurrence of olive trees showing symptoms of OQDS (ie, leaf, twig, and branch wilting or crown collapse). Before starting with the sampling, based on the direct survey or information obtained by the owner of the orchard, a notification form was compiled to reveal some basic features such as name of the cultivar, age of trees, soil management, occurrence of “olive knot,” possibly caused by *Pseudomonas savastanoi* pv *savastanoi*, and “olive leprosy,” possibly caused by *Colletotrichum* spp. Concerning *P. savastanoi* pv *savastanoi*, the typical “knot” symptoms on twigs and branches were scored, whereas for *Colletotrichum* spp, the symptoms were scored on the drupes. In the case of trees showing decline symptoms within a single plot, samples were taken from the tree showing the most extensive symptoms and from the 4 trees surrounding it. In the case of asymptomatic trees, the samples in the plot were taken at random from 1 single tree. For pathogen detection, 8 single leaf samples were taken from each selected tree according to the cardinal directions and at 2 different heights. To avoid involuntary spread of insect vector(s), each sample was checked for absence of any insect. Leaf samples were labeled, georeferenced, and placed into plastic bags. Two bags per each sample were used. Refrigerated containers were used to store the samples. A thorough check of the car to avoid the transportation of insects was performed before leaving the plot. Car windows were closed during the monitoring surveys. In the laboratory, samples were stored in refrigerated chambers maintained at 4°C ± 2°C. Mature leaves taken from woody twigs were used for detection. To ascertain the occurrence of *X. fastidiosa*, an ELISA test and subsequently, quantitative real-time PCR assay for confirmation were performed. The official procedures established by the EPPO for *X. fastidiosa* detection were followed. After the analyses’ completion, both the remaining plant parts and plastic bags and tubes were sterilized using autoclave.

Results

Occurrence of Dieback Symptoms Among the Olive Cultivars

A total of 220 279 olive trees were taken into account for checking decline symptoms and the possible occurrence of *X. fastidiosa*. In the surveys, olive trees not necessarily grown in specialized orchards were also monitored. The extensive monitoring allowed us to ascertain either the occurrence of a very diverse olive germplasm cultivated in the containment and buffer areas or the incidence of decline symptoms recorded for these cultivars. A total of 33 different olive cultivars are grown to different extents in the surveyed areas. The monitoring recorded the occurrence of putative OQDS symptoms on 17 755 trees, 8.06% of the total trees assessed. The occurrence

of decline symptoms on the major cultivars grown in the areas was as follows: “Nociara” (16.31%), “Cima di Melfi” (13.08%), “Cellina di Nardò” (10.92%), “Leccino” (8.45%), “Coratina” (7.96%), “Ogliarola salentina” (7.65%), “Ogliarola barese” (7.41%), and “Frantoio” (6.95%).

Age of Trees, Soil Management, Olive Diseases, and Dieback Symptoms

Tree age appears to be related to the incidence of decline symptoms since older trees (more than 100 years old) showed more decline symptoms (10.85%) when compared to those of younger trees (less than 50 years old), which showed decline in 6.53% of trees. Soil management could also play a role in relation to the occurrence of tree decline. Olive trees growing in well-managed soils (regular harrowing and removal of weeds) showed less symptoms (7.42%) than trees cultivated in farms where such agronomic techniques are not regularly performed (13.78%). Symptoms possibly caused by *P. savastanoi* pv *savastanoi* (olive knot) were observed on 10 180 trees, with 2351 of these trees also showing decline symptoms. The number of trees showing symptoms attributable to *Colletotrichum* spp (olive leprosy) was 3533, with 273 also showing decline. Symptoms attributable to some “disorder” of unknown origin (not OQDS) were found on 10 360 trees.

Detection of *X. Fastidiosa* in Olive Trees

ELISA and quantitative PCR analyses were applied to detect *X. fastidiosa* in samples taken from the surveyed areas. A total of 13 706 trees were analyzed, among which 8328 trees were asymptomatic and 5378 showed symptoms of decline to different extent. *X. fastidiosa* was detected in 2078 samples taken from symptomatic trees and 1653 samples obtained from asymptomatic trees. In 3300 samples taken from symptomatic trees, the pathogen was not detected (61.3% of symptomatic trees).

Discussion

These surveys allowed us to obtain useful information on some epidemiological aspects related to symptoms that could be putatively associated with the OQDS in Apulia. Leaf scorch and twig and branch dieback are among the most common symptoms associated with both OQDS and *X. fastidiosa* subsp *pauca* observed in the olive trees of the infected area (Lecce province) and are believed to precede subsequent extensive crown collapse and plant death.⁴ Leaf scorch and twig and small branch diebacks were found on 8.06% of the 220 279 olive trees monitored in those areas, and some cultivars largely grown in the surveyed areas apparently showed more symptoms than others. Indeed, the cultivars “Nociara,” “Cima di Melfi,” and “Cellina di Nardò” showed a higher dieback occurrence than that of other cultivars grown in the same area such as “Ogliarola salentina,” “Leccino,” “Coratina,” “Ogliarola barese,” and “Frantoio.”

It should be said that such symptoms are not specific to a single disease and cannot be attributable with certainty to a specific pathogen and/or insect without performing proper isolations and pathogenicity tests. Apart from *X. fastidiosa*, among the phytopathogens frequently associated with olive dieback in the Apulia region. *Phaeoacremonium* spp spread either in southern⁵ or northern⁶ Apulia as well as *Neofusicoccum parvum* and *Pleurostomophora richardsiae*, with the latter considered to be the primary cause of the decline in northern Apulia.⁷ In addition, either *P. savastanoi* pv *savastanoi*, the causal agent of olive knot or, to lesser extent, *Colletotrichum* spp, causal agents of olive leprosy, can cause diebacks in olive trees. These latter 2 phytopathogens were reported in many cases of the monitored olive trees showing or not showing leaf scorch or twig and branch diebacks. It should be noted that in the initial phase of their plant colonization, both pathogens can incite knot and leprosy symptoms without causing wilting. Aspecific diebacks in olive trees can also be caused by insects such as *Zeuzera pyrina* (leopard moth) and *Phloeotribus scarabaeoides* (olive bark beetle).

Therefore, simple visual assessment of dieback symptoms is not enough to conclude which is the causal agent of the disease. Within this context, it should be said that 3300 olive trees showing decline symptoms tested negative for the occurrence of *X. fastidiosa* upon performing the detection assay in the laboratories. In contrast, 1653 asymptomatic trees tested positive for the occurrence of the bacterium, thus confirming the sensitivity of the current detection techniques. It should be said that in the infected area (Lecce province), where *X. fastidiosa* has been present for many years and has had the possibility to spread over a very large portion of the territory, there is a higher occurrence of the pathogen in trees showing OQDS symptoms.

To note that in the infected area, for olive trees older than 70 years, from the appearance of visual symptoms (leaf, twig, and small branch dieback) and the complete collapse of the plant caused by *X. fastidiosa* subsp *pauca* could occur over a period of approximately 2 to 4 years.⁸ From an epidemiological point of view of a plant disease, this is a long period, and during this time, other phytopathogens can cocolonize the trees weakened by the bacterium, thus potentially contributing to the final collapse. Ad hoc studies taking into account adult trees and sub-sequential coinfections performed with different olive pathogens during different periods of the year would seem useful to elucidate this facet of OQDS.

Reliable detection of this bacterium in areas not still heavily infected is fundamental for the identification of olive trees infected by *X. fastidiosa*. The detection efficiency could be augmented by increasing the number of trees sampled in each single plot. Currently, 5 trees per plot are sampled for further analyses in the laboratory to ascertain the occurrence of *X. fastidiosa* in trees showing decline symptoms. The utilization of portable detection tools allowing for in situ sampling and detection of the pathogen, if adequately validated on a large scale especially for specificity and sensitivity, could increase the number of trees monitored per plot. Both real-

time loop-mediated isothermal amplification (real-time LAMP)⁹ and an electrochemical transduction method using a portable chip¹⁰ could potentially be utilized. In addition, in situ presymptomatic detection of the bacterium could potentially be performed by a portable instrument that analyzes mRNA of plant genes linked to OQDS (ie, biomarkers) using an in-field qRT-PCR assay.¹¹ In this case, the detection tool is strictly related to host response at the transcript level upon infection. If the field reliability of such techniques is confirmed by parallel analyses performed using real-time PCR, it would be possible to screen a larger number of trees per plot, thus obtaining more robust results. In addition, taking into account the long infection process characterizing *X. fastidiosa* subsp *pauc*a in olive trees, another possibility to increase the reliability of detection is to perform an additional screening on “suspected” trees a few months after the first screening.

This extensive monitoring also allowed us to observe that tree age could be related to an increase in decline symptoms. A higher incidence of OQDS for older trees has also been observed in the Gallipoli area, where the decline was first observed. Tree age appears to be related to the differential production of primary and secondary compounds linked to plant metabolism, including the defense system,¹² and differential mutation loads in meristematic tissues.¹³ However, specific studies concerning the *X. fastidiosa*–*Olea europaea* pathosystem are currently lacking.

A neglect of soil management would also seem related to a higher occurrence of olive tree decline in both the containment and buffer areas. In the farms of these areas where soil harrowing is rarely carried out or not practiced at all, there was a higher occurrence of decline symptoms. It should be stressed that limiting the spread of *X. fastidiosa* subsp *pauc*a within and between the olive orchards through the mechanical removal of weeds during the end of winter and early spring plays a fundamental role since it could drastically reduce the number of bacterium vectors in their juvenile phase.¹⁴ This agronomical practice is fundamental for limiting the vector populations.

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Not applicable

Statement of Human and Animal Rights

Not applicable

Statement of Informed Consent

Not applicable

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