Experience Gained from Efforts to Contain an Olive Decline in Southern Italy and Research Needs to Manage it in the Mediterranean Region

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Abstract

Yaseen, Thaer. 2018. Experience Gained from Efforts to Contain an Olive Decline in Southern Italy and Research Needs to Manage it in the Mediterranean Region. Arab Journal of Plant Protection, 36(1): 64-74.

Xylella fastidiosa (Xf), a xylem-limited and vector-transmitted bacterium, has subspecies (*fastidiosa, multiplex, pauca*) which are known to induce several diseases in woody and herbaceous plants (more than 360), mainly in the American continent. In 2013 the subsp. pauca strain CoDiRO, vectored by *Philaenus spumarius*, was found in Apulia region (Italy), causing the quick decline of a million olive trees with severe economic, environmental and social consequences. The Italian government and the EU Commission soon declared a state of emergency strengthening phytosanitary measures. In the demarcated area, which includes the infected and buffer zones, intensive monitoring, eradication and containment measures, vector control, movement restrictions of plants, and planting prohibition of host plants are carried out. More than 200,000 plants were tested to assess the presence and spread of the infection, which currently affects approximately 16 per cent of the national olive growing area. Sampled and infected plants were mapped, management of monitoring data was fully computerized, and several initiatives were carried out for awareness campaigns and capacity development (www.emergenzaxylella.it). In this contest, an innovative model for the surveillance of Xf was developed and provided to the Plant Protection Service to support institutional decision making. This model is multidisciplinary, multifunctional and includes multiple actors. It allows the traceability, storage, management, and analysis of different types of data using a web-based software (XylWeb). The tool combines remote sensing data, obtained through the photointerpretation of high-resolution aerial images for rapid identification of suspected symptomatic trees, with field data acquired accurately with the application XylApp. The model includes on site methods for early detection of the pathogen (real time LAMP and DTBIA) in plant material and 'spy insects'. This model is under improvement through current research initiatives.

Introduction

The quick decline syndrome of olive (OQDS) is a disease that appeared suddenly some years ago in a *Olea europaea* grove near the city of Gallipoli, on the Ionian coast of the Salento peninsula (south-east Italy), and began spreading fast in lower Salento. OQDS has been the object of several reviews (14) which readers are referred to for a more exhaustive information. These trees, especially the centuries-old ones, are often pruned heavily, forcing them to push new growth which, eventually, will wither and desiccate. The investigations carried out at Bari by the local University, a phytopathological unit of the National Reseach Council of Italy (CNR), the CIHEAM Mediterranean Agronomic Institute of Bari (MAIB) and the Centro di Ricerca, Sperimentazione e Formazione in Agricoltura Basile Caramia at Locorotondo (Bari), disclosed that in addition to other putative disease agents, OQDS-affected trees consistently hosted *Xylella fastidiosa* (Xf), a most feared quarantine pathogen, which had never been detected in any of the European Union (EU) countries. Xf is a Gram-negative bacterium of the family *Xanthomonadaceae*, that enters the xylem vessels of the hosts and is transferred from plant to plant by xylem fluid feeding insects of the family Cicadellidae. Colonization of the xylem vessels results in their clogging by the bacterial biofilm which impairs water uptake. Xf is subdivided in subspecies, four of which, Xf fastidiosa, Xf multiplex, Xf pauca and Xf sandyi, are currently retained as taxonomically valid. These subspecies have a different geographic distribution in the American continent. This seems to be the case of Salento, as suggested by the outcome of the studies underway at Bari, whose major results are listed hereafter.

i. First identification of *Xylella fastidiosa* in different plants (olive, almond, oleander) showing leaf scorch symptoms in the Salento peninsula (22).
ii. Different fungal species colonize the wood of declining olive trees of the Salento peninsula (6, 16).
iii. Finalization of serological (ELISA, DTBIA, immunoluorescence) and molecular (PCR, Real time PCR, LAMP) procedures for the reliable identification of Xf in host plants and vector (4, 7, 12, 26).
iv. Isolation in axenic culture of strain CoDiRO from olive and other naturally infected plant species (4, 8).
v. Identification of CoDiRO as a strain of Xf pauca. Molecular evidence of its identity with a bacterial isolate (ST53) of the same subspecies present in Costa Rica, a country from which it may have landed in Salento with an un identified ornamental plant (9, 12).
vi. Complete sequence of the genome of strain CoDiRO, a DNA molecule of 2.46 MB (9).

* This symposium was sponsored by the FAO Near East and North Africa Regional Office, and organized as part of the 12th Arab Congress of Plant Protection held in Hurghada, Egypt, 5-9 November 2017.

http://dx.doi.org/10.22268/AJPP-036.1.064074

vii. Identification of the spittlebug *Philaenus spumarius* (family Aphrophoridae) as the main, if not the only, vector of strain CoDiRO, and determination of its biological cycle (5, 23).

viii. Elecron microscopic detection and identiication by gold immunolabelling of the bacterium in xylem vessels of infected plants and in the foregut of the spittlebug vector (4, 5).

ix. Identification of 22 alternative hosts of strain CoDiRO in the province of Lecce out of more than 600 trees, shrubs and weeds analysed, including grapevines and citrus (18).

x. Experimental evidence that upon mechanical inoculation with bacterial cultures, strain CoDiRO does not infect grapevines (cv. Cabernet sauvignon) and citrus (orange Madame Vinous and Navelina, mandarin, grapefruit Duncan, citranges Carrizo, Troyer and C35), whereas it multiplies readily in olive seedlings and in rooted cuttings of cv. Cellina di Nardù and other olive cultivars (Coratina, Frantoio, Leccino), and oleander (23).

xi. Complete sequence of the genome of CO33, a coffee-infecting isolate of *Xylella fastidiosa* intercepted in northern Italy, a DNA molecule of 2.68 MB (9).

xii. Host plants exposed to infective *Philaenus spumarius* in the field are infected at different rates. Xf was detected by laboratory assays in still symptomless olive plants as soon as six months after caging with infective vectors (20).

xiii. When bait plants of young trees of olive, oleander, citrus, grapevine and almond were planted in diseased olive orchards for exposure to infective vectors, only olives and oleanders became infected within 12 months and started to show symptoms 16-18 months after planting (20).

xiv. Koch's postulates were fulfilled upon mechanical inoculation of different hosts (olive, Polygala myrtifolia, oleander) with pure cultures of strain CoDiRO (20). The Salentinian strain of Xf pauca is a primary pathogen causing desiccation and necrosis of inoculated susceptible hosts.

xv. Comparative analysis of the transcriptome of infected and healthy plants of cvs Leccino and *Ogliarola salentina* showed that genes coding for receptor-like kinases (RLK) and receptor like proteins (RLP) involved in plant defence responses are differentially expressed in the two cultivars. Partial resistance of cv. Leccino to strain CoDiRO seems to be expressed essentially through a remarkable reduction of the bacterial population, i.e. 130.000 CFU/ml of tissue extract in cv. Leccino vs 2,094,000 CFU/ml in cv. *Ogliarola salentina* (10).

Being a quarantine pathogen, Xf is regulated by EU Directive 2000/29/CE, which must be enforced in all member States, Italy included. This Directive dictates the protective measures to be implemented against the introduction and spreading of such pathogens in the EU territory. Eradication is mandatory or, should this be no longer possible, measures must be adopted for restraining pathogen dissemination. Based on the knowledge acquired with the above-listed investigations, a plan was envisaged by the Italian Ministry of Agriculture and Forestry for confining the contagion within the province of Lecce, its current boundaries, through the control of *P. spumarius*, the OQDS vector:

(i) mechanical weeding against the larval stages;
(ii) chemical treatments against the adults;
(iii) uprooting alternative hosts and infected olive trees in newly identified foci. Stumbling blocks have prevented the enforcement of this plan, thus the disease is moving north, and has reached the neighbouring provinces of Brindisi and Taranto.

From science to policy to manage *Xylella fastidiosa* emergency

Given the very serious threat to the agriculture and environment, *Xylella fastidiosa* was added to the EPPO A1 list of pests recommended for regulation as quarantine pests in 1981. Following the recent detection of the bacterium in Italy and France, the EPPO (European and Mediterranean Plant Protection Organization) member countries have agreed to start several activities under the coordination of the EPPO Secretariat. In October 2013, in view of the high profile of the outbreak of *X. fastidiosa* in Europe, the EPPO Working Party on Phytosanitary Regulations agreed that the EPPO Diagnostic protocol on *X. fastidiosa* should be revised (previous version dated from 2004) and two Inspection Standards on *X. fastidiosa* should be prepared. The three standards were prepared and sent to members for approval through an oficial EPPO country consultation. The National Plant Protection Organisations will provide their feedbacks on the documents whose content will be amended accordingly. The Standards on Phytosanitary procedures for inspection of places of production and on Phytosanitary procedures for inspection of consignments have been prepared under the leadership of the EPPO Panel on Phytosanitary Inspections. The first document describes the procedures for inspection of places of production of plants for planting which are susceptible to *X. fastidiosa* for export or for internal country movements. The second one describes the procedures for inspection of consignments for detection of *X. fastidiosa* on host plants and insect vectors. The main content of these Standards is presented below. Descriptions of symptoms in the main host plants are presented to support visual inspection and selection of plant material. Recommendations on how to sample are also provided. These recommendations are as follows. In the case of symptomatic plants, the sample should consist of branches/cuttings representative of the symptoms seen on the plant and containing at least 10 to 25 leaves (depending on leaf size). The Standard recommends that symptomatic plant material should preferably be collected from a single plant; however, a pooled sample may also be collected from several plants showing similar symptoms. For asymptomatic plants, the sample should be representative of the entire aerial part of the plant. Foliage, branch lets, leaves and all accessible container surfaces, including loor or walls, should be examined to look for live insect vectors. The size of the unit of inspection (minimum number of individuals to be

examined) to be selected for inspection at a specified level of infection in a specified lot size, is given according to ISPM no. 31 Methodologies for sampling of consignments. To maximize the likelihood of detection, inspections and sampling during the period of active growth and after warm periods is recommended. For outdoor plants in Europe this period is usually between late spring up to autumn. For tropical plant species grown indoors such as coffee plants, sampling all year round is considered appropriate. Sampling after warm periods (e.g. late summer-early autumn) increases the probability for an accurate bacterial detection.

An Expert Working Group was formed for the revision of the EPPO Diagnostic protocol on *Xylella fastidiosa* (PM 7/24). In this Standard, recommendations for the preparation of the sample in the laboratory are provided, based on the type of sample (individual plants, composite samples, dormant plants and cuttings) and on the host plants and type of tissue (petioles, midribs, leaves, etc.). The screening tests described in the Standard are either serological (immunoluorescence, direct tissue blot immunoassay -DTBIA-, enzyme-linked immunosorbent assay -ELISA-) or molecular (conventional PCR, real-time PCR, loop mediated isothermal ampliication -LAMP-). Testing for asymptomatic plants in an outbreak area or a buffer zone around an outbreak often implies that a high number of tests need to be performed. In such a situation and given that the concentration of the bacterium is expected to be higher than in an area thought to be pest free, a single test including serological tests (e.g. ELISA) may be performed. Unlike other EPPO protocols for bacteria, isolation is not recommended as a screening test as the bacterium is very difficult to isolate. Subspecies determination by molecular tests (PCR for multi locus sequence typing, conventional PCRs, multiplex PCR) and/or sequencing analysis should then be performed. Validation data for most of the tests included in the EPPO Diagnostic protocol are available from the EPPO Diagnostic Expertise Database http://dc.eppo.int/validationlist.php. Regular meetings are organised with other international organisations sharing an interest in *X. fastidiosa*, such as the International Plant Protection Convention Secretariat, the Near-East Plant Protection Organization, the European Commission, the European Food Safety Agency and the International Olive Council, to avoid duplication of efforts.

Insect vectors of *X. fastidiosa* in Italy

The transmission of *X. fastidiosa* is not specific, and all xylem sap sucking insects are considered potential vectors. Furthermore, transmission efficiency varies depending on insect vector species, *X. fastidiosa* genotype and host plant (19). Recently, Saponari *et al.* (22) and Cariddi *et al.* (4) detected, isolated and confirmed the presence of *X. fastidiosa* in olive trees, oleander and almond in south-eastern Italy as the first record in the European Union. Pathogenetic tests confirmed the bacterium responsibility for a new disease: the olive quick decline syndrome (OQDS or CoDiRO) (20). The syndrome begins with severe leaf scorch and scattered twigs desiccation of the upper part of the canopy. Later, the symptoms expand on the plant until the host death (14). The disease is lethal, and knowledge of the candidate and actual *X. fastidiosa* vectors are crucial for a correct risk assessment of this threat. The xylem sap-sucking insect species present in Europe are seven sharpshooters (Cicadellidae, Cicadellinae), twenty-six spittlebugs (Aphrophoridae), seven Cercopidae, fifty-four cicadas (Cicadidae and Tibicinidae) (3). Of these, only two species were considered potential vectors of the bacterium in Europe, *Philaenus spumarius* (L.) and *Cicadella viridis* (L.). Surveys over the infected or diseased olive groves found three homopteran species positive to *X. fastidiosa*. Namely, two species belonging to Aphrophoridae: *P. spumarius* and *Neophilaenus campestris* Fallèn and one species of Cicadellidae: Euscelis lineolatus Brullè (2, 8). Transmission tests with insects collected in infected olive groves showed the ability of *P. spumarius* to acquire and transmit the bacterium among olive trees and other host plants (5, 23). By the time, *P. spumarius* was the only vector of *X. fastidiosa* in Europe. On-going studies will help understand the role of other xylem-sap feeder vector species candidates in the spread of the pathogenic bacterium.

State of the art of the research on *X. fastidiosa* in Puglia

Taxonomic position of the Puglia strain- Multilocus sequence typing (MLST) for *X. fastidiosa*, first introduced by Scally *et al.* (24) and then reined by (27), has been successfully used to study *X. fastidiosa* diversity at the species/subspecies level, and to infer the phylogenetic placement of newly identified isolates. The MLST data have resulted in a robust taxonomy for the species and for the classification of isolates into sequence types (STs) (unique genotypes based on the 7 loci used in MLST) (1).

MLST analysis of the *X. fastidiosa* isolates recovered from different infected olive trees found in the first outbreak (Gallipoli district) proved that all isolates collected belonged to ST53 (8, 12). Until to now, isolates with the same ST are known to occur only in Costa Rica, associated to *X. fastidiosa* infections on oleander and coffee plants (17). The phylogenetic network derived from all MLST data publicly available indicated that ST53 clusters with *X. fastidiosa* subspecies pauca. Further molecular investigations assessed that this ST is the only allelic profile associated with the isolates causing infections in olive trees located in geographically distant foci in Puglia and in different naturally infected hosts found in the Puglia infected area (12, 13, 21).

Host range and pathogenicity tests- Preliminary data on the susceptibility of ornamentals (i.e. oleander and Polygala myrtifolia) important tree species (i.e. olive, stone fruit, citrus, grape and holm oak) in the EU to the Puglia strain of *X. fastidiosa* were obtained by conducting small-scale experiments under controlled and field conditions. Needle inoculations with the *X. fastidiosa* strain CoDiRO and exposure of plants to natural infective vector populations have provided critical information that substantiated the field observations made in the last 2 years after the discovery of the first Puglia outbreak. In summary:

(i) olives appear to be highly susceptible to infections caused by isolates of the subspecies pauca, and in particular to the strain CoDiRO;
(ii) olive cultivars display a differential response to *X. fastidiosa* infection, multiplication and movement;
(iii) upon systemic infections, symptoms similar to those observed in the affected ield (desiccation and dieback) were observed on inoculated olive plants;
(iv) amongst the cultivars tested, Cellina di Nardù clearly resulted as the most susceptible to the CoDiRO strain (20).

Field experiments with exposure of young plants to natural inoculum and to naturally infective specimens of *P. spumarius* resulted in successful infection of the known host plants (olive, oleander and *P. myrtifolia*); conversely no transmission occurred on grapevines, citrus and holm oak. So far no symptoms have been observed on the infected plants under field conditions.

Major research outcomes - Sequence analyses on the *X. fastidiosa* isolates recovered from different hosts and foci in Puglia showed that all infected samples harboured a single ST, denoted as ST53, with phylogenetic relationships with the subspecies pauca. The finding that isolates with the same ST53 have been so far identified only in Costa Rica, provided evidence toward the better understanding of the introduction pathways in the EU, and supports the single introduction hypothesis associated to the Puglia outbreak.
Artificial inoculations confirmed that olives, oleander and myrtle-leaf milkwort support systemic infections of *X. fastidiosa* strain CoDiRO, and develop symptoms resembling those observed in the outbreak area. These results contributed to disclosing the etiology of the olive quick decline syndrome and the role of *X. fastidiosa* strain CoDiRO in this novel olive disease.

Upon artificial inoculations and the ield exposure of citrus, grapevines and holm oak, no successful systemic infections were detected on these plant species, which so far appear to be not susceptible to *X. fastidiosa* strain CoDiRO.

Legislative aspects for the mandatory control of *X. fastidiosa* in Puglia and in Italy- Puglia is the main olive-growing region in Italy (32% of the national olive-growing area) and olive trees cover 29% of the Puglia agricultural surface. In summer 2013, after the presence of the quarantine bacterium *Xylella fastidiosa*, subsp. pauca, strain CoDiRO as the main cause associated to the OQDS. Following the indications of the EU Directive 2000/29 (08.05.2000), the Regional Plant Protection Service of Puglia (RPPS) immediately communicated the finding to the Ministry of Agriculture, Food and Forestry Policies (MiPAAF) and to the European Commission; a series of measures and actions against *X. fastidiosa* were soon taken (Table 1).

A large scale monitoring of the pathogen was conducted soon after the finding in the entire Region by analysing over 16,000 host plant samples (primarily olive trees) and delimiting the infected and buffer zones. The movement of plants from the infected area was blocked and strict measures were adopted for nurseries and producers. The dramatic nature of the emergency and the increasing extent of the infection prompted MiPAAF in 2014 to adopt urgent measures for the containment of the bacterium across the whole Puglia. In February 2015, the Italian Council of Ministers declared a state of emergency and appointed a Commissioner (head of the Civil Protection department) and a national scientific committee for advising technical decisions. The Commissioner adopted all available means in order to prevent the pathogen from further spreading, thereby endangering olive cultivation in Puglia, in Italy, in Europe and the whole Mediterranean region. Several emergency measures were then implemented, with regard to territorial management and in response to the measures of the European Commission. An Action Plan for the rapid implementation of the mandatory control measures against *Xylella*, as indicated in the Ministerial Decree (MD) no. 2777 (26.09.2014) was applied throughout 2015. The main measures were: the elimination of infected plants, in order to reduce possible pathogen inoculum, and the containment of the insect vector population, *Philaenus spumarius*, also known as “meadow spittlebug”, which is the only confirmed vector in Puglia.

Following the MD issued on 19.06.2015, official investigations were conducted on host plants and insect vectors in all Italian regions, primarily in areas considered at higher risk of introduction of Xylella (nurseries, garden centres and production sites). A total of 17186 sites were inspected and 13766 analyses were performed without any finding of the infection. In the region of Puglia, a similar number of sites were also inspected (17124 sites) but with a higher number of analysed samples (50.488 samples).

Following the EU Commission Implementing Decision 2015/789, phytosanitary measures were issued based on the new demarcated area, which includes the infected zone and buffer zone (10km surrounding the infected zone). Intensive monitoring, eradication and containment measures, vector control, movement restrictions of plants, planting prohibition of host plants are the main actions conducted in the buffer zone and in the infected zone surrounding the buffer zone (a 20km-wide strip). As for the buffer zone, in addition to the infected plants, all pathogen host species are removed in a radius of 100mt around the infected plant/s, regardless of their health status.

Since its first discovery in 2013, more than 200.000 plants have been tested mainly in the buffer and containment areas with the aim of determining the presence and spread of the infection for the application of eradication/containment measures. The spread of the infection covers approximately 180000 ha, i.e. 16% of the national olive-growing area. Sampled and infected plants in the demarcated area have been mapped and the management of the monitoring data has been fully computerized. The graphical representation of the areas monitored and their results are available on the official website of the Puglia Region (www.emergenzaxylella.it). A series of different activities took place in order to raise awareness such as dedicated website, meeting with farmers, distribution of 16.000 informative leaflets and others. Plant Protection Service, Forestry and Municipality police (about 500 units) have been employed in the application of the phytosanitary measures as indicated in the contingency plan against *X. fastidiosa*.

Table 1. Synthesis of the main actions taken after the first finding of Xylella fastidiosa in EU (2013-2016) by Regione Puglia, Ministero Italiano delle Politiche Agricole, Alimentari e Forestali (MiPAAF) and European Commission

2013	
October	OFFICIAL COMMUNICATION OF *XYELLA FASTIDIOSA* FINDING IN PUGLIA REGION Measures for the movement of host plants Temporal Blocking of plants movement from the Province of Lecce (Apulia) Provisions on the implementation measures for *X. fastidiosa* in Puglia region
November	1st Monitoring of *X. fastidiosa* for the definition of the delimited areas
2014	
February	COMMISSION IMPLEMENTING DECISION (EU) 2014/87 as regards measures to prevent the introduction into and the spread within the Union of *X. fastidiosa*
March April	Removal of infected trees in Apulia region Definition of the outbreak area of Apulia region
July	COMMISSION IMPLEMENTING DECISION (EU) 2014/497 as regards measures to prevent the introduction into and the spread within the Union of *X. fastidiosa*
July September	Definition of the infected and buffer zones Regional Council Deliberation of Puglia n. 1824 – Declaration of the extraordinary phytosanitary emergency for *X. fastidiosa* Ministerial Decree – Establishment of the national Technical Scientific Committee of *X. fastidiosa* Ministerial Decree – Emergency measures for the prevention, control and eradication of *X. fastidiosa* in the Italian territory Guidelines for the containment of the spread of *X. fastidiosa* sub. pauca , strain CoDiRO in Puglia region
October	2nd Monitoring of *X. fastidiosa* for the definition of the delimited areas in Puglia region
2015	
February March	Appointment of a special Commissioner for emergency of *X. fastidiosa* Action plan for implementing measures for *X. fastidiosa* in Puglia region
March	Definition of the delimited areas for *X. fastidiosa* in Puglia region COMMISSION IMPLEMENTING DECISION (EU) 2015/789 as regards measures to prevent the introduction into and the spread within the Union of *X. fastidiosa*
June September	Ministerial Decree-National surveys and updating of the demarcated area in Apulia New Action Plan for *X. fastidiosa* in Puglia region Removal of spontaneous/host plants Control of vectors Pruning of olive trees removing symptomatic parts Strengthening of checks on the movement of specified plants Surveys activities Removal of infected plants and host plants in 100mt radius
December	Definition of the delimited areas for *X. fastidiosa* in Puglia region Ministerial Decree – Extension of financial contribution to the farmers
December	COMMISSION IMPLEMENTING DECISION (EU) 2015/2417 amending Implementing Decision (EU) 2015/789 as regards measures to prevent the introduction into and the spread within the Union of *X. fastidiosa*
2016	
February	Ministerial Decree – Official recognition of pest-free areas in all Italian regions, with the exception of the demarcated area in Apulia
May	COMMISSION IMPLEMENTING DECISION (EU) 2016/764 amending Implementing Decision (EU) 2015/789 as regards measures to prevent the introduction into and the spread within the Union of *X. fastidiosa*
September	3rd Monitoring of *X. fastidiosa* for updating the demarcated areas

Innovative tools for early detection methods of *X. fastidiosa*

The severe threat posed by *X. fastidiosa* in Italy prompted the Italian Ministry of Agriculture to declare a state of emergency for *X. fastidiosa*. To this aim, a special Commissioner was soon appointed and a national scientific committee was established for advising technical decisions.

An action plan was established for implementing measures as for the:

(i) removal of host plants located near roads, canals, green areas;
(ii) control of young stages of the vectors on ground vegetation;
(iii) phytosanitary treatments for the control of adult vectors;
(iv) removal of infected plants;

(v) destruction of host species in nurseries; and other horizontal activities.

Based on the infection status, a demarcated area was established, which includes the infected zone and buffer zone (10km surrounding the infected zone). In the infected zone, where the pathogen is considered established, measures concern planting prohibition of EU host plants, while intensive monitoring and the removal of infected plants are restricted to a 20km-wide strip at the border with the buffer zone. In the buffer zone, where the pathogen is not present, measures concern the intensified monitoring of specified plants, vector control, movement restrictions out of the buffer zone. In all the above zones, the infected plants have been mapped and the management of the monitoring data has been fully computerized. The graphical representation of the areas monitored and their results are available on the official website of the Puglia Region (www.emergenzaxylella.it).

The early surveillance and detection of *X. fastidiosa* is so difficult that it is necessary to develop an efficient and sustainable management of the infection. It should be based on a thorough knowledge of the: territory (e.g. cartography, land cover), time and space evolution of the infection since its first outbreak, priority risky sites to be monitored, diagnostic protocols to be applied, etc..

The surveillance low of both qualitative and quantitative data should be managed properly in order to provide accurate indications to the National Plant Protection Body for the application of control measures. Relatively new approaches as the remote sensing coupled with the availability of large-scale datasets, the rapid development of computer technology and biotechnology, are leading to considerable improvements in strategic and tactical decision making on plant disease surveillance and management. To this aim, the system developed by CIHEAM Bari for the official surveillance of *X. fastidiosa* in South of Italy is aimed to early detect the pathogen integrating innovative tools for: territorial analyses (e.g. photointerpretation of aerial images), accurate onsite data acquisition (XylApp), rapid on-site pathogen detection (DTBIA, real-time LAMP) in plant material and spy insects. This surveillance system, which is multidisciplinary, multifunctional and multi-actors, allows the traceability of different types of data which converge in a central server (XylWeb) for their rapid storage and analysis. The main components of this system are here after briefly described.

The assisted photo interpretation of high resolution aerial images was developed for the rapid recognition of olive trees showing OQD-like symptoms on a large scale, being this species the primary host of the CodiRO strain in Puglia region. This approach allows the implementation of precision intervention at local and territorial levels. All suspected infected sites were investigated by visual observations for confirming OQD symptoms and assessing the presence of the pathogen. This method can also provide indications on: the presence of symptoms in olive trees which have been pruned before visual inspections; the application of some measures included in the action plan (e.g. soil tillage in spring to significantly reduce vector juvenile's populations), etc.

XylApp is an application for android systems, designed and developed with the aim of facilitating, optimizing and rationalising collection, geolocalisation and storage of data related to plant (e.g. OQDS photointerpreted trees) and/or insect samples (vectors and spy insects) collected in the field during the monitoring phase. The application consists of five independent modules for on-site data acquisition by inspectors: Sampling, Explore & Sampling, Find, Archive and Vademecum.

The spy insects approach is based on the monitoring of insect vectors or potential vectors of *X. fastidiosa*, which have been assessed to harbour the pathogen as *P. spumarius*, the only assessed vector, *Neophilaenus campestris* and *Euscelis lineolatus* (2, 8). The detection of the pathogen in these insect species can early reveal the presence of the infection before symptom development in the buffer zone and in the pathogen free area. Due to the different dynamics of seasonal population of the spy insects in Puglia region, their monitoring can be carried out during the whole year.

On-site rapid detection of *X. fastidiosa* has been developed using the real time LAMP (loopmediated isothermal ampliication) and DTBIA (Direct Tissue Blot ImmunoAssay). The real time LAMP can be totally performed on site in plants and 'spy insects' using a ield device (26) while, in the case of DTBIA, membranes can be printed in the ield with plant material and processed in laboratory (7). However, in both cases the movement of infected plant material in Xylella-free areas for pathogen testing can be avoided.

XylWeb is a web-based software for the collection, storage and management of surveillance data for X. *fastidiosa*. This software represents the core of the surveillance system in which all data converge, e.g. daily data acquired by XylApp are transmitted in real time to XylWeb. XylWeb allows data traceability and real time analyses for producing reports and other elaborates. The application consists of the following independent modules: Sample; Processing; Browse; Management; Downloads; and Links. Its implementation with the regional cartography provides a clear map on the distribution of the samples, infected plants, etc..

In the framework of this surveillance system in South of Italy, more than 100 000 diagnostic tests have been conducted (in the buffer zone and the 20km-wide strip of the infected zone surrounding the buffer zone) with the aim of determining the presence and spread of the infection, thus applying eradication/containment measures as indicated in the Commission Implementing Decision EU 2015/789.

IT platform based on smart device and web application for the survey of *X. fastidiosa*

The assessment of the bacterium in the host plants, primarily on olive trees which show the Olive Quick Decline Syndrome (OQDS), and the control of its spread throughout Puglia have raised attention on the importance of early identification of the infection in the surveillance programme on large scale. Therefore, the timely collection of information on the host plant species, their geographical position, presence of symptoms, presence of the vector(s),

characteristics of the surrounding environment and pathogen detection is the first step for the survey and statistical forecast of the infection. At the beginning, the survey on *X. fastidiosa* in Puglia was managed through the traditional approach based on field data collection with the use of GPS devices, maps, etc. This method brought up wrong data in time and space, inconsistent statistics mainly due to the huge mass of geographic and alphanumeric information acquired and their processing by operators. A new approach was set up on the use of Information and Communication Technology (ICT) for the management and processing of survey data for *X. fastidiosa* with a focus on the sample collection, coding, transmission, storage, phytosanitary analysis, management and visualization on a map. The IT architecture is made up of two specific types of software, XylApp and XylWeb, developed for Android mobile clients and for the web-based data collection unit (server), respectively, as reported in the Figure 1.

XylApp, installed on tablet/smartphone, combines the acquisition of field data with the GPS-GLONASS satellite positioning; it is thus possible to overlap vectorial maps or rasters, grids having different cartographic scale or official demarcation areas, which are available in the mobile terminals both off-line and on-line (2G/3G/4G/WiFi). Once data are acquired, the application generates an encryption, stores it and transfers it to the server in real time through functions, which make the app user-friendly, robust and accurate.

The Spy Insects approach for monitoring *X. fastidiosa* in absence of symptomatic plants

The monitoring of the infection in absence of symptomatic hosts in the buffer zone and pathogen-free areas is difficult and requires a randomised sampling for pathogen detection. Due to the quick dissemination of *X. fastidiosa* in Puglia, an effective approach was therefore developed for the early detection of the bacterium in the symptomless areas. The three Auchenorrhyncha specimens *P. spumarius*, *N. campestris* and *E. lineolatus* are used as spy insects, i.e. as indicators of the presence of *X. fastidiosa* in apparently uncontaminated areas (2). They have a different seasonal population density which allows the possibility to monitor the pathogen through the whole year. From spring to early autumn, *P. spumarius* followed by *N. campestris* are the most numerous for sampling, while *E. lineolatus* is more frequent in autumn and winter months. A site/submesh is identified and georeferenced, selecting areas with high presence of pathogen host plants. Insects are mainly collected from the ground vegetation or from the host plants using about 10 sweeps with the sweeping net. However, a D-Vac or yellow sticky traps may be also used but are less efficient. Adults of spy insects are carefully collected by aspiration directly in loco, put in small tubes containing 70% ethanol, codified and brought to the laboratory for testing and, eventually, identification. If few specimens or no specimen are collected, it is preferred to change the site or combine the collection of 2 sites for a total amount of about 10 adults/site.

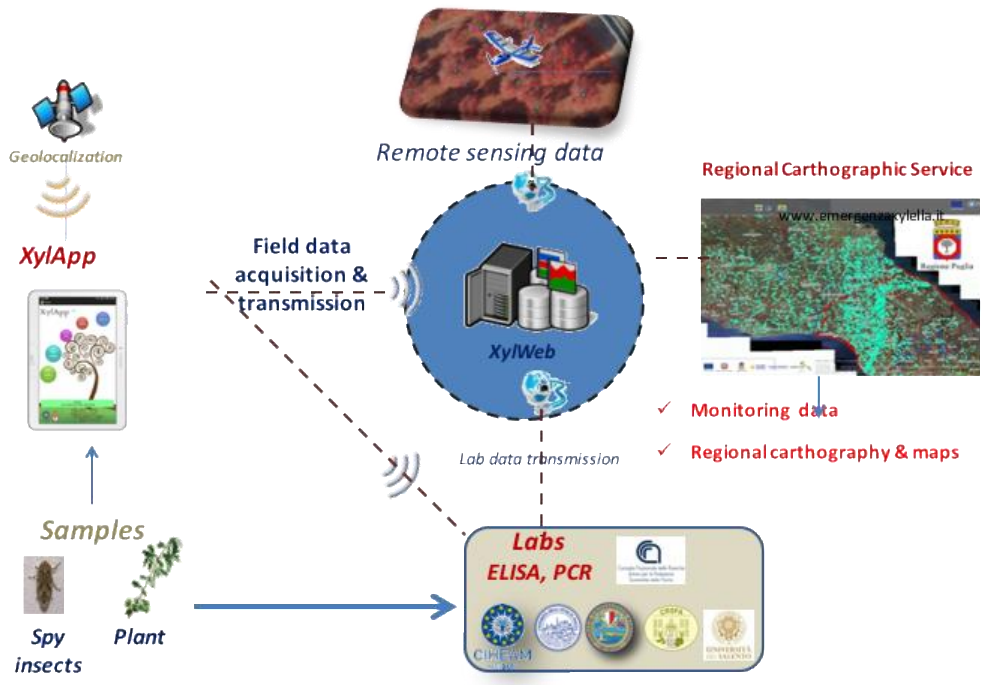

Figure 1. Innovative model for the surveillance of *Xylella fastidiosa*

The list of the samples and relative code numbers is sent as excel file through XylApp, the application used for field data acquisition, to the laboratory for analyses and to the central web server, XylWeb.

The bacterium is successfully detected in insects by molecular assays (real time PCR and real time LAMP). Nonetheless, real time LAMP is the preferred method because it is fast and accurate; moreover, the use of the field device allows the on-site detection of *X. fastidiosa* in insects and plant material (26). After testing results, only the positive insects were identified. Once a positive insect is found, the monitoring of the infection is carried out in a more capillary way in a 100mt radius from the positive sampled site, either collecting plant material from all plant hosts either or capturing other insects specimens.

The presence of infected insects has two possible explanations: the first one is that the insects have acquired the bacterium from symptomless infected host plants present in the apparently Xf-free area; the second one is that the insects could have acquired the bacterium in the outbreak area and moved to the pathogen-free area also through indirect transport.

This approach is effective for the early detection of the pathogen in the buffer zone and in the pathogen-free areas. Sampled site for insect captures should be located in the risky points of introduction (e.g. existing trade patterns, traffic ways, nurseries and sites where plants originating in risky areas are grown or kept).

Real time Loop-Mediated Isothermal Amplification (RT-LAMP) system: simple and rapid user-friendly detection method of *X. fastidiosa* from plant materials, plant sap and insect vectors

In Apulia region, a large-scale monitoring campaign was implemented by the Regional Plant Protection Service in order to demarcate the contaminated area boundaries and to implement adequate control measures. To this aim, Enzyme linked Immuno Sorbent Assays (ELISA) and Polymerase Chain Reaction (PCR) assays were largely used. Since the movement of large amounts of infectious materials to the laboratories for testing greatly exposes "*X. fastidiosa*-free areas" to the risk of contamination, the use of rapid and on-site detection methods was highly desirable. The suitability of a new Real time Loop-mediated isothermal amplification (RT-LAMP) system (Enbiotech s.r.l.- Italy), composed of a portable instrument (icgene mini) and a ready to use diagnostic kit denominated "Xylella Screen Glow" (Enbiotech s.r.l.- Italy), was therefore evaluated in this study for the detection of *X. fastidiosa* in host plants and insects. To this aim, its specificity and sensitivity were compared with those of PCR and real-time qPCR assays.

For PCR assay, *X. fastidiosa* RNA polymerase gene was amplified using RST31 and RST33 specific primers (15). Quantitative Real-time PCR (qPCR) assay was performed to amplify the *X. fastidiosa rimM* gene (11). Real-time LAMP assay was carried out using Enbiotech's LAMP system, the system envisages a rapid preliminary nucleic acid extraction from the sample, genetic amplification using LAMP technology, detection of the fluorescence emitted and automatic interpretation of results. The kit contains ready to use 0.2ml strip with extraction buffer and another with dried primers. Ready to use LAMP master mix stable at room temperature is placed in separate 2ml tube. Amplification conditions were set at 65°C for 25 min.

For the specificity tests through PCR, real-time qPCR and real-time LAMP methods, pure cultures of *X. fastidiosa* and a group of 19 non-target bacterial species and/or patovars were used. All bacterial species were grown on Nutrient Agar media while *X. fastidiosa* was grown on buffered cysteine-yeast extract (BCYE) agar medium (25) as a control. The bacterial DNAs were extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich s.r.l., Milan, Italy) and quantified by spectrophotometric absorbance at 260 nm (A260).

Identical results were obtained for all three techniques adopted, as only *X. fastidiosa* DNA was amplified and no aspecific amplification was observed. Moreover, using the real-time LAMP system, *X. fastidiosa* was detected after only 15 min, while qPCR and PCR required about 30 min and 1.5 h, respectively.

Sensitivity tests were performed on DNA obtained from *X. fastidiosa* pure cultures harvested from BCYE agar medium and from artificially inoculated olive plants. Serial decimal DNA dilutions from 10 ng/µl to 1 fg/µl were prepared and analysed through all three diagnostic techniques.

Healthy olive extract used for diluting the bacteria suspension was obtained following two different procedures. In the first one, excised petioles and midribs (0.3-0.5 g) were extracted in the presence of Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer and homogenized. The serial diluted extracts were heated at 65°C for 30 min and centrifuged at 10.000 rpm for 5 min. Then the DNA was extracted from the supernatant by mixing in an equal volume of chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol, after incubation at -20°C for 1h. In the second procedure, sap was extracted from olive cuttings by injecting with a syringe 100µl extraction buffer through the plant shoot vessels. The serial dilutions of *X. fastidiosa* were directly used for the real-time LAMP reaction, after incubation at 65°C for 10 min. The remaining part of the extract was purified through a QIA shredder mini spin column (DNeasy Plant Mini kit, Qiagen, Milan, Italy), for using in the Real-time PCR and PCR sensitivity assays.

X. fastidiosa DNA extracted from pure culture successfully amplified by PCR and qPCR until a DNA concentration of 0.1 pg/µl; only real-time LAMP was able to detect up to 10 fg/µl concentration.

Real-time LAMP system allowed to detect the presence of *X. fastidiosa* DNA at a very low concentration within a shorter reaction time (20 min instead of 40 and 90 min required by qPCR and PCR assays, respectively), RT-LAMP demonstrates to be the only method able to detect *X. fastidiosa* using the cud extract from the plant or from insect vector. In other hand, using the crud extract qPCR and PCR assays were not able to detect the DNA of the bacterium, because of the presence of PCR reaction inhibitors, those inhibitors did not influence RT-LAMP reaction.

Using sap extracted from plant tissue, real-time LAMP method allowed the detection of *X. fastidiosa* DNA from a concentration of 10^5 up to 10 CFU/ml with both purified and non-purified sap extracts, similarly to qPCR analysis. Conversely, the PCR assay showed to be highly influenced by the extraction method adopted, since it was unable to detect *X. fastidiosa* DNA from olive sap extracts in the samples containing less than 10^4 CFU/ml.

Unlike the other two techniques, the sensitivity of Enbiotech's real-time LAMP system showed to be not affected by the grade of purity of DNA samples and required shorter amplification times. In addition, the Enbiotech's real-time LAMP system did not require laborious sample preparation and expensive equipment, thus being applied also by non-specialized personnel.

These results, together with the simplicity of the extraction procedure and the brief reaction time required, make the Enbiotech's real-time LAMP, user friendly, easy to use, and highly suitable system for *X. fastidiosa* detection directly in the field, thus minimizing the risk of carrying infectious plant material (and vectors) in disease-free areas.

الملخص

ياسين، ثائر. 2018. الخبرة المكتسبة من الجهود المبذولة لاحتواء مرض تدهور أشجار الزيتون في جنوب إيطاليا والاحتياجات البحثية لإدارة المرض في منطقة البحر المتوسط. مجلة وقاية النبات العربية، 36(1): 64-74.

Xylella fastidiosa (XF) هي بكتريا ممرضة محدودة الإنتشار في خشب النبات وتنتقل عبر النواقل الحشرية. للبكتريا عدة تحت أنواع (*X. fastidiosa*، *X. multiplex*، *X. pauca*) المعروفة بأنها تسبب عدداً كبيراً من الأمراض في أكثر من 360 من النباتات الخشبية والعشبية، وبشكل خاص في القارة الأمريكية. في عام 2013، تم الكشف عن وجود تحت النوع باوكا، سلالة سميت بالكوديرو (CoDiRO) من هذه البكتريا في منطقة بوليا (إيطاليا)، والتي تنقل بحشرة *Philaenus spumarius*، مما تسبب في تدهور سريع للحالة الصحية للملايين من أشجار الزيتون مع عواقب اقتصادية وبيئية واجتماعية خطيرة. أعلنت الحكومة الايطالية والمفوضية الأوروبية فوراً حالة الطوارئ مع تعزيز تدابير الصحة النباتية وفي المنطقة المرسمة، والتي تشمل المناطق المصابة والمنطقة العازلة، تم القيام بمراقبة مشددة وتدابير استئصال واحتواء للمرض، ومكافحة ناقلات المرض، وتقييد نقل النباتات العائلة و حظر زرع النباتات العائلة. كما و قد تم اختبار أكثر من 200,000 نبات لتقويم وجود وحصر انتشار العدوى. تمثل المنطقة المصابة حالياً ما يقرب من 16 في المائة من مساحة زراعة الزيتون في إيطاليا. تم أخذ عينات ووضع خرائط للنباتات المصابة، كما وقد تم إدارة بيانات الرصد حاسوبياً بشكل كامل، كما تم القيام بعدة مبادرات من أجل حملات التوعية وتنمية القدرات (www.emergenzaxylella.it). تم في هذه النطاق، تطوير نموذج مبتكر لمراقبة XF تم تقديمه إلى مديرية وقاية النباتات في منطقة بوليا لدعم اتخاذ القرارات المؤسساتية. يعد هذا النموذج متعدد التخصصات، متعدد الوظائف ويشمل العديد من الجهات الفاعلة. حيث يسمح بتتبع، تخزين، إدارة، وتحليل جميع أنواع البيانات باستخدام تطبيق مبنى على الشابكة (XylWeb). هذا التطبيق هو عبارة عن أداة تجمع بيانات الاستشعار عن بعد، التي يتم الحصول عليها من خلال الإستشعار باستخدام الصور الجوية عالية الدقة لتحديد الأعراض المشابهة للتدهور السريع للأشجار، مع البيانات الميدانية المكتسبة بدقة على الأرض باستخدام تطبيق XylApp. ويتضمن النموذج المبتكر أيضا طرائق جديدة للكشف المبكر عن العامل الممرض في الحقل مثل (real time LAMP) في المواد النباتية والحشرات الحاملة للعامل الممرض المسماه "بالحشرات الجاسوسة". يخضع هذا النموذج المبتكر لتعديلات و تحسينات جديدة بناء على نتائج البحوث الحالية.

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