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60th MPU Anniversary Special Section - Review

Recent research accomplishments on early detection of *Xylella fastidiosa* outbreaks in the Mediterranean Basin

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Summary. *Xylella fastidiosa* is a major transboundary plant pest, causing severe socioeconomic impacts. Development of preventive strategies and methods for surveillance, early detection, monitoring, and accurate diagnosis of *X. fastidiosa* and its vectors, are keys to preventing the effects of this plant pathogen, and assist timely eradication or optimisation of containment measures. This review focuses on approaches for early detection of *X. fastidiosa* in the Mediterranean Basin, including development of climatic suitability risk maps to determine areas of potential establishment, and epidemiological models to assist in outbreak management through optimized surveillance and targeted responses. The usefulness of airborne hyperspectral and thermal images from remote sensing to discriminate *X. fastidiosa* infections from other biotic- and abiotic-induced spectral signatures is also discussed. The most commonly used methods for identifying *X. fastidiosa* in infected plants and vectors, and the molecular approaches available to genetically characterize *X. fastidiosa* strains, are described. Each of these approaches has trade-offs, but stepwise or simultaneous combinations of these methods may help to contain *X. fastidiosa* epidemics in the Mediterranean Basin.

Keywords. Climatic suitability risk maps, diagnostics, molecular approaches, quarantine plant pathogens, sequential adaptive survey.

INTRODUCTION

Xylella fastidiosa is a plant-associated bacterium that is a major transboundary plant health threat, and is a serious plant pathogen in terms of socioeconomic impacts. The bacterium causes diseases on a wide host range of plants including crops of economic importance in agriculture and forestry, and with cultural and heritage value. Diseases caused by *X. fastidiosa* include Pierce's Disease (PD) of grapevine, Citrus Variegated Chlorosis (CVC),

Almond Leaf Scorch (ALS), and Olive Quick Decline Syndrome (OQDS) (EFSA, 2022). A recent study by the Joint Research Center (JRC) and European Food Safety Authority (EFSA) (Sanchez *et al*., 2019) has identified *X. fastidiosa* as the quarantine pathogen with the greatest potential impact in the European Union (EU), in all economic, social, and environmental domains. That study estimated that *X. fastidiosa* could ultimately cost the EU over €5.5 billion per year due to production losses, and €0.7 billion per year in export losses, since the bacterium has the potential to affect 70% of EU production value of full-productive old (>30 years) olive trees, 13% of almond, 11% of citrus, and between 1 to 2% of grape production, in a full spread scenario across the EU.

Early detection of *X. fastidiosa* is important for taking timely measures for its eradication, containment or management (Almeida 2016; Zarco-Tejada *et al.* 2018). Consequently, development of efficient methods and strategies for surveillance, early detection and monitoring of *X. fastidiosa* have been the foci of several EU research projects such as XF-ACTORS (https://www.xfactorsproject.eu/) and POnTE (https://www.ponteproject.eu/), and grants by the EFSA. A major effort is currently being made in the EU to detect and assign *X. fastidiosa* strains from outbreaks to subspecies and, within them, to Sequence Types (STs). This is to infer relationships between ST and host range, and to trace back the possible origins of the introductions. Accurate diagnoses of *X. fastidiosa* at subspecies levels in the EU is essential for regulatory measures on outbreak response and management (e.g., removal of plants and replanting) (Regulation EU 2021/1688; EC, 2021). Currently, methods for *X. fastidiosa* identification are based on the European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocol (EPPO, 2019).

The present review is based on an oral presentation entitled 'Current situation of *Xylella fastidiosa* impacts in Spain: ongoing research initiatives to understand and tackle this pathogen', which was presented at the 16th Congress of the Mediterranean Phytopathological Union in April 2022, Limassol, Cyprus (Landa, 2022). This review describes research outputs from the framework of the EU projects POnTE and XF-ACTORS for early detection of *X. fastidiosa*, together with current knowledge reported in *X. fastidiosa* literature and experienced gained during current EU outbreaks of the pathogen in the Mediterranean Basin. It is not intended, however, to provide a detailed state of the art summary on this topic. Topics covered include development of climatic suitability risk maps, how they can assist to determine areas of potential *X. fastidiosa* establishment, and how epidemiological models and surveillance strategies can help to track the outbreaks of *X. fastidiosa* and their potential spread. Other topics include the usefulness of remote sensing to support surveillance and monitoring of areas affected by *X. fastidiosa* outbreaks, and how to discriminate *X. fastidiosa* infections from other biotic and abiotic-induced stresses. Also provided are an extended description of the most commonly used methods to identify *X. fastidiosa* in infected plants and vectors, molecular approaches available to characterize *X. fastidiosa* strains at subspecies and ST level, and future directions for efficient molecular diagnostics and genetic characterization of *X. fastidiosa* strains.

Although the procedures for field sampling of plant material, in places of production and in consignments, as well as laboratory sample preparation, are all essential for obtaining reliable diagnostic results, description of these procedures is beyond the scope of this review. Detailed information on these aspects can be found in PM 7/24 Diagnostics for *Xylella fastidiosa* (EPPO, 2019), PM 3/81 Inspection of consignments for *Xylella fastidiosa* (EPPO, 2022a), PM 3/82 Inspection of places of production for *Xylella fastidiosa* (EPPO, 2022b), Methodologies for Sampling of Consignments ISPM 31 (IPPC, 2008), and in D'Onghia *et al.* (2022) and Loconsole *et al.* (2021).

CLIMATIC SUITABILITY RISK MAPS TO ESTIMATE REGIONS FOR POTENTIAL ESTABLISHMENT OF *XYLELLA FASTIDIOSA*

Xylella fastidiosa occurs in a variety of climatic zones, although it is particularly prevalent in the tropics and sub-tropics. The pathogen is also found in areas where climatic conditions are similar to those prevailing in Mediterranean climate zones, such as California, and in various European regions including Corsica in southern Italy, the Côte d'Azur in France, southern Portugal and the Balearic Islands and the Valencian Community in Spain. Records of diseases caused by *X. fastidiosa* also occur from much colder climates, such as New Jersey and Washington in the United States of America and the Niagara Peninsula, southern Ontario, British Columbia, Saskatchewan and Alberta in Canada (EFSA, 2015).

Different approaches have been used to infer areas with favourable climatic conditions for *X. fastidiosa*. Feil and Purcell (2001) used isotherms of winter minimum temperatures to propose the following severity levels and thermal ranges (minimum winter temperatures) for PD in grapevine: severe impact, >4.5°C; moderate, from 1.7 to 4.5°C; occasional, from 1.7 to -1.1°C; and rare, <-1.1°C. However, Anas *et al.* (2008) established areas at risk for PD at much lower temperatures, based on the number of days with minimum temperature below

-12.2°C or -9.4°C. Following this criterion of minimum temperatures in winter, most southern European countries have climatic conditions that would allow survival of *X. fastidiosa,* and these regions overlap with the production areas for several crops relevant to the EU economy, including olive and grapevine. All of these regions where *X. fastidiosa* has been described in Europe, including the outbreaks in Italy, France, Portugal and Spain, have climatic conditions that are considered favourable for *X. fastidiosa* survival, demonstrating the validity of this criterion. In contrast, Hoddle (2004) used the CLIMEX model to map the potential distribution of PD, and its Californian vector *Homalodisca vitripennis*, based on data from Feil and Purcell (2001), and concluded that regions with tropical, semi-tropical, temperate and moderate Mediterranean climates are suitable for both organisms. These additional criteria indicate that most wine-growing regions of southern France, central and southern Spain and Italy have climatic conditions suitable for PD. Conversely, low winter temperatures would exclude this disease from vineyard-growing areas in France, northern Spain, and Italy (Hoddle, 2004).

In 2019, the Plant Health Panel of EFSA evaluated the potential for establishment of *X. fastidiosa* in the EU. In that study, Köppen-Geiger climate matching revealed that most parts of the EU could be suitable for establishment of the bacterium, excluding only some higher altitude and northern EU regions. However, analyses using species distribution ensemble modelling identified areas in southern Europe to be at more risk, mainly in southern regions of Portugal, Spain, France, Italy, Greece, Malta, and Cyprus (EFSA, 2019), as well as coastal regions of Morocco, Algeria, Tunisia, Libya, Turkey, Syria, and Israel within the Mediterranean Basin (POnTE project, 2019). These results agree with those of Cardone *et al.* (2022), who evaluates risks of establishment and spread of *X. fastidiosa* in the EU, the Balkans and the Middle East and North Africa regions. They identified Malta, followed by Lebanon, Greece, Portugal, Algeria, Spain, Turkey, Egypt, Morocco, and Albania, as the most vulnerable countries with respect to climate suitability. The North European and Arabian Gulf countries were less vulnerable to the spread of the bacterium. When developing these models at pathogen subspecies level, it has been estimated that *X. fastidiosa* subsp. *multiplex* presents the greatest potential for establishment in the EU, compared with that predicted for subspp. *fastidiosa* and *pauca,* with subsp. *multiplex* able to establish the furthest north in the EU (EFSA, 2019).

Using species distribution ensemble modelling, Arias-Giraldo *et al.* (2022) determined relationships between sample location for *X. fastidiosa* with associated regional environmental variables for Andalusia, Southern Spain; the area with the largest olive production in the world. They analyzed ecological requirements for the three main *X. fastidiosa* subspecies, and estimated that the Eastern part of Andalusia was at the greatest relative risk.

Future directions for this research include development of modelling tools that integrate the main components of *X. fastidiosa* epidemics at different spatial and temporal scales, including the effects of environmental drivers (increased precision of weather and land use, and inclusion of insect vector distribution databases), and of climate change. These models will allow prioritization of surveillance programmes for *X. fastidiosa*, based on risk levels in areas free of the pathogen or with recent outbreaks (Arias-Giraldo *et al.*, 2022).

EPIDEMIOLOGICAL MODELS TO ASSIST OUTBREAK RESPONSE PROGRAMMES FOR *XYLELLA FASTIDIOSA*

After an outbreak of *X. fastidiosa* in a region, official surveys are implemented, initially to delimit the infested area, and then to maintain the pest-free status of a surrounding buffer zone (Commission Implementing Regulation (EU) 2020/1201; EC, 2020). Surveillance is a large proportion of the resources required in outbreak response programmes, so several methods have been developed for optimizing survey efficiency. A sequential adaptive delimiting survey for *X. fastidiosa* with increasing spatial resolution was evaluated, using occurrence data of *X. fastidiosa* in Alicante, Spain (Lázaro *et al.*, 2021). Inspection and sampling intensities were adjusted using an optimization algorithm, considering the results obtained in a previous coarse spatial resolution, with three-phase or two-phase designs. With this sequential adaptive survey strategy, it was possible to delimit the distribution of *X. fastidiosa* in the study area, with substantial reduction of the total number of samples to be collected and tested. With some adjustments, this approach could also be used to optimize delimiting surveys in other *X. fastidiosa* outbreaks in Europe and elsewhere.

Effects of climatic and spatial factors on the geographic distribution of *X. fastidiosa* in Lecce, Italy, and Alicante, Spain, were studied with Bayesian hierarchical models (Cendoya *et al.*, 2020). These two outbreaks represent different, but simple, epidemiological scenarios, one with OQDS, caused by *X. fastidiosa* subsp. *pauca* ST53, in Lecce (Morelli *et al.*, 2021; Saponari *et al.*, 2013), and other with ALS, caused by *X. fastidiosa* subsp. multiplex ST6, in Alicante (Landa *et al.*, 2020; Marco-Noales

et al., 2021). The climate covariates presented low variabilities and were not relevant in the resulting models, so they were not related with the distribution of *X. fastidiosa* in the study areas. These results indicate that climate is not likely to stop the spread of the pathogen from outbreaks to adjacent areas. Furthermore, the models were mainly driven by the spatial components, so probability of *X. fastidiosa* presence substantially increased with proximity to infested area. Overall, these results highlight the importance of implementing control measures based on reduction of inoculum and vector populations, to limit further disease spread from outbreak areas.

In epidemiological models, spatial dependence is often considered as direction-invariant and uniform (i.e., isotropic and stationary). However, these assumptions do not hold when there are elements limiting disease spread. This is the case when geographic barriers are present, or control measures are implemented to contain disease spread. Using the outbreak in Alicante, Spain, as a case study, *X. fastidiosa* occurrence data were analyzed through stationary and nonstationary models (Cendoya *et al.*, 2022). The nonstationary models considered a cordon sanitaire surrounding the infested area, where host plants were removed and measures applied to impede disease spread. The mean value of the spatial range of the stationary model indicated that host plants closer than 4 km to the infested area would be at risk of *X. fastidiosa* infections. Consequently, the plant health authority increased by 10 km the minimum width of 2.5 km established by the Regulation (EU) 2020/1201 (EC, 2020) for buffer zones surrounding infested zones (Generalitat Valenciana, 2020). The nonstationary models with the cordon sanitaire resulted in a substantial reduction of the probability of *X. fastidiosa* presence outside the infested area. Nevertheless, these models assume that barriers are completely impermeable to pathogen spread, which is not realistic for those causing most plant diseases. Further methodological research is thus required to consider realistic barriers with different levels of permeability.

AIRBORNE HYPERSPECTRAL AND THERMAL IMAGES FROM REMOTE SENSING TO DETECT *XYLELLA FASTIDIOSA* INFECTED HOST PLANTS

Remote sensing studies on *X. fastidiosa* have manily focused on development of algorithms for early detection of symptoms induced by infections either using unmanned (e.g., Castrignanò *et al.*, 2021) or manned (e.g., Zarco *et al.*, 2018; 2021b) aerial vehicles. Zarco-Tejada *et al.* (2018) studied the Italian *X. fastidiosa* outbreak, evaluating more than 7000 olive trees using high-resolution hyperspectral and thermal imagery. This revealed that pre-visual detection of *X. fastidiosa*-infected trees was feasible using radiative transfer modelling and spectral plant-trait retrievals from imaging spectroscopy and thermal data. Their study showed that changes in specific plant functional traits detected using hyperspectral and thermal imagery could reveal *X. fastidiosa* infections occurring months before symptoms were visible. Important inputs identified for *X. fastidiosa* detection included spectral ratios in the blue region, plant traits such as leaf anthocyanin and carotenoid pigment content estimated using a radiative transfer model inversion, tree temperature, and estimates of solar-induced chlorophyll fluorescence emission. Later Poblete *et al.* (2020) used high-resolution hyperspectral and thermal imagery to assess performance of spectrally constrained machine-learning algorithms to measuring multispectral bandsets, selected from the original hyperspectral imagery, that were compatible with large-scale monitoring from unmanned platforms and a manned aircraft, as well as the contribution of solar–induced chlorophyll fluorescence (SIF) and the temperature-based Crop Water Stress Index (CWSI) retrieved, respectively, from hyperspectral and thermal imaging. This research demonstrated that large-scale *X. fastidiosa* monitoring could be supported using airborne platforms carrying multispectral and thermal cameras with limited numbers of spectral bands (e.g., six to 12 bands with 10 nm bandwidths), as long as the bands were selected for their sensitivity to distinguish *X. fastidiosa* symptoms.

Although these studies have shown that spectral screening methods can detect non-visual symptoms of early infection by *X. fastidiosa*, and can help prevent pathogen spread, the subtle pathogen-induced host physiological alterations detected by imaging spectroscopy can be entangled with dynamics of abiotic stresses. Zarco-Tejada *et al.* (2021b) used airborne spectroscopy and thermal scanning to monitor different EU areas covering more than one million trees, including different host species (olive and almond), affected by two vascular pathogens (*X. fastidiosa* and *Verticillium dahliae*), and comprising a gradient in water stress levels. This study demonstrated the existence of divergent pathogen- and host-specific spectral pathways, that could disentangle biotic-induced symptoms, and showed that uncoupling biotic and abiotic spectral dynamics diminished uncertainty in *X. fastidiosa* detection to less than 6% across different hosts (almond and olive). The study also assessed these deviating pathways against *V. dahliae,* another vascular pathogen that produces symptoms analogous to *X. fastidiosa*, and showed that the divergent

routes remained pathogen- and host-specific, with detection accuracies exceeding 92% across the pathosystems.

Recent studies have also correlated chemical compounds closely associated with *X. fastidiosa* infection in olive plants (i.e., higher contents of malic acid, formic acid, mannitol and sucrose and lower contents of oleuropein; Jlilat *et al.*, 2021) with hyperspectral signals, by identifying specific wavelength packages also associated with bacterial infection. This combined spectro-metabolomic approach may represent a new paradigm for reliable detection of *X. fastidiosa* by remote sensing at the early stages of bacterial infection (Ahmed *et al.*, 2021; A.M. D'Onghia, *personnal communication*).

The research described above has shown that early detection of *X. fastidiosa*-induced symptoms is feasible with high-resolution hyperspectral and thermal imagery and physically based plant trait retrievals. New research (Poblete *et al.*, *in press*) has demonstrated that high resolution multispectral satellite imagery failed to detect early symptoms of infection, but was able to monitor medium and advanced severity levels at large scales. Results from these studies are essential for implementation of effective management of plant diseases, using airborne, drone and satellite based remote sensing technologies. These imaging methods could also contribute to future operational monitoring of infected crop areas at large scales, well beyond what is possible from field surveys and laboratory analyses (Zarco-Tejada *et al.*, 2021a).

MOLECULAR DIAGNOSTIC TESTS FOR EARLY DETECTION AND SUBSPECIES DETERMINATION OF *XYLELLA FASTIDIOSA*

The EPPO Diagnostic protocol PM 7/24 (4) for *X. fastidiosa* is the most complete source for current screening tests available for detection of this bacterium and subspecies determination (EPPO, 2019), which includes a detailed description of each screening test with validation data. Among all the screening tests described, those based on molecular approaches are the most sensitive and rapid for *X. fastidiosa* detection and will be the focus of this review.

Most molecular tests used for *X. fastidiosa* diagnoses are based on conventional end-point PCR or real-time PCR (qPCR). However, for some of the PCR protocols, if the amplicon product is sequenced it can also provide information for subspecies assignation. The Commission Implementing Regulation (EU) 2021/1688 (EC, 2021) indicates which tests from those described in the EPPO Diagnostic protocol PM 7/24 should be used for official surveys and identification of *X. fastidiosa* and its subspecies.

There are several conventional PCR tests for *X. fastidiosa* diagnoses, and one of the most commonly used is that developed by Minsavage *et al.* (1994). This test is based on amplification of the RNA polymerase sigma-factor 70 (*rpo*D). A search on Google Scholar using 'Minsavage and *Xylella fastidiosa*' retrieved more than 800 publication records. This test can also be used to assign *X. fastidiosa* at the subspecies level, by sequencing the *rpo*D amplicon, and comparing its sequence using the Basic Local Alignment Search Tool (BLASTN; http://www.ncbi.nlm.nih.gov/) available at the National Center for Biotechnology Information, against the RefSeq Genome database of *Xylella fastidiosa* (taxid: 2371) as the search organism. This tool also allows a distance tree of results to be obtained, that clusters the target sequence with those *rpo*D sequences included in the reference genomes of different strains of *X. fastidiosa* belonging to different subspecies (Figure 1).

Other conventional PCR diagnostic tests are based on the gene encoding the β-subunit polypeptide of the DNA gyrase (*gyrB*). Although this methodology is not included in the EPPO Diagnostic protocol PM 7/24, it was used for developing a mini sequencing or single-nucleotide primer extension (SNuPE) approach for the multiplex amplification of six *X. fastidiosa gyrB* sequences targeting subspp. *fastidiosa, multiplex* and *sandyi*, and three genotypes within subspecies *pauca* present in the EU territory, and including strains from coffee and citrus from Brazil, and the type isolate infecting olive in Italy (Montes-Borrego *et al.*, 2015; Saponari *et al.*, 2016).

Several qPCR tests are available and have been validated at different laboratories (EPPO, 2019), including tests described by Harper *et al.* (2010), Francis *et al.* (2006), Ouyang *et al.* (2013), and Li *et al.* (2013). The target sequences for these tests are, respectively, the 16S rRNA processing RimM protein, the hypothetical protein HL gene, the cobalamin synthesis protein-coding gene, and the 16S rRNA. Because of their greater analytical sensitivity than other molecular tests, the use of qPCR is recommended for detection surveys to substantiate pest freedom in areas where *X. fastidiosa* is not known, and for asymptomatic plants (EPPO, 2019). These qPCR tests have been widely used in studies assessing distribution and host range of *X. fastidiosa* in Europe (e.g., Saponari *et al.*, 2013; Jacques *et al.*, 2016; Moralejo *et al.*, 2020; Olmo *et al.*, 2021), potential insect vectors of *X. fastidiosa* (e.g., Elbeaino *et al.*, 2014; Cavalieri *et al.*, 2019; Cunty *et al.*, 2020; Moralejo *et al.*, 2020; Marco-Noales *et al.*, 2021), and in remote sensing studies (e.g., Zarco-Tejada *et al.*, 2018; Poblete *et al.*, 2020; Zarco-Tejada *et al.*, 2021b; Camino *et al.*, 2022) to outline examples of their usefulness.

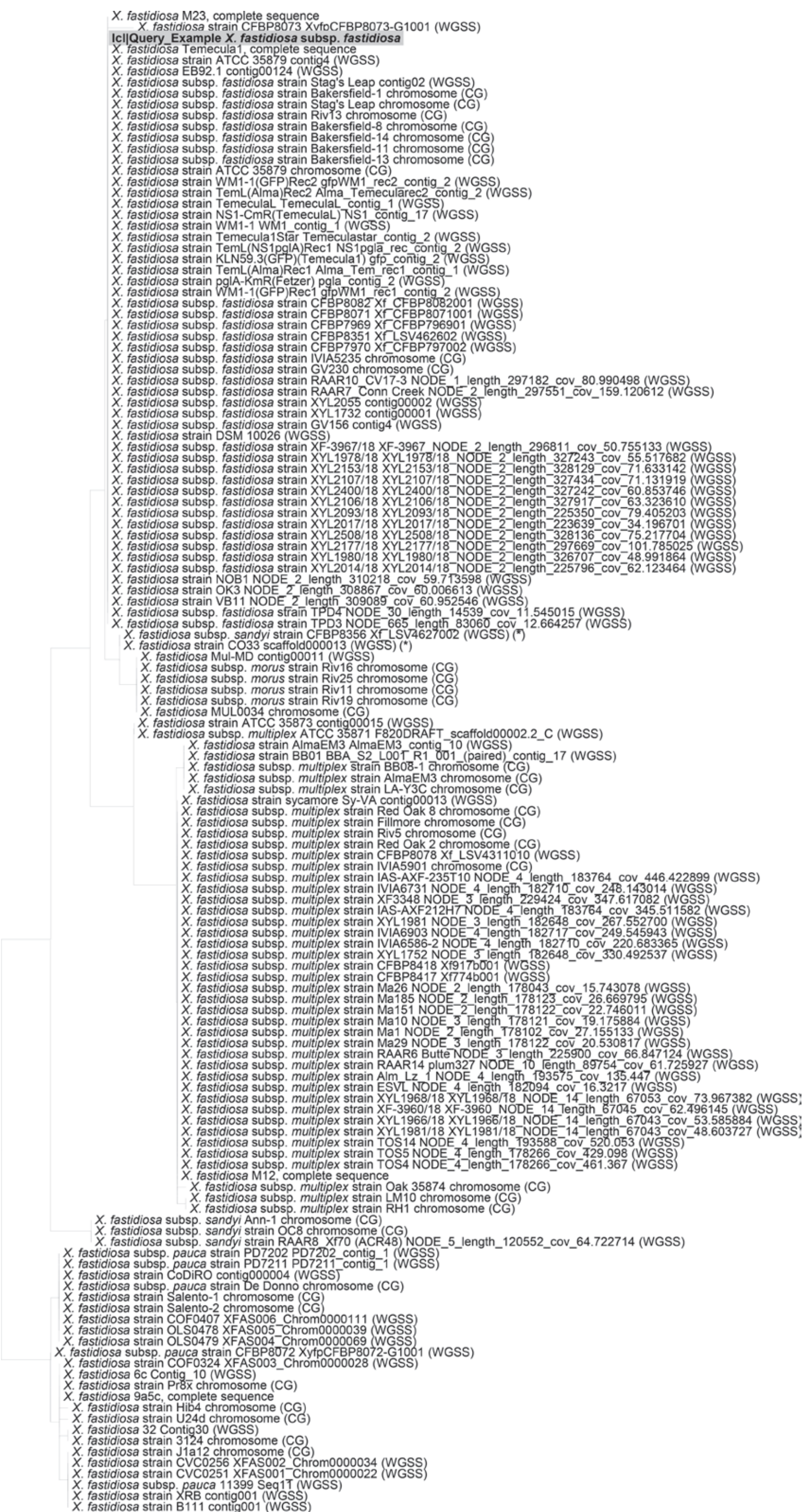

Figure 1. Phylogenetic distance tree of RNA polymerase sigma-factor 70 (*rpo*D) partial sequences obtained after BLAST analysis (http://www.ncbi.nlm.nih.gov/) of a query sequence against the RefSeq Genome database of *Xylella fastidiosa* (taxid: 2371). The different clusters correlate with main *X. fastidiosa* subspecies. lcl|Query= Represents an example of a query made for the *rpo*D sequence of a *X. fastidiosa* subsp. *fastidiosa* strain. (CG)= Complete Genome; (WGSS)= Whole Genome Shotgun Sequence. (*)= recombinant subspecies *morus/sandyi*.

Where a positive result is obtained in areas outside of demarcated areas (i.e., pest-free areas), the Commission Implementing Regulation (EU) 2020/1201 (EC, 2020) indicated that presence of *X. fastidiosa* must be confirmed by two tests targeting different parts of the bacterial genome, as recommended by EPPO (2019). To facilitate diagnosis of *X. fastidiosa* with two tests, Bonants *et al.* (2019) implemented a triplex qPCR test based on the primers and probes included in Harper qPCR and Ouyang qPCR tests, and an additional primer pair and probe for internal controls. This test facilitates two diagnostic tests simultaneously, saving time and resources, and can provide the same analytical sensitivity as each test independently (Bonants *et al.*, 2019).

Results from test performance studies (TPS) and proficiency tests (PT) performed in the frameworks of XF-ACTORS and POnTE, Euphresco PROMODE projects, have compared the performance characteristics of qPCR tests when used with plant or insect mock-inoculated matrices, and these results are available at the EPPO Database on Diagnostic Expertise (https://dc.eppo.int; EPPO, 2023). Results from these validation tests have indicated that all the qPCR diagnostic protocols were robust, and were suitable for the diagnoses of *X. fastidiosa* in plant and insect materials. However, although most qPCR protocols produced good performance values, their analytical sensitivity was slightly different when using mock-inoculated samples. A recent study has also shown greater analytic sensitivity of the Harper qPCR test compared to the Francis qPCR test, when using DNA samples extracted from naturally infected almond trees (Anguita-Maeso *et al.*, 2021).

For *X. fastidiosa* subspecies assignment, the Commission Implementing Regulation (EU) 2020/1201 (EC, 2020) determines Multilocus Sequence Type (MLST) analysis to be used (Yuan *et al.*, 2010), especially for new records (i.e., for a new outbreak or new hosts). This test is based on amplification and sequencing of seven housekeeping gene (HKG) loci (*cys*G, *glt*T, *hol*C, *leu*A, *mal*F, *nuo*L, *pet*C). Analysis of *rpo*D and *mal*F or *cys*G and *mal*F sequences have been shown to be sufficient for assignment of sample pathogens to subspecies (EPPO, 2019); whereas the sequences of the seven loci are needed to assign samples into STs. Originally, MSLT analysis was designed to be used with DNA extracted from *X. fastidiosa* pure cultures (Yuan *et al.*, 2010), and when used with plant DNA samples is partly efficient. To improve sensitivity, Cesbron *et al.* (2020) developed a direct nested-MLST assay for plant and insect extracted DNA, based on the same seven targeted HKG loci as those used in the Yuan *et al.* (2010) test. This nested-MLST assay improved detection threshold by 100 to 1000 times, compared to conventional MLST. Using this nested-MLST assay, plants that were previously not considered hosts (giving high or inconclusive Ct values in qPCR assays) tested positive, and novel alleles were revealed in France. In samples from Spanish outbreaks, the nested-MLST assay allowed to identify the *X. fastidiosa* subspecies or ST infecting new hosts in Europe at that time (Cesbron *et al.* 2020). This nested-MLST assay has been used to type *X. fastidiosa* positive samples to the subspecies and ST level in the outbreaks in Spain. These include three *X. fastidiosa* subspecies and four STs in the Balearic Islands: *X. fastidiosa* subsp. *fastidiosa* ST1 and *X. fastidiosa* subsp. *multiplex* ST7 in Mallorca, *X. fastidiosa* subsp. *pauca* ST80 in Ibiza, and *X. fastidiosa* subsp. *multiplex* ST81 in Mallorca and Menorca (Olmo *et al.*, 2021). Only a single subspecies and ST (i.e., *X. fastidiosa* subsp. *multiplex* ST6) was identified in the outbreak in Alicante (Marco-Noales *et al.*, 2021).

Some qPCR tests have been developed to specifically detect subspecies of *X. fastidiosa* (e.g., the tests of Burbank *et al.*, 2018; Dupas *et al.* 2019; Hodgetts *et al.*, 2021). These tests are based on Taqman probes, designed to specifically and simultaneously target one or several *X. fastidiosa* subspecies. The advantage of these protocols is, while the presence of the bacterium is detected, even at low concentration, the subspecies is also defined. The Burbank qPCR test was used by Moralejo *et al.* (2020) to track the *X. fastidiosa* DNA inside growth rings of infected almond trees, through dendrochronological analysis. The protocol allowed differentiation between subsp. *fastidiosa* and *multiplex* in rings of 25 trees, with nine infected by subsp. *fastidiosa*, and 19 infected by subsp. *multiplex,* and three trees had mixed infections. This qPCR test combined with the conventional and nested-MLST tests (described above) enabled dating infections back to 1998 for *X. fastidiosa* subsp. *fastidiosa* ST1, and before 2000 for *X. fastidiosa* subsp. *multiplex* ST81 (Moralejo *et al.*, 2020), indicating that the bacterium was introduced to the Balearic Islands earlier than previously thought.

Time and portability are also important factors in pathogen diagnoses, especially for quarantine plant pathogens (Aglietti *et al.*, 2019). Isothermal nucleic acid amplification tests have been developed for *X. fastidiosa* field diagnoses. These included loop-mediated isothermal amplification (LAMP) based on primers developed by Harper *et al.* (2010) that were modified by Yaseen *et al.* (2015), and the AmplifyRP® XRT+ test, using isothermal amplification based on recombinase polymerase amplification (RPA) (Kersting *et al.*, 2014), based on the protocol of Li *et al.* (2016). Both tests have kits and specific portable devices that are commercially available,

but other equipment can also be used. For instance, the AmplifyRP® XRT+ test uses the battery powered AmpliFire® Portable Fluorometer device, that is easy to transport, and does not require DNA extraction, since the test can be performed with the crude plant macerate and the amplification takes 20 min. This test is easy to use by untrained laboratory staff, and is well adapted to the field for preliminary on site screening, as it requires no particular expertise (Cesbron *et al.*, 2022).

A constraint of these tests is that detection limits are greater than for qPCR tests. Nevertheless, negative or doubtful results can be verified with another, more sensitive test. During a field campaign in Mallorca, Spain, almond trees in 14 orchards under rainfed and irrigated conditions were visually scored for the presence of ALS. A total of 356 symptomatic and symptomless trees were sampled and analyzed using the AmplifyRP® XRT+ test and the AmpliFire® device by two operators in less of 5 days (Camino *et al.*, 2022). When comparing results of this test with that of the Harper qPCR test using the same almond plant branch samples, 92.8% agreement for infected samples was obtained for the two methods. The samples that were negative by the AmplifyRP® XRT+ test showed cycle thresholds (Cts) >31 in the Harper qPCR test (Landa *et al.*, unpublished).

Digital PCR (dPCR) is an innovative PCR tool based on partitioning of PCR reagents and DNA samples into thousands of droplets or microchambers (depending on thermocycler brand), that allows increased precision, sensitivity and absolute quantification without requirements for reference samples or standard curves. Detection of phytopathogenic bacteria by droplet PCR has provided successful results for pathogen diganoses, due to its detection efficiency at low pathogen concentrations and tolerance to PCR inhibitors (Dreo *et al.* 2014). Dupas *et al.* (2019) developed a droplet ddPCR protocol based on the Harper qPCR test. Both protocols showed the same detection limits for olive, *Polygala myrtifolia* and *Rosmarinus officinalis*, but the Harper qPCR test allowed better detection of 0.5 log for *Lavandula angustifolia*, and droplet dPCR allowed better detection of 0.5 log for *Quercus ilex*.

Investigation of pathogen strain origins in a new disease outbreaks requires whole genome sequencing (WGS) of pure bacterial cultures to resolve phylogenetic reconstruction. This is a challenge for *X. fastidiosa* due to its fastidious nature. For all the available nucleic acid based amplification methods described above for the detection of *X. fastidiosa,* the target sequence is a single locus, making the assays prone to false-positive or false-negative results (Bonants *et al.*, 2019). High throughput Next Generation Sequencing (NGS) technologies allow sequencing of total DNA in samples, potentially providing detection of *X. fastidiosa* and characterization to subspecies and strain levels, without requirement for pathogen cultivation. NGS based on Illumina (Bonants *et al.*, 2019; Román-Reyna *et al.*, 2022) or Oxford Nanopore technologies (Johnson *et al.*, 2022) have been explored for fast detection and identification of *X. fastidiosa*. The potential advantages and disadvantages of both of these technologies are beyond the scope of this review.

Using Illumina sequencing, Bonants *et al.* (2019) analyzed DNA extracts, by WGS, for presence of *X. fastidiosa* in artificially inoculated host plants and from naturally infected plants sampled or intercepted in different European countries. In all samples, even in samples with low infection levels, some DNA reads specific for *X. fastidiosa* were detected, and in several cases the pathogen could be identified to the subspecies level. Only for one sample was the whole genome assembled and the ST determined. Samples in which more *X. fastidiosa* genomic information was obtained corresponded to those with low Ct values (i.e., high *X. fastidiosa* titres). Thus, alinear relationship is found between the Log number of *X. fastidiosa* reads obtained by NGS and the Cts from Harper and Ouyang qPCR tests, and the time for positivity in a LAMP assay (Figure 2). Similarly, Anguita-Maeso *et al.* (2021) found a linear relationship between the Ct values obtained for the Harper and Francis qPCR tests and the Log of *X. fastidiosa* reads. Román-Reyna *et al.* (2021) developed a metagenomics pipeline using in-house short read Illumina sequencing to analyze samples from different plant species originating from Europe and the United States, and naturally infected by *X. fastidiosa*. They identified *X. fastidiosa* to the strain level in single and mixed infected plant samples at concentrations of 1 pg of bacterial DNA per gram of plant tissue, and in samples previously considered inconclusive when using qPCR (Ct >35), the protocol was able to confirm infection by *X. fastidiosa*. These results indicate that using the NGS approach, only in cases where DNA has been extracted from highly infected material, and where high genome coverage is used during sequencing, is possible to identify *X. fastidiosa* at the strain level.

To overcome this limitation, we have developed a Targeted Sequence Capture Enrichment (TSCE), in combination with High Throughput Sequencing (HTS). This uses an Illumina platform to provide adequate bacterial genome information for identification of *X. fastidiosa* at strain level (Velasco-Amo *et al.*, 2021). Results indicated that although <0.25% of *X. fastidiosa* reads were detected by direct WGS of host DNA, this was increased by 41–73% when using the TSCE-HTS approach, for indi-

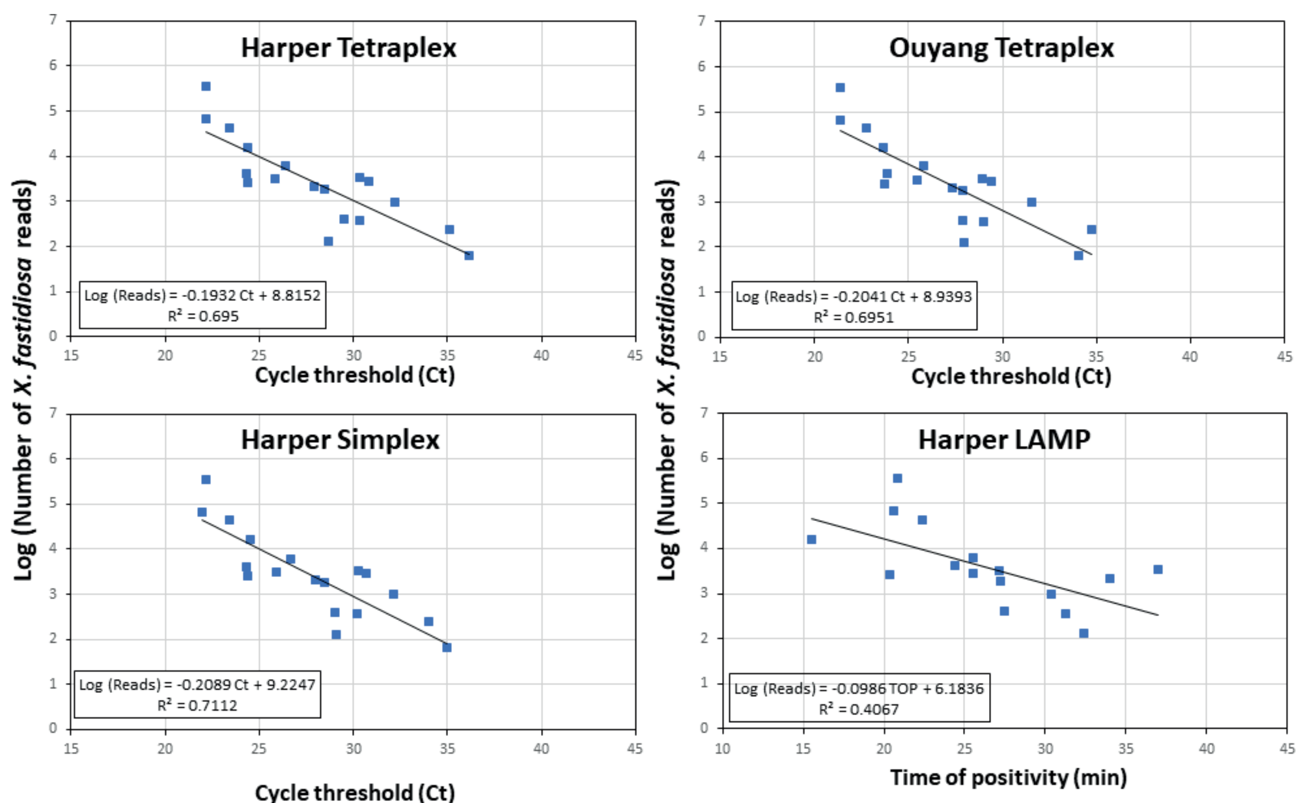

Figure 2. Linear regressions between Log [*Xylella fastidiosa* reads] obtained by next generation sequencing (NGS) and Ct values from quantitative polymerase chain reaction (qPCR) protocols of Ouyang tetraplex and Harper tetraplex (Bonants *et al.*, 2019), Harper Simplex (Harper *et al.* 2010), and LAMP test based in Harper *et al.* (2010). Data were obtained from Table 7 of Bonats *et al.* (2019), and represent the mean Ct value obtained for each sample.

vidual samples or in mixtures of up to four plant samples. The protocol was also validated using a range of insect and plant samples from different naturally infected host plants, and with levels of *X. fastidiosa* ranging from very high (CT >20) to close to the detection limit for Harper qPCR assay. More importantly, 80–100% of the 140 target sequences used to design the baits were captured, which allowed phylogenetic reconstruction of the *X. fastidiosa* strains infecting the samples, and identifying these at strain level (Landa *et al.*, 2021). This methodology may be useful for studies of *X. fastidiosa* introductions at outbreak stages, since a limited number of genetic markers do not provide sufficient phylogenetic resolution to determine dispersal paths or relationships among strains that are of biological and quarantine relevance.

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