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An integrated strategy for pathogen surveillance unveiled *Xylella fastidiosa* ST1 outbreak in hidden agricultural compartments in the Apulia region (Southern Italy)

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Abstract Annual surveillance programs for the plant pathogenic bacterium *Xylella fastidiosa* are implemented in Europe as part of the preventive phytosanitary strategies enforced to face the emergence of the detrimental infections reported in olives in southern Italy. The programs include inspections and sampling of host plants by prioritizing those showing suspicious symptoms or those known to be highly susceptible to different strains and subspecies of the bacterium. In the framework of these programs numerous outbreaks have been unraveled, with several strains and subspecies found to infect a relatively large host range in France, Spain, Portugal and Italy. Here we report the results of an integrated surveillance approach implemented in the Apulia Region (southern Italy), where a conventional survey program on host plants is complemented by monitoring and testing for *X. fastidiosa* the insect vectors. In the framework of this program, bacterium-positive spittlebugs were intercepted in the *Xylella*-free area of the region, close to one of the most relevant Italian table grape production district. Such findings prompted further investigation to identify the bacterial reservoir in the host plants. Almond and grapevine were found to be the most frequently infected hosts, with infections caused by isolates of *X. fastidiosa* subspecies *fastidiosa* ST1. Investigations are ongoing to assess the extent and history of the outbreak, to assess and estimate the potential impacts, and define the best options for its containment.

Keywords Xylella · Grape

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Short communication

The history of *Xylella fastidiosa* in the old Continent begins in 2013, when the fastidious bacterium was first reported and later identified as the causal agent of the detrimental Olive Quick Decline Syndrome (OQDS) (Saponari et al., 2019). Since then, a large number of genetically distinct bacterial isolates

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belonging to three subspecies (*fastidiosa*, *multiplex* and *pauca*) have been detected in four countries (France, Italy, Portugal and Spain). Assessment of genetic relationships clearly proved that many of these isolates originated from American countries, most likely introduced via plant propagating materials (Landa et al., 2020). Given that *X. fastidiosa* is ranked as one of the quarantine priority pests, strict preventive phytosanitary measures are implemented in Europe and in several Mediterranean countries, such as compulsory monitoring programs. To this end, developing an effective early detection surveillance strategy for *X. fastidiosa* is hampered by the need to survey large and heterogeneous areas of landscape, and to perform massive laboratory tests on potential host species to early detect infections. Moreover, while certain host plant-*X. fastidiosa* combinations result in overt diseases, in several areas the bacterium establishes and spreads in landscape compartments as latent infections or is misidentified with other vascular diseases (Landa et al., 2022; Moralejo et al., 2020; Soubeyrand et al., 2018). On the other hand, monitoring the occurrence and prevalence of the bacterium in insect vector populations for identifying new bacterial outbreaks might represent a more cost- and time-effective first-screening approach, i.e. it does not require inspections of large numbers of host plants and it is not affected by the host-range of the bacterial genotype present in the surveyed area, given that no strain-vector species specificity has been so far demonstrated (Cornara et al., 2019; Lopes et al., 2009).

Currently, the EU legislative framework (https://eur-lex.europa.eu/eli/reg_impl/2020/1201/2021-10-11) defines Europe-wide inspections and surveys to be carried out for both outbreaks (demarcated areas) and for *Xylella*-free areas. In the first case, the plant species monitored are those known to be susceptible to the subspecies identified in the outbreak, while in the *Xylella*-free areas the absence of *X. fastidiosa* is assessed at species level, thus including a large number of host plant species in the target population so as not to exclude any *X. fastidiosa* subspecies or lineage.

In the Apulia region (southern Italy), one of the European areas classified as at high risk for this pathogen due to the epidemics of OQDS, caused by isolates of *X. fastidiosa* susbsp. *pauca* ST53 (Xfp-ST53), xylem-sap feeding insect vectors are included in the official regional monitoring program along with the host plants susceptible to strains of the subspecies *pauca*. Vectors surveillance aims to monitor the developmental stage, population abundance and dynamics of xylem-sap feeder, in order to provide indications to farmers about the best timing for vector control. Furthermore, a percentage of the insects collected in the *Xylella*-free and buffer zones is tested for the presence of the bacterium.

In detail, the monitoring program in the Apulia Region is based on inspections and sampling of host plants known to host Xfp-ST53, accounting mainly for olive, oleander, almond and a list of ornamentals. The scheme adopted for the plant surveillance is based on the survey guidelines and statistical software RiBESS + (EFSA, 2020). Insect vectors surveillance is yearly carried out from the beginning of March to the end of October in 90 olive orchards (at approx. 10 days interval) across the Apulia Region. In addition, adults of xylem-sap feeder species are monitored in 90 sampling sites in the *Xylella*-free area, selected alongside the main roads and highways, where insects are captured in the proximity of parking areas, packing houses and gas stations.

The presence and abundance of xylem feeders (mainly 3 aphrophorid species, *Philaenus spumarius* (L., 1758), *Philaenus italosignus* (Drosopoulos & Remane, 2000), and *Neophilaenus campestris* (Fallen, 1805)) is determined following the protocol described by Bodino et al. (2019), i.e. using quadrat sampling for juveniles and sweeping net for adults. Briefly, ground cover is scrutinized for nymphs, with five quadrats per hectare, taking notes of nymphal age, abundance per plant/quadrat, and host plant species. Adults are collected by sweep net from ground cover (18 randomly selected points/hectare, four sweeps per point), olive canopies (10 plants/ hectare) and trees and shrubs surrounding the orchard (12 plants/ hectare).

Adults are identified, counted, and sorted by sex in the field, and then released (conservative sampling), except for a small percentage (ca. 1–3% of the insects, roughly corresponding to 400–500 specimens/year) which is stored in 90% EtOH, and in the laboratory identified at species level and tested for *X. fastidiosa* by real time quantitative (q) PCR with the *Xylella*-specific primers/probe designed by Harper et al. (2010). Insect samples yielding doubtful qPCR results are amplified by nested PCR assays targeting the bacterial gene *HOLC* according to Cruaud et al.

(2018). The samples displaying the specific amplicon on agarose gel are considered positive.

In late summer 2022 and 2023, positive specimens of *P. spumarius* were detected in the municipality of Triggiano in the province of Bari, approximately 20 km northwest from the demarcated areas for Xfp-ST53, thus in the *Xylella*-free area. Specifically, in October 2022 one positive *P. spumarius* (male) was firstly found in an olive orchard, then following an additional sampling within a radius of 500 m, a second positive individual (male) was detected out of the 79 specimens collected in this area (2.53% infectivity). In autumn 2023, 13 bacterium-positive *P. spumarius* out of 474 specimens captured (2.65% infectivity) were intercepted between September and October in semi-natural habitats and olive orchards in an area of approx. 600 m of radius and located at 1 km from the olive orchard where the first *Xylella*-positive spittlebug was detected in 2022 (Fig. 1).

Following the detection of the first two positive specimens in 2022, the area was intensely inspected to search for host plants serving as bacterial reservoir for the spittlebugs. Field inspections did not reveal symptoms putatively associated with *Xylella*-infections, and none of the thirty-one olive samples initially collected in the neighboring area tested positive for the bacterium. Following the high number of positive spittlebugs detected in late 2023, an extensive sampling campaign on host plants was carried out in early 2024, with a total of 432 individual plant samples collected from 8 different plant species, namely olive (n. 397), almond (n. 20), *Artemisia* spp, *Asparagus acutifolius, Laurus nobilis*, *Prunus avium*, *Rubus* spp and *Salvia rosmarinus* (one to four samples per species). None of the olive trees or the other evergreen species showed putative bacterial symptoms, while almond trees were at the end of the dormancy stage with buds just starting to sprout.

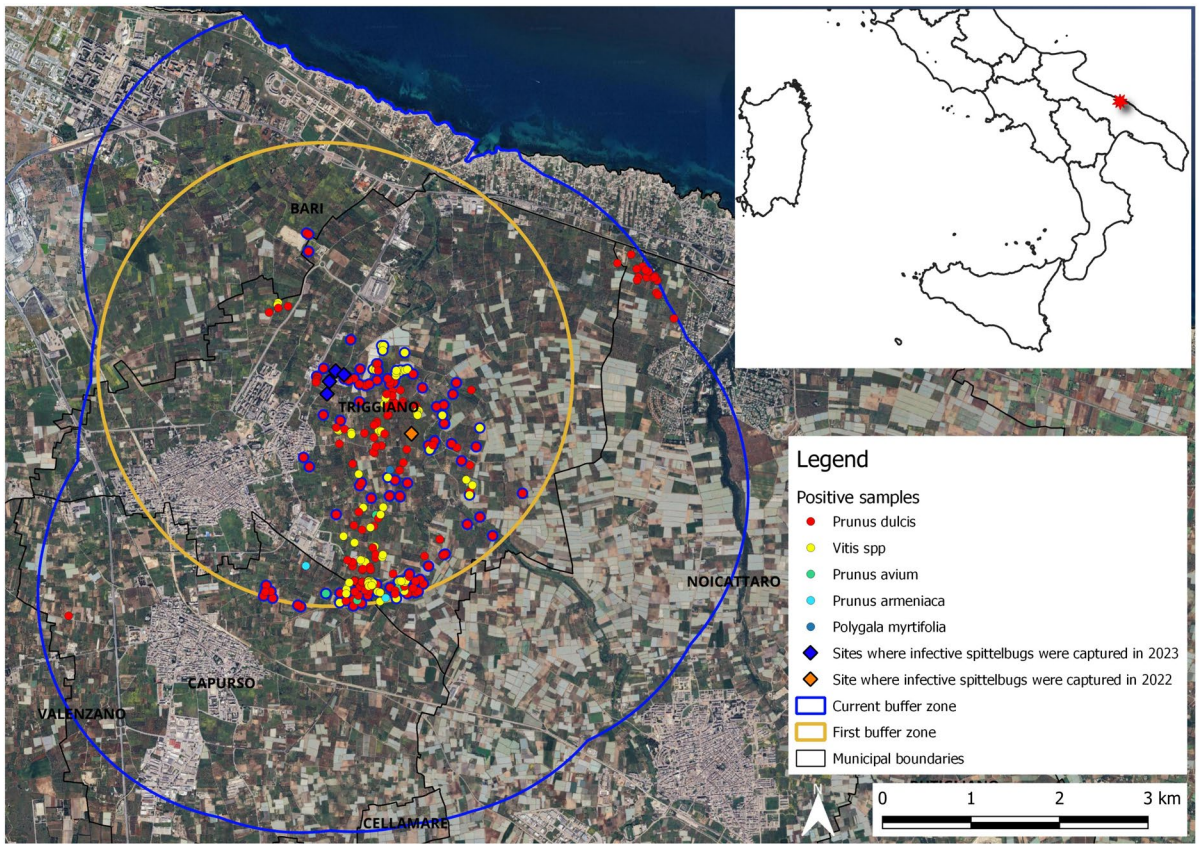

Fig. 1 Map showing the outbreak of *Xylella fastidiosa* subspecies *fastidiosa* in Apulia region (southern Italy). Locations where positive spittlebugs were captured and infected host plants detected are reported, along with the delimitation of the buffer zone

Diagnostic tests were performed following the standard procedures adopted in the framework of the regional contingency plan. Specifically, diagnostic tests for olive and evergreen species are performed all year around by analyzing DNA extracted from small pieces of semi-hardwood cuttings or leaf petioles, while for almond and other deciduous species, slices of debarked cuttings and branches are used, except in late summer, when leaf petioles are also excised from mature leaves.

Plant tissues consisting of 0.5–1 g of wood tissues were homogenized in CTAB-based buffer (1:10 w:v) using the Homex 6 apparatus. An aliquot of 1 ml of CTAB-sap was subjected to plant DNA purification using the Maxwell® RSC PureFood GMO and Authentication Kit (Promega Corporation, Madison, WI, USA). Duplex real time PCR was then set up using the *Xylella*-specific primers/probe designed by Harper et al. (2010) and the plant endogenous gene cytochrome oxidase (*COX*) as internal control (Weller et al., 2000). For *Prunus* spp. and asparagus, the *COX* internal control was set up in a separate simplex qPCR assay with an annealing temperature of 56 °C. Primer mismatches occurring with the target gene sequences of these species did not generate efficient amplification when qPCR was carried out at 62 °C, the optimal annealing temperature for the primers/probe designed by Harper et al. (2010).

Six almond trees out of 432 plants tested gave positive qPCR reactions for *X. fastidiosa*, with an average quantitation cycle (Cq) of 28.28 (SD 2.28) (Cq ranging from 25.70 to 31.93), while none of the other species monitored including olives tested positive for the bacterium. Quantitation cycle for the endogenous *COX* gene ranged from 14.63 to 20.47 for olives, and from 17.61 to 27.46 for almonds, confirming the suitability of the recovered total plant DNA for qPCR reactions. The lack of bacterial detection in olives, the most commonly infected host plant in the Xfp-ST53 demarcated areas, suggested that the infections detected in almond trees could have possibly originated from a different bacterial genotype. To confirm this hypothesis, the DNA from infected almond plants and bacterium-positive insects was subjected to conventional PCR using the primers described by Pooler and Hartung (1995), which selectively amplify isolates of the subspecies *pauca*. PCR reactions consisted of 2 µl of total DNA, 180 nM of each primer in 2X concentrated solution of GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA). PCR conditions included an annealing temperature of 55 °C, lower than that reported in the original paper. Gel electrophoresis analysis showed no amplification products, neither in the positive insects nor in the infected almond trees, indicating that the bacterial DNA did not belong to isolates of the subspecies *pauca*. To characterize the isolates associated with the positive detection, a pool of DNAs from the qPCR-positive insects collected in 2022 and 2023 and two almond samples were subjected to multi-locus sequence typing (MLST) (Yuan et al., 2010). BLAST sequence analyses using the dedicated PubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa) unraveled that the allelic profile of all typed positive samples (both insect and almond) corresponded to the sequence type 1 (ST1), clustering within the subspecies *fastidiosa*, reported as the causal agent of Pierce's Disease (PD) of grapevine (Hopkins & Purcell, 2014).

The confirmation of the finding of *Xylella*-infections in almond trees, associated with bacterial isolates genetically and biologically distinct from Xfp-ST53, implied the demarcation of infected and buffer zones around the X. *fastidiosa* subsp. *fastidiosa* ST1 (Xff-ST1) outbreak, distinct from the demarcated Xfp-ST53 areas. Initially, a buffer zone of 2.5 km in radius surrounding the six infected almond trees was delimited. In this buffer zone a reinforced surveillance program targeting host plants known to be susceptible to Xff was carried out. The sampling unit corresponded to each single hectare, in which host plants were visually inspected and one composite sample (consisting of a pool of 4–7 trees) for each host species was collected and tested by qPCR in bulk. The results indicated the occurrence of several positive host plants on the border of the buffer zone, requiring a new delimitation of the outbreak, with the buffer zone extended for additional 2.5 km in radius from the outermost newly discovered positive findings (Fig. 1). Two months after the first Xff-ST1 detection, a total of 21,089 samples representing the 40 species/genera listed in the EU regulation, including almond and other *Prunus* spp., grapevine (*Vitis vinifera*), and fig (*Ficus carica*) have been sampled and tested (Table 1).

The workflow of the diagnostic tests includes a first test on bulked samples, and then the bulk testing

Table 1 List of plant species monitored in the Xff-ST1 outbreak from February to early May 2024. For each species the number of samples subjected to molecular test, those testing positive and those for which the identification of the subspecies (Dupas et al., 2019) has been completed are reported. Host species found infected under field conditions are indicated in bold

Host species	Total samples collected and tested	Samples positive to *Xylella fastidiosa*	Positive samples analyzed for the identification of the subspecies
Acer L	1	0	
Ambrosia artemisiifolia L	1	0	
Asparagus acutifolius L	2	0	
Calicotome spinosa (L.) Link	1	0	
Cercis occidentalis Torr	32	0	
Citrus limon (L.) Osbeck	868	0	
Citrus paradisi Macfad	4	0	
Citrus reticulata Blanco	478	0	
Citrus sinensis (L.) Osbeck	854	0	
Citrus spp. L	14	0	
Erysimum L	1	0	
Ficus carica L	3962	0	
Juglans regia L	87	0	
Laurus nobilis L	2	0	
Lupinus aridorum McFarlin ex Beckner	1	0	
Magnolia grandiflora L	13	0	
Malvarosa (*Pelargonium graveolens)* L	7	0	
Morus L	266	0	
Myrtus communis L	17	0	
Nerium oleander L	560	0	
Olea europaea L	415	0	
Pelargonium x *fragans* Willd	1	0	
***Polygala myrtifolia* L**	**62**	**1**	**1***
***Prunus armeniaca* L**	**913**	**1**	
***Prunus avium* L**	**638**	**4**	**4***
Prunus dulcis (Mill.) D.A. Webb	**5723**	**197**	**162****
Prunus L	503	0	
Prunus persica (L.) Batsch	403	0	
Rhamnus alaternus L	77	0	
Rubus rigidus Sm	66	0	
Ruta chalepensis L	13	0	
Salvia rosmarinus Spenn	301	0	
Sambucus L	3	0	
Spartium junceum L	77	0	
Strelitzia reginae Aiton	42	0	
Ulex europaeus L	5	0	
Ulmus americana L	1	0	
Vinca L	41	0	
***Vitis vinifera* L**	**4579**	**86**	**73*****
Other species	55	0	
Total	**21089**	**289**	**240**

*In all samples the subspecies *fastidiosa* has been identified

**In 150 samples the subspecies *fastidiosa* has been identified. The remaining 12 samples generated high Cq values and could not be assigned to any of the tested subspecies

*** In 71 samples the subspecies *fastidiosa* has been identified. The remaining 2 samples generated high Cq values and could not be assigned to any of the tested subspecies

positive or doubtful are subjected to a second round of tests on individual samples.

So far by the end of April 2024, a total of 289 infected host plants have been detected in the Xff-ST1 demarcated areas: 197 almond trees, 86 grapes (including both vine and table grapes), 4 cherry trees, 1 apricot tree and 1 *Polygala myrtifolia*. None of these infected plants were found with symptoms at the time when sampling and testing were performed (Fig. 2). No infections were recorded on the remaining host species sampled (Table 1). In April, when new shoots were well developed, 10 infected almond trees and 10 grapes were re-sampled for further tests by purifying the total plant DNA from both the xylem tissues and the leaf petioles and midribs, to monitor the detectability of the bacterium in different plant tissues. Results confirmed that at this vegetative developmental stage, the bacterium could only be detected in the xylem tissues recovered from mature cuttings. These results are in line with those reported by Zecharia et al. (2024) in almond trees in Israel, indicating that isolation of viable *X. fastidiosa* cells during the vegetative growth stage was successful from shoots and branches but not from leaves.

One or two representative positive samples for each species, except apricot, were subjected to MLST analysis which confirmed the subspecies *fastidiosa* and sequence type ST1. Indeed, all positive samples are going to be tested by a triplex qPCR assay (Dupas et al., 2019) with the primers/probe combinations for the identification of the subspecies *fastidiosa, multiplex* and *pauca* to rapidly identify the subspecies. So far, the triplex qPCR has been completed on a total of 240 positive samples, whose results indicated the occurrence of Xff in 226 samples, while the remaining 14 samples (12 from almond, 2 from grapevines and 1 from apricot) generated high Cq values (above the limit of detection of the test) and could not be univocally assigned to any of the three subspecies. These samples were also associated to high Cq values when tested by Harper et al. (2010).

The bacterium has been successfully isolated from 20 infected plants, representing different Xff-ST1 host species, except apricot. Cultured isolates have been

Fig. 2 Infected almond tree (on the left) and grapevine (on the right) testing positive for *Xylella fastidiosa* subspecies *fastidiosa.* Photos taken in April 2024

recovered by homogenization of the scraped xylem tissues (almond, cherry and grape) or leaf petioles for *P. myrtifolia* in PBS buffer (1:10 w:v) prior to plate aliquots of the 10-serial dilution prepared from the crude sap on periwinkle wilt-GelRite (PWG) medium or Pierce Disease medium (PD3). For almond, isolation was also attempted by performing fresh-cut imprints from the cuttings on Buffered Charcoal Yeast Extract (BCYE) agar medium. Isolates were more efficiently cultured on PWG, with typical colonies starting to become visible within 5 days from plating, in contrast with 10–12 days required to observe growing cells on PD3 or in the imprints on BCYE medium. *Xylella fastidiosa* colony forming units (CFU) recovered from almond and *P. myrtifolia* were in the range of 10^7–10^6 CFU/ml, with colonies recovered also from almond trees showing high Cq values (i.e. > 28–29). While high bacterial populations were constantly recovered from almond trees, active growing cells from grapes and cherry were very limited.

Overall, the results of the molecular assays and the successful isolation indicate that, under the climate conditions occurring in the region, the bacterium can be effectively detected in the dormancy stage during the winter-early spring season, which notoriously is not the optimal period for bacterial detection in the deciduous trees. The successful establishment of the axenic cultures for almost 20 bacterial isolates will help to produce massive sequence dataset to support molecular studies aimed at elucidating the pathway of introduction and tracing back the sequence type introduction and establishment in the area.

The finding of Xff-ST1 in Apulia follows the one in cherry, grapes and almond in Mallorca (Balearic Islands, Spain) (Moralejo et al., 2019; Olmo et al., 2017), the report in almond and grapes in Israel (Zecharia et al., 2022) and more recently in grape and citrus in Portugal (EPPO, 2023). The field data collected in the first two countries so far indicate that infections are more frequent and widespread in almond than in grapes, and similarly the impact is higher in almond than grapes.

Although data are still limited, in table grape vineyards infected plants are mainly located on the external rows, often close to infected almond trees, suggesting similar epidemiological conditions of those described in North America with the sharpshooters, i.e. primary infections occurring from reservoir outside the vineyards and lack of grape-to-grape transmission.

While sharpshooters have very limited occurrence and distribution in Europe and the main American *X. fastidiosa* vector species have not been reported so far, spittlebugs play key role in European epidemics (such as those affecting olives and almond), and this report also suggests they were able to transmit the bacterium to grapes. Although, PD epidemics are widely associated to different sharpshooters (Krugner et al., 2019; Shih et al., 2013), some studies have also investigated the role of spittlebugs in the PD epidemics (Beal et al., 2021; Cornara et al., 2016), i.e. along the California North Coast grape-growing region, suggesting that abundant populations of spittlebugs may contribute to the persistence of PD epidemics where sharpshooters occur at low population level. Thus,

the finding of the causal agent of PD in one of the most important table-grape districts in Europe urgently calls for research aimed at answering fundamental epidemiological questions such as the role of almond and other alternative hosts, the transmission ecology of spittlebugs in vineyards, the influence of the growing system and management practices commonly adopted for table grapes productions (i.e. the "tendone" trellis system and the plastic cover used throughout the whole vegetative and ripening season). Likewise, it is crucial to estimate the impact of this outbreak:the detection occurred in the winter season when it was not possible to inspect the infected plants for the presence of symptoms, even so farmers and farm advisors did not report the occurrence of suspicious severe symptoms in the area, neither on grapes or almond.

The finding of Xff-ST1 in the Apulia Region also highlights the importance of adopting an integrated surveillance approach for *X. fastidiosa* large-scale surveys. Indeed, given the absence of vector species-bacterial strain specificity, vector surveillance may unveil the presence of the bacterium independently of the bacterial sequence type and its related host range. The efforts for molecular analysis of thousands of sampled insects can be scaled out by replacing tests on insects with transmission experiments in which a relevant number of specimens can be caged on a single recipient plant, which will be then tested.

Area-wide integrated surveys based on inspections carried out in areas categorized with high risk, by sampling main known susceptible host plants (i.e.

susceptible to a wide range of bacterial strains) and vectors, can allow to effectively spot bacterial outbreaks especially in hidden agricultural and landscape compartments.

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Declarations

The authors have no relevant financial or non-financial interests to disclose.
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