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BEYOND XYLELLA

Report on potential new host plants and pathogenicity of *Xylella fastidiosa* strains relevant for EU and the Mediterranean Basin

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1. INTRODUCTION

This deliverable provides a consolidated assessment of new knowledge generated within the project on the host range, pathogenicity, and genetic diversity of *Xylella fastidiosa*, a quarantine pathogen of major relevance to the European Union and the Mediterranean Basin. The results presented here contribute directly to improving the biological and epidemiological understanding required to support surveillance, risk assessment, and management strategies under EU plant health policy.

The primary objectives of this deliverable were: (i) to characterize the pathogenicity and colonization behaviour of a genetically diverse collection of *X. fastidiosa* strains under controlled conditions, (ii) to document host responses across key Mediterranean crops and selected forest and shrub species, (iii) to analyse *X. fastidiosa* occurrence, host associations, and outbreak dynamics based on field surveys in participating countries, and (iv) to integrate genomic and in silico analyses to expand knowledge on *X. fastidiosa* diversity and potential host range. This deliverable comprises four main components:

- **Pathogenicity experiments under controlled conditions** – Experimental inoculations performed across multiple host plants to evaluate susceptibility, symptom development, bacterial colonization, and strain-dependent virulence.
- **Surveys in countries participating in the project** – Field surveillance activities aimed at detecting *X. fastidiosa*, identifying new outbreaks, documenting host plants, and characterizing circulating genotypes.
- **Sentinel plots** – Establishment and monitoring of sentinel plantings to support early detection, epidemiological understanding, and long-term surveillance.
- **Botanic gardens and arboreta** – Targeted surveillance in high-diversity plant collections to identify potential new hosts and refine host prioritisation frameworks.

The work presented in this deliverable reflects the collaborative efforts of multiple project partners. Contributions included experimental design, strain preparation and distribution, inoculation trials, molecular diagnostics, field surveys, genomic analyses, and data interpretation. The partners involved and their specific roles are summarized in Table 1.1.

Table 1.1. Participation of BeXyl partner members in the different deliverable activities

Partners	P1-CSIC	P2-IVIA	P5-UdG	P6-CNR	P8-INRAE	P9-AU	P11-ARO	P15-APTA	P16-UCR	P17-CRSFA	P21-IRFAP	P27-INTA	P30-FRC	P31-BCGI
Pathogenicity experiments	√	√	√	√	√	√	√	√	√	√	√	√	√	
Surveys in BeXyl countries	√	√		√	√	√	√	√	√	√	√	√		
Sentinel plots	√	√	√	√	√	√		√	√	√	√	√		
Botanic gardens	√				√									√

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2. PATHOGENICITY EXPERIMENTS UNDER CONTROLLED CONDITIONS

2.1 Background

This section of the deliverable presents the results of an extensive experimental assessment aimed to evaluate the potential host range, pathogenicity and colonization patterns of a wide collection of *X. fastidiosa* strains. The study focused primarily on strains isolated and adapted to the climatic conditions of Europe and the Mediterranean Basin, but also including other strains isolated on other regions of the world to account for broader bacterial genetic diversity.

A wide collection of *X. fastidiosa* strains isolated from different hosts and geographical regions (Table 2.1), was artificially inoculated under controlled environmental conditions onto the main Mediterranean crops of high economic relevance, including grapevine, almond, and olive. Other important crops, such as blueberry and cherry, were also evaluated to assess their potential susceptibility. The study further included several forest species and typical Mediterranean shrubland plants, which are considered to be potential hosts and may influence pathogen ecology and epidemiology. Furthermore, *Nicotiana benthamiana* was inoculated as a model plant. Endemic strains from the demarcated area of Alicante, as well as strains isolated in Costa Rica and Brazil were evaluated on selected economically important crops to evaluate their pathogenic potential and host adaptation under controlled conditions.

Symptom development was systematically monitored across all experiments, and plant material was periodically sampled to quantify bacterial population levels in the different hosts. The information generated by these studies provides a comprehensive experimental framework for a better understanding of the host range of *X. fastidiosa* and the differential susceptibility of key Mediterranean plant species to genetically diverse *X. fastidiosa* strains.

2.2 Material and methods

2.2.1 Bacterial strains and culture conditions

A total of 59 strains were included to assess their pathogenicity and patterns of colonization in several host plants. Strains were selected to capture broad genetic and biological diversity, ensuring representation of all described subspecies and major sequence types. The dataset comprised isolates from diverse host species and geographic regions, including European Union Member States and non-EU countries (Table 2.1).

Bacterial strains were prepared and distributed to project partners after obtaining the required phytosanitary authorization (Letter of Authorization, LoA) from the competent plant health authority. This procedure ensured the use of harmonized bacterial material across research facilities for pathogenicity experiments.

All bacterial strains were cultured from glycerol stocks on PD3 (Davis et al. 1981), BCYE (Wells et al. 1981), PW (Davis et al. 1981), PD2 (Davis 1980) or PCYE (Lopes and Torres 2006) agar, depending on the most suitable medium for each strain. Cultures were incubated at

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28°C under dark conditions. After 6 to 8 days, colonies were subculture onto new plates and cultured for 6 additional days before use them in the experiment. Stocks of *X. fastidiosa* cultures were stored in broth medium or phosphate-buffered saline (PBS) plus 50 % glycerol at - 80°C.

Table 2.1. *Xylella fastidiosa* strains selected with indication of subspecies, sequence types (ST), geographical origin, host plants, and responsible entities for multiplication and distribution to research partners

Strain[a]	Subspecies	ST[b]	Geographic origin	Host	Source[c]
Temecula	*fastidiosa*	1	USA (Georgia)	Grapevine	AU
CCPM1	*fastidiosa*	1	USA (Georgia)	Grapevine	AU
IVIA5235	*fastidiosa*	1	Spain (Mallorca)	Cherry	IVIA
IVIA5770	*fastidiosa*	1	Spain (Mallorca)	Grapevine	IVIA
IVIA5387.2	*fastidiosa*	1	Spain (Mallorca)	Almond	IVIA
XYL2014/18	*fastidiosa*	1	Spain (Mallorca)	Almond	IVIA
B80 RO19-1	*fastidiosa*	1	Israel (Ram-On)	Almond	AU
B3_SN17-2, B3_SN17-4	*fastidiosa*	1	Israel (Sde Nehemia)	Almond	AU
WM1-1	*fastidiosa*	2	USA (Georgia)	Grapevine	AU
BA_GR31*	*fastidiosa*	1	Italy	Grape	-
M23 *	*fastidiosa*	1	USA (California)	Almond	-
ALS10T14*	*fastidiosa*	nd	USA (California)	Almond	-
ALS17T10*	*fastidiosa*	nd	USA (California)	Almond	-
Med1*	*fastidiosa*	nd	USA (Georgia)	Blueberry	-
XF-68*	*fastidiosa*	nd	Costa Rica	Guava	-
XF-1094*	*fastidiosa*	nd	Costa Rica	Vinca	
XF-1090*	*fastidiosa*	nd	Costa Rica	Coffee	
XF-1093*	*fastidiosa*	nd	Costa Rica	Coffee	-
XF-1105*	*fastidiosa*	nd	Costa Rica	Coffee	-
CFBP8073	*fastidiosa/ sandyi*	75	Mexico (intercepted in France)	*Coffea canephora*	CFBP
IVIA5901	*multiplex*	6	Spain (Alicante)	Almond	IVIA
ESVL	*multiplex*	6	Spain (Alicante)	Almond	IVIA
IVIA6629	*multiplex*	6	Spain (Alicante)	Almond	IVIA
CFBP8418	*multiplex*	6	France (Corsica)	*Spartium junceum*	CFBP
LSV52.20	*multiplex*	6	France (Occitanie)	*Lavander*	CFBP
CFBP13620, CFBP8430	*multiplex*	6	France (PACA)	*Polygala myrtifolia*	CFBP
CFBP8416	*multiplex*	7	France (Corsica)	*Polygala myrtifolia*	CFBP
Am327*	*multiplex*	6	Brazil (Rio Grande do Soul)	Plum	-
ALS15T2*	*multiplex*	nd	USA (California)	Almond	AU
ALS6*	*multiplex*	nd	USA (California)	Almond	AU
Dixon*	*multiplex*	6	USA (California)	Almond	-
LM10*	*multiplex*	7	USA (California)	Olive	-
M12*	*multiplex*	7	USA (California)	Almond	-
CFBP8173*	*multiplex*	41	Georgia (EEUU)	Plum	-
BB08-1*	*multiplex*	41	USA (Georgia)	Blueberry	-
AlmaEM3	*multiplex*	42	USA (Georgia)	Blueberry	AU
BBI64*	*multiplex*	42	USA (Florida)	Blueberry	-
XYL1966/18	*multiplex*	81	Spain (Mallorca)	Wild olive	CSIC
XYL466/19	*multiplex*	81	Spain (Mallorca)	Wild olive	CSIC
XYL468	*multiplex*	81	Spain (Mallorca)	Wild olive	CSIC

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XF3348	*multiplex*	81	Spain (Mallorca)	Almond	CSIC
TOS4	*multiplex*	87	Italy (Tuscany)	Almond	CNR
DeDonno	*pauca*	53	Italy (Salento)	Olive	CNR
CIST-18	*pauca*	53	Italy (Salento)	Olive	CNR
GP-18	*pauca*	53	Italy (Salento)	Olive	CNR
CN-28	*pauca*	53	Italy (Salento)	Olive	CNR
CFBP8498	*pauca*	73	Costa Rica (intercepted in Netherlands)	Coffee	CFBP
XYL1961/18	*pauca*	80	Spain (Ibiza)	Olive	CSIC
IAS-XYL1513-1	*pauca*	80	Spain (Ibiza)	Almond	CSIC
IVIA11529	*pauca*	80	Spain (Ibiza)	Lavander	IVIA
9a5c	*pauca*	13	Brazil (Macaubal)	Citrus	-
J1a12*	*pauca*	11	Brazil (São Paulo)	Citrus	-
Copl4p5*	*pauca*	16	Brazil (São Paulo)	Coffee	-
XF361*	*pauca*	16	Brazil (Minas Gerais)	Olive	-
CFBP8356	*sandyi*	72	Costa Rica (intercepted in France)	Coffee	CFBP
CO33	*sandyi*	72	Costa Rica (intercepted in Italy)	Coffee	CFBP

[a] (*) Strains used only by the indicated partner.

[b] (nd) Sequence type not determined

[c] AU: Auburn University; IVIA: Instituto Valenciano de Investigaciones Agrarias; CFBP: Collection Française de Bactéries associées aux Plantes; CSIC: Consejo Superior de Investigaciones Científicas; and CNR: Consiglio Nazionale delle Ricerche.

2.2.2 Plant species

The selected strains were inoculated into a range of potential host plants, including major crops, such as grapevine, olive, almond or blueberry, as well as forest and shrub species typical of Mediterranean ecosystems, including several oak species, *Spartium junceum* (Spanish broom), or *Helichrysum italicum* (Mediterranean strawflower). Tobacco (*Nicotiana benthamiana*) plants were also included to evaluate *X. fastidiosa* virulence as a model plant host (Baró et al. 2022). Table 2.2 summarizes the inoculated plant species, the strains inoculated in each plant species and the project partner that developed each study.

Plants were maintained in pots filled with either a standardized peat–silt–perlite mixture or a commercial growing substrate. In EU research facilities, plants were cultivated under controlled temperature conditions in PBSL2 biosafety-confined greenhouses or growth chambers officially authorized to work with *X. fastidiosa*. All experimental procedures complied with EPPO biosafety guidelines and EU phytosanitary regulations. Plants were watered to field capacity and fertilized as necessary.

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Table 2.2. Plant species, *Xylella fastidiosa* strains and partner responsible of each pathogenicity experiment

Plant species	Strains	Partner[a]
Grapevine (*Vitis vinifera*)	AlmaEm3, ALS10T14, ALS15T2, ALS17T10, ALS6, B3_SN17-2, B3_SN17-4, BB08-1, Dixon, ESVL, IVIA5387.2, IVIA5770, IVIA5901, M12, M23, Temecula, XF3348	P9-AU
Grapevine (*Vitis vinifera*)	Temecula, WM1-1, XYL2014/18, IVIA5235, IVIA5770, CFBP8416, CFBP8418, LSV47.12, LSV52.20	P8-INRAE
Grapevine (*Vitis vinifera*) (table and wine cultivars)	BA_GR31	P5-CNR
Olive (*Olea europaea*)	IVIA5387.2, CFBP8416, TOS4, XYL468/19, CFBP8498, CIST-18, IAS-XYL1513-1, IVIA11529, XYL1961/18, 9a5c, ESVL ESVL, IVIA5901 OLI17A	P1-CSIC P2-IVIA P27-INTA
Almond (*Prunus dulcis*)	AlmaEm3, B3_SN17-2, CCPM1, CFBP8073, CFBP8416, CFBP8418, CFBP8498, CIST-18, DeDonno, ESVL, IAS-XYL1513-1, IVIA11529, IVIA5235, IVIA5387.2, IVIA5770, IVIA5901, IVIA6629, LSV47.2, LSV52.20, Temecula, TOS4, WM1-1, XF3348, XYL1961/18, XYL1966/18, XYL2014/18, XYL466/19, XYL468/19	P2-IVIA
Prunus dulcis & Prunus cerasifera	Field-infected materials of Xfm-ST26 (isolates were not cultured, but infected materials used directly for graft-inoculate potted plants	P5-CNR
Blueberry (*Vaccinium* spp.)	AlmaEm3, AlmaReb1, AlmaReb3, B3_SN17-2, BB08-1, BBI64, CCPM1, CFBP8173, CFBP8073, CIST-18, CO33, CFBP8356, DeDonno, ESVL, IVIA11529, IVIA5235, IVIA5387.2, IVIA5770, IVIA5901, IVIA6629, Med1, Temecula, TOS4, WM1-1, XF3348, XYL1961/18, XYL1966/18, XYL2014/18, XYL466/19, XYL468/19	P9-AU
Nicotiana benthamiana	WM1-1, XYL2014/18, IVIA5770, IVIA5235, IVIA5387.2, CFBP8073, AlmaEm3, CFBP8416, CFBP8418, CFBP8498, CIST-18, ESVL, TOS4, XF3348, XYL1966/18, XYL466/19, XYL468/19, CFBP13620, 9a5c, CO33, CFBP8356	P1-CSIC
Nicotiana benthamiana	B3_SN17-2, CFBP8416, CFBP8430, CN-28, DeDonno, GP-18, IAS-XYL1513-1, IVIA11529, IVIA5387.2, IVIA5901, IVIA6629, IVIA5770, LM10, LSV52.20, M12,Temecula, XYL1961/18	P4-UdG
Persimmon (*Diospyros kaki*) / Carob (*Ceratonia siliqua*) / Dragon fruit (*Hylocereus* spp.)	ESVL, IVIA5901	P2-IVIA
***Quercus ilex / Q. suber / Fraxinus angustifolia / Arbutus unedo / Juniperus* sp.**	XYL1961/18, CoDiRo	P1-CSIC
Quercus robur, Prunus avium, Corylus avellana	Temecula, De Donno, IVIA5235, CFBP8173	P30-FCRA
Cistus monspeliensis, Helichrysum italicum, Spartium junceum	LSV52.20, CFPB8418, CFBP8416, XYL468, TOS4, IVIA5770, DeDonno, IVIA11529	P8-INRAE

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Coffee (*Coffea arabica*) / Avocado (*Persea americana*) / Sweet orange (*Citrus sinensis*) / Guava (*Psidium guajava*) / Sour guava (*Psidium friedrichsthalium*) / Basil (*Ocimum basilicum*)	XF-68, XF-1094, XF-1090, XF-1093, XF-1105	P16-UCR
Spartium junceum	9a5c, J1a12, Copl4p5, XF361, Am327	P15-APT

[a] AU: Auburn University; INRAE: Institut National de la Recherche Agronomique; CSIC: Consejo Superior de Investigaciones Cientificas; IVIA: Instituto Valenciano de Investigaciones Agrarias; INTA: Instituto Nacional De Tecnologia Agropecuaria; UdG: Universidad de Girona; FCRA: Forestry Commission Research Agency; UCR: Universidad de Costa Rica; APTA: Agencia paulista de Tecnologia dos Agronegocios.

2.2.3 Pathogenicity experiments

To prepare the bacterial inoculum, colonies from each strain were scraped from plates containing the appropriate culture medium and dispersed in culture medium or phosphate-buffered saline (PBS) to an optical density (OD_{600}) of 0.3 to 2, corresponding to a bacterial load of 10^8 to 10^9 CFU/ml. All plant species, except tobacco plants and some forest species, were inoculated using the pin-prick method (EPPO, 2023). Briefly, 10 to 20 µl of cell suspensions (depending on the host plant) were placed at the basal part of a secondary stem, at the first node (corresponding to the first pair of leaves) of the stem, using a micropipette and introduced into the tissue by pricking several times with a fine needle until the suspension was fully absorbed. At least three inoculation points were established per plant. Inoculation was performed in the main stem for small plants and in secondary branches for woody plants.

Forest species (from CSIC) and tobacco plants (CSIC and UdG) were inoculated using a high precision microinjector equipped with a 250 µl Hamilton glass syringe with a bevelled needle tip, following the procedure described by Baró et al. (2021, 2022). In this method, the needle was introduced approximately half of the stem diameter, ensuring penetration into the vascular system and 10 µl was inoculated at each inoculation point.

Except for tobacco plants, a second inoculation was generally performed two to three weeks after the first inoculation, applied to the same branches and at the same inoculation point or at the node immediately above the initial inoculation point, following the same procedure. This additional inoculation was to promote adequate bacterial colonization. The number of plants inoculated per species ranged from five to eighteen. Mock-inoculated control plants were treated with culture medium, sterile water, or PBS, depending on the study.

2.2.4 Detection of *Xylella fastidiosa* in inoculated plants

Plants were monitored for symptom development and colonization dynamics. Inoculated plants were periodically inspected and evaluated for symptoms compatible with *X. fastidiosa* infection, such as leaf scorch or branch desiccation.

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Colonization dynamics were evaluated by sampling leaves, pieces of branches or main stem sections, depending on the host plant. Samples were collected at different distances above and, in some cases, below inoculation point depending on the host plant. The distance from inoculation point and the time of sampling were adjusted according to host plant species. These details are described further within each corresponding result section.

In general, sections of the petiole and lower midrib of leaves were removed and chopped into small pieces and placed into 2 mL microcentrifuge tubes containing beads before being ground at high speed using a high-speed benchtop homogenizer. Branches or stem sections were processed in an extraction bag containing CTAB extraction buffer and the content was ground with a homogenizer. Genomic DNA was extracted either using a CTAB-based procedure following the EPPO protocol PM7/24 (5) (EPPO 2023) or using commercial kits for DNA extraction, according to laboratory-specific procedures.

Bacterial detection was determined using molecular methods in accordance with laboratory-specific procedures based on the protocols described in EPPO PM7/24-5 (EPPO 2023). Table 2.3 summarizes all the molecular techniques and tests used for the detection of *X. fastidiosa* in the collected samples. For additional details, see EPPO protocol PM7/24 (5) (EPPO 2023)

Table 2.3. Summary of molecular protocols and detection and identification level for *Xylella fastidiosa*.

Detection method	Primers	Detection Level	Protocol
Conventional PCR	RST31 / RST33	Species level	Minsavage et al. 1994
Real-time PCR (qPCR)	HL5 / HL6/probe		Francis et al. 2006
	XF16Sf/XF16Sr/XF16Sp		Li et al. 2013
	XF-F/XF-R/XF-P		Harper et al. 2010 (and erratum 2013)
	Xf.Csp6F/Xf.Csp6R/Xf.Csp6P		Ouyang et al. 2013
	XF-F/XF-R/XF-P; Xf.Csp6F/Xf.Csp6R/Xf.Csp6P		Bonants et al. 2019
Tetraplex Real-time PCR	XF-F/XF-R/XF-P; XFF-F/XFF-R/XFF-P; XFFSL-F/XFFSL-R/XFFSL-P; XFM-F/XFM-R/XFM-P; XFMO-F/XFMO-R/XFMO-P; XFP-F/XFP-R/XFP-P	Subspecies level	Dupas et al. 2019
Tetraplex Real-time PCR	*pilA*,histone-like protein, hypothetical protein, putative hemagglutinin-related protein	Subspecies level	Hodgetts et al. 2021
Nested Multilocus sequence typing (PCR)	External and internal primers for genes leuA; petC; malF; cysG; holC; nuoL; gltT	Sequence type level	Cesbron et al. 2020
Digital droplet PCR (ddPCR)		Species level (High sensitivity)	Dupas et al. 2019

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2.3 Results

2.3.1 Host response in grapevine

The susceptibility of grapevine cultivars Chardonnay (AU partner) and Cabernet Sauvignon noir (INRAE partner) to 17 and 11 *X. fastidiosa* strains, respectively (Table 2.2), was evaluated by monitoring symptom development using a disease severity scale from 0 to 5 (Guilhabert and Kirkpatrick, 2005) (Figure 2.1). Additionally, plant samples were collected at the end of the experiment to assess pathogen load and the ability of the tested *X. fastidiosa* strains to colonize the grapevine cultivars. Several petioles were collected above the inoculation point in the cultivar Chardonnay and a stem section approximately 30 cm from the inoculation point was sampled from Cabernet Sauvignon. The strain BA_GR31 isolated recently in Apulia was inoculated to assess its pathogenicity on several grapevine cultivars.

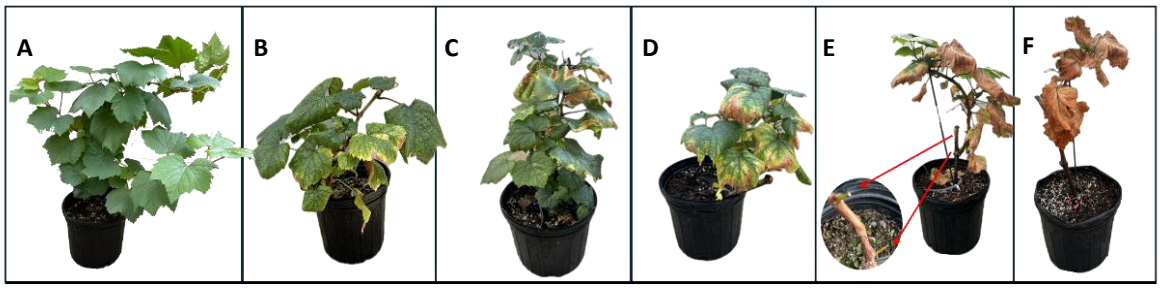


Figure 2.1. Symptom development caused by *Xylella fastidiosa* strains in susceptible grapevine. Mock-inoculated control or asymptomatic plant (A) and plants infected with *X. fastidiosa* strains, representing disease severity ratings from 1 to 5 (B–F). Symptoms were assessed using a visual scale from 0 (healthy, no scorched or abscised leaves) to 5 (severe scorching with numerous matchsticks and leaves restricted to the cane tip).

In the cultivar Chardonnay disease severity increased gradually over time for most inoculated plants, with variation among *X. fastidiosa* strains (Figure 2.2A). Strains IVIA5770, B3SN17-4, B3SN17-2, ALS10T14, and ALS17T10 induced the highest Disease Severity Index (DSI) values, exceedingly 60-70% by week 9. TemeculaL, IVIA5387.2, and M23 showed moderate symptom development with DSI around 50% by week 9, while several strains, including ALS15T2, BB08, ESVL, XF3348, ALS6, Dixon, M12, and the PBS control displayed limited or no disease progression with DSI 25% or less. Differences among strains became pronounced after week 4, indicating varying degrees of virulence and host adaptation within the strain set.

Similarly, analysis of the area under the disease progress curve (AUDPC) (Campbell and Madden, 1990) revealed significant variation in disease development among *X. fastidiosa* strains (Figure 2.2B). Strains IVIA5770 and B3SN17-4 exhibited the highest mean AUDPC values, indicating the most aggressive disease progression, followed closely by B3SN17-2, ALS10T14, IVIA5387.2, and ALS17T10. Moderate AUDPC values were observed for strains M23 and TemeculaL (positive control), while several others, including IVIA5901, AlmaEm3, ALS15T2, BB08, ESVL, XF3348, ALS6, Dixon, M12, and PBS (negative control), showed significantly lower disease intensities. Statistical groupings at 5% level of significance indicated that the most virulent strains (IVIA5770, B3SN17-4, B3SN17-2, ALS17T10, ALS10T14, IVIA5387.2) differed significantly from the least virulent or non-pathogenic strains.

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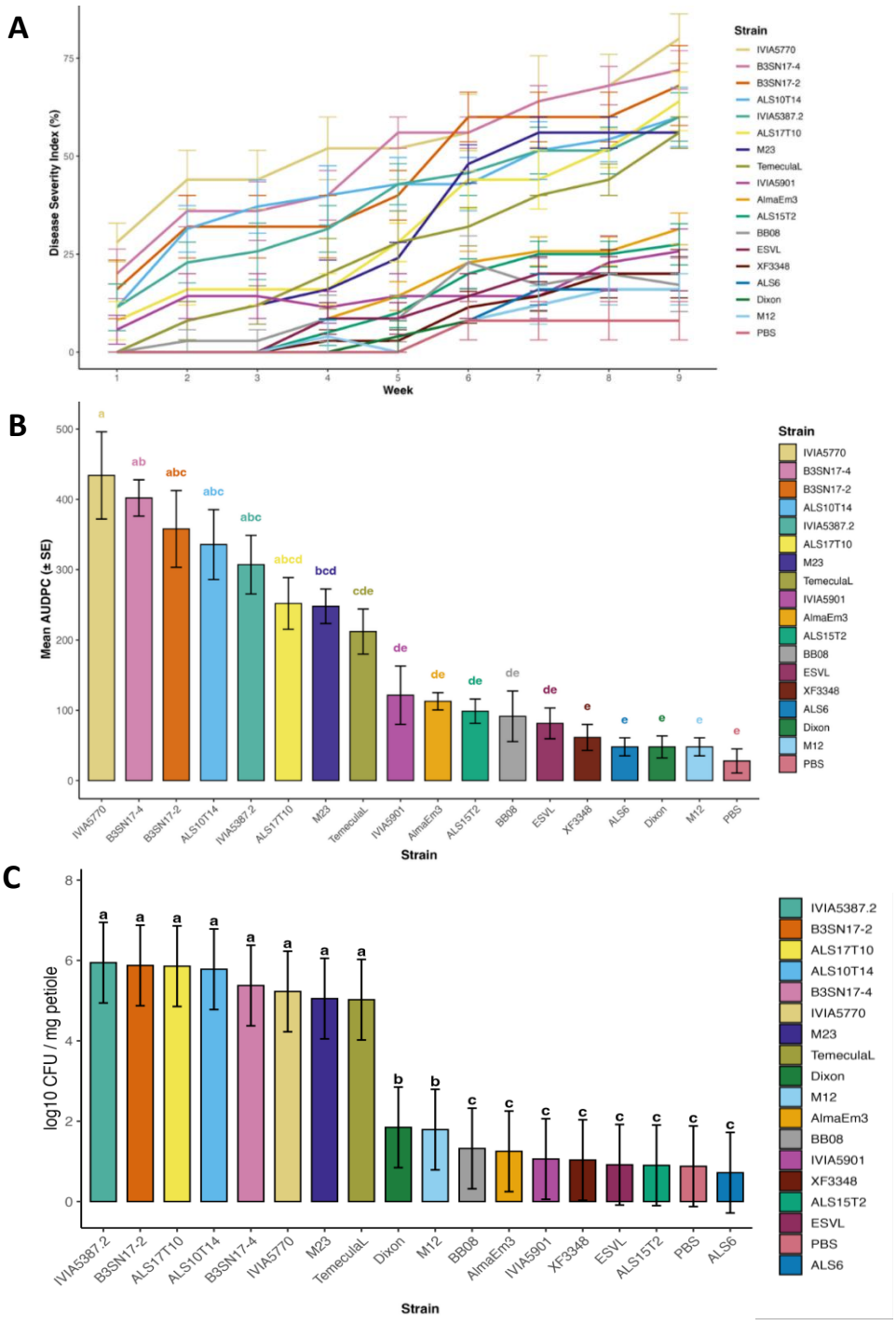


Figure 2.2: Disease progression (A) through the Disease Severity Index (DSI), Area under the disease progress curve of (AUDPC) (B) and estimated bacterial population levels of multiple *X. fastidiosa* strains (log_{10} CFU mg^{-1} petiole) (C) of several *X. fastidiosa* strains inoculated on *Vitis vinifera* 'Chardonnay'. Bars show estimated marginal means (EMMs) from linear model with strain as a fixed effect (for AUDPC) and from a linear mixed-effects model (fixed: strain; random: plant, leaf nested within plant, and plate) (for (log_{10} CFU mg^{-1}). Distinct lowercase letters indicate Tukey HSD groupings at $\alpha = 0.05$ using compact letter display (CLD). n = 7 plants per treatment. DSI was scored weekly for 9 weeks after symptom onset, was calculated from a 0–7 rating scale as (score / 7) × 100. DSI, %; mean ± SE. AUDPC ± SE. log_{10} CFU mg^{-1} ± 95% CI

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Quantification of *X. fastidiosa* populations in grapevine petiole tissues by qPCR revealed clear differences among strains (Figure 2.2C). The strains IVIA5387.2, ALS17T10, B3SN17-2, ALS10T14, B3SN17-4, IVIA5770, TemeculaL, and M23 exhibited the highest bacterial loads, averaging approximately 5–6 $\log_{10}$ CFU mg^{-1} tissue, and did not differ significantly from each other, while strains Dixon, M12, IVIA5901, AlmaEm3, BB08, ALS6, XF3348, ALS15T2 and ESVL had significantly lower bacterial loads compared to the first group.

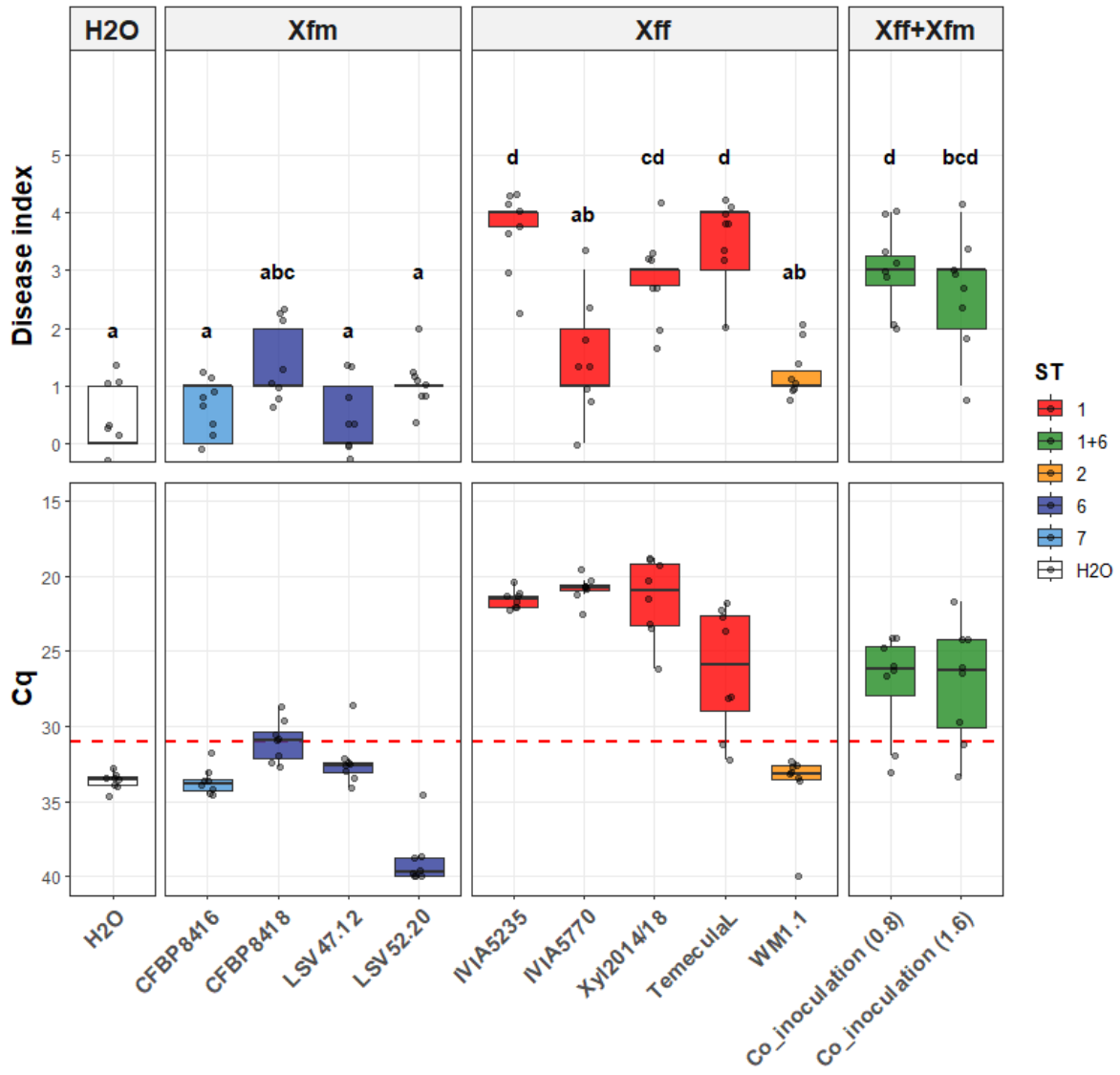


Figure 2.3. Pathogenicity of nine *Xylella fastidiosa* strains in grapevines 18 weeks post-inoculation. The top panel displays disease index scores assessed on a scale of 0 (healthy) to 5 (severely diseased). Different letters indicate statistically significant differences according to a Kruskal-Wallis rank sum test followed by Dunn's posthoc test with Benjamini-Hochberg adjustment. The bottom panel shows bactebrial titers (Cq values) for each treatment. The dashed red line indicates the threshold of Cq=31, below which a plant was considered uninfected. Boxplots are coloured based on Sequence Type (ST) and represent the median (midline) and the interquartile range of eight biological replicates (n=8). Each dot represents one biological replicate. Xfm = *X. fastidiosa* subsp. *multiplex*, Xff = *X. fastidiosa* subsp. *fastidiosa*.

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In the experiment conducted on the cultivar *Cabernet Sauvignon noir*, nine *X. fastidiosa* strains were evaluated. Two additional treatments consisted of a co-inoculation with one strain from subspecies *fastidiosa* (TemeculaL) and a strain from *multiplex* (CFBP8418) in order to investigate whether competition with a non-virulent *multiplex* strain might alter the virulence of Pierce's disease-associated *fastidiosa* strains. The strain TemeculaL (subsp. *fastidiosa* ST1) induced severe symptoms of Pierce's disease, reaching a median disease index of 4 and high bacterial titers (Cq 21-32) (Figure 2.3). Similar results were obtained for the Spanish ST1 strains IVIA5235 and XYL2014/18, which reached similar disease scores and Cq values of 21-22 and 18-26, respectively.

In contrast, the third Spanish ST1 strain IVIA5770 caused unexpectedly low disease scores that were not statistically different from the mock-inoculated control group, despite carrying similar bacterial loads to its Spanish counterparts (Cq 19-22). The strain WM1-1 (ST2) was the only member of the subspecies *fastidiosa* that failed to show clear evidence of colonization by week 18 (Cq 33-40) (Figure 2.3).

As expected, the four strains of subspecies *multiplex* did not cause disease on grapevine at 18 weeks post-inoculation. Nonetheless, a few plants showed evidence of plant colonization with Cq values <31, a threshold close to the detection limit to be considered positive. This was the case for 3/8 plants inoculated with the strain CFBP8418 (ST6 from Corsica) and for 1/8 plants inoculated with LSV47.12 (ST6 from PACA), which reached Cq values of 28-30 (Figure 2.3). This also resulted in slightly higher disease scores for CFBP8418 compared to the control group, but these differences were not statistically significant (Figure 2.3). It is important to note that the two strains are closely related, but LSV47.12 carries a conjugative plasmid that is absent from CFBP8418. There was no evidence of colonization for any of the other *multiplex* strains – LSV52.20 (ST6 from Occitanie) or CFBP8416 (ST7 from Corsica) – as they did not cause visible symptoms and their mean Cq values ranged from 34-40 and 32-34, respectively (Figure 2.3).

In a competition experiment, the plants co-inoculated with the strains TemeculaL (subsp. *fastidiosa* ST1) and CFBP8418 (subsp. *multiplex* ST6) at bacterial concentrations of OD_{600} = 0.8 and OD_{600} = 1.6 produced similar disease scores and qPCR titers to those observed when the strain TemeculaL was inoculated alone (Figure 2.3). Moreover, the tetraplex qPCR detected only the subsp. *fastidiosa* 18 weeks post-inoculation. These findings indicate that the co-inoculation of the non-virulent strain CFBP8418 did not result in detectable persistence in the plants and failed to attenuate the progression of Pierce's disease caused by the virulent strain TemeculaL.

The strain BA_GR31, recovered from the recent *X. fastidiosa* subsp. *fastidiosa* outbreak in Apulia, was used to assess its pathogenicity on table grape (cvs. Vittoria, Baresana, Italia, Cardinal), wine grape (Cabernet Franc, Primitivo, Falangina, Malvasia Nera), and the rootstock 140RU. Self-rooted plants were needle-inoculated by placing 3 drops of 10µl of a highly concentrated bacterial suspension at three nodes. Most of the artificially inoculated vines began to exhibit Pierce's disease-like symptoms 9-10 weeks after inoculation with a certain variability among plants. Leaf scorch first appeared at the base, near the inoculation sites and moved upwards, as the disease progressed. After six months, severe symptoms were observed in all cultivars and in the rootstock, with the only

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exception of the plants of cultivar Vittoria, which displayed milder symptoms, suggesting a delayed in disease progression in this variety (Figure 2.4).

Diagnostic tests carried out using quantitate PCR (Harper et al. 2010) were consistent with symptom development. Table 2.4 summarizes the diagnostic results; most likely the use of high bacterial inoculum dose (three inoculation points) may have promoted a fast bacterial colonization and severe symptom expression. However, similarly to what was observed for the symptoms, diagnostic tests for the cultivar Vittoria showed a lower proportion of plants fully colonized by the bacterium (70% vs 100% in the remaining cultivars) (Table 2.4).

Table 2.4. Results of the diagnostic tests carried out by quantitative PCR, 6 months post inoculation on the apical portions of the inoculated grapevine plants.

Cultivar	% of positive plants detected 6 months post inoculation	Average quantitation cycles recorded on the apical portions of the inoculated plants
Baresana	90%	20.16±1.37
Italia	100%	21.71±2.34
Vittoria	70%	23.50±4.42
Primus	100%	20.23±1.76
Cardinal	100%	19.75±2.48
Cabernet Franc	90%	20.73±1.47
Primitivo	100%	20.18±1.92
Malvasia Nera	100%	19.48±0.83
Falanghina	100%	19.91±2.60
140RU	100%	20.21±1.60

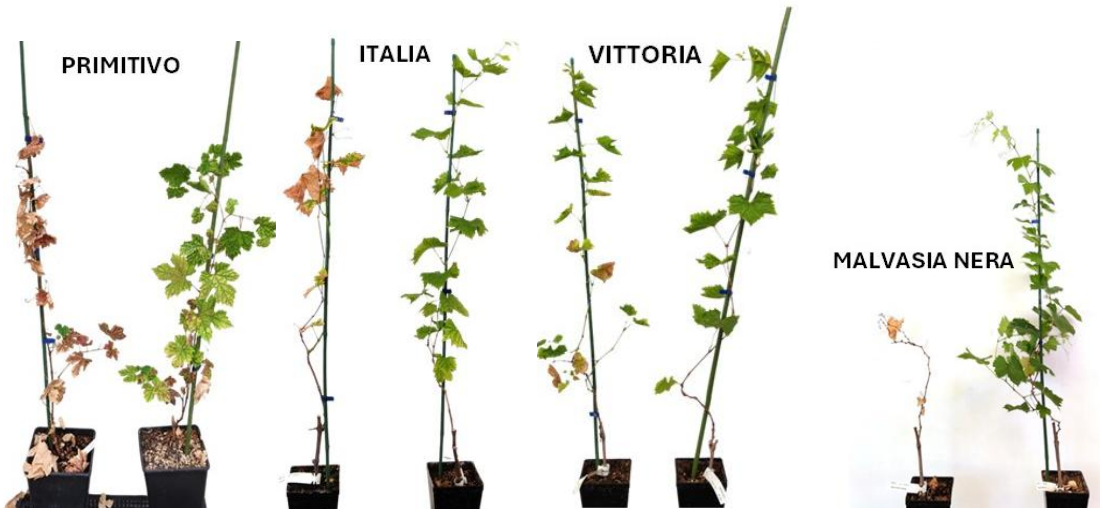


Figure 2.4. Symptom development caused by *Xylella fastidiosa* subsp. fastidiosa ST1 in different grapevine cultivars. Mock-inoculated controls are pots on the right, while representative infected plants are on the left.

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2.3.2 Host response in olive

The response of olive cv. Arbequina to ten inoculated *X. fastidiosa* strains (Table 2.2) was evaluated by sampling a secondary branch at 4 months post inoculation (mpi) to study infection dynamics. The secondary stem was divided into two sections: at 5-10 cm and at 15-20 cm above the inoculation point. The frequency of detection and bacterial load of the different *X. fastidiosa* strains, was estimated using the qPCR protocol of Bonants (Table 2.3).

Frequency of detection in branch samples did not show significant differences among the main factors: *X. fastidiosa* strain, plant section sampled or their interaction ($F<0.11$, $P>0.9955$). Across both branch sections, the frequency of detection was higher ($P\geq0.05$) for the strains 9a5c and CFBP8416, reaching 91.7±0.08%, although differences were not statistically significant ($P\geq0.05$), compared with the strains IVIA11529 and IVIA5387.3, which showed the lowest detection frequencies (58.5±14.86% and 50.0±15.08%, respectively). The remaining strains exhibited intermediate detection frequencies, ranging from 66.7±14.21% (strains TOS4, CIST/18 and XYL1961/18) to 75.0±0.13% (strains CFBP8498, IAS-XYL1513-1, XYL4468) (Figure 2.5A).

X. fastidiosa load, estimated by qPCR Cq values, was significantly affected by strain ($F=9.61$, $P<0.0001$), the plant section sampled ($F=79.03$, $P<0.0001$), and their interaction ($F=2.83$, $P=0.0253$). Among the evaluated strains, XYL1961/18 exhibited the lowest ($P<0.05$) Cq values at 27.2±1.36, indicating the highest population levels, which differed significantly from most other strains except CFBP8498 (31.0±1.36). The lowest bacterial load was detected for the strain 9a5c (36.1±0.31), which did not differ significantly ($P\geq0.05$) from the strains CIST/18, XYL468, IVIA5787.2, IAS-XYL1513-1 and TOS4, with Cq values ranging from 35.0±0.68 to 32.8±0.66, respectively. Intermediate values were detected for the strains CFBP8416 and IVIA11529 (32.3±0.59 and 32.0±1.38, respectively), which did not show significant differences ($P\geq0.05$) from the remaining strains, except for XYL1961/18.

Bacterial population levels were significantly higher ($P<0.0001$) in the branch section closest to the inoculation point (10AIP; Cq = 31.4±0.44) compared with the branch at 20 cm above the IP (20AIP) with Cq values of 34.9±0.49 (Figure 2.5B). Regarding the interaction between the two factors, the highest bacterial load was detected in the 10AIP section for strains XYL1961/18 (26.5±0.89) and CFBP8498 (28.0±0.67). Intermediate bacterial loads were observed for XYL1961/18 in the 20AIP section and for IVIA11529, CFBP8416 and IVIA5387.2 in the 10AIP branch section, with Cq values ranging from 29.4±0.68 to 31.0±0.26, showing no significant differences from the strains XYL1961/18 and CFBP8498. The lowest bacterial loads were generally detected in the 20AIP section, except for the strain 9a5c, which showed low bacterial loads (high Cq values) in both plant sections. The strain IVIA5387.2 had the highest Cq in this section (37.1±0.03), followed by the strains IVIA5387.2, CIST/18, 9a5c, XYL468, IVIA11519 and CFBP8498, with Cq values ranging from 37.0±0.54 to 34.8±1.42 (Figure 2.5B).

The results indicate that all strains inoculated into the olive cv. Arbequina, known to be susceptible to *X. fastidiosa* (Boscia and Altamura, 2021), were capable of infecting this host, with no significant differences among the ten strains inoculated from the different subspecies and isolated from different host plants. However, significant differences were observed among strains in terms of bacterial load and colonization capacity within this olive cultivar. The highest colonization and bacterial load were detected for the strain XYL1961/18

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from subspecies *pauca* and originally isolated from olive in Ibiza Island, followed by strain CFBP8498, also belonging to subspecies pauca and isolated from coffee.

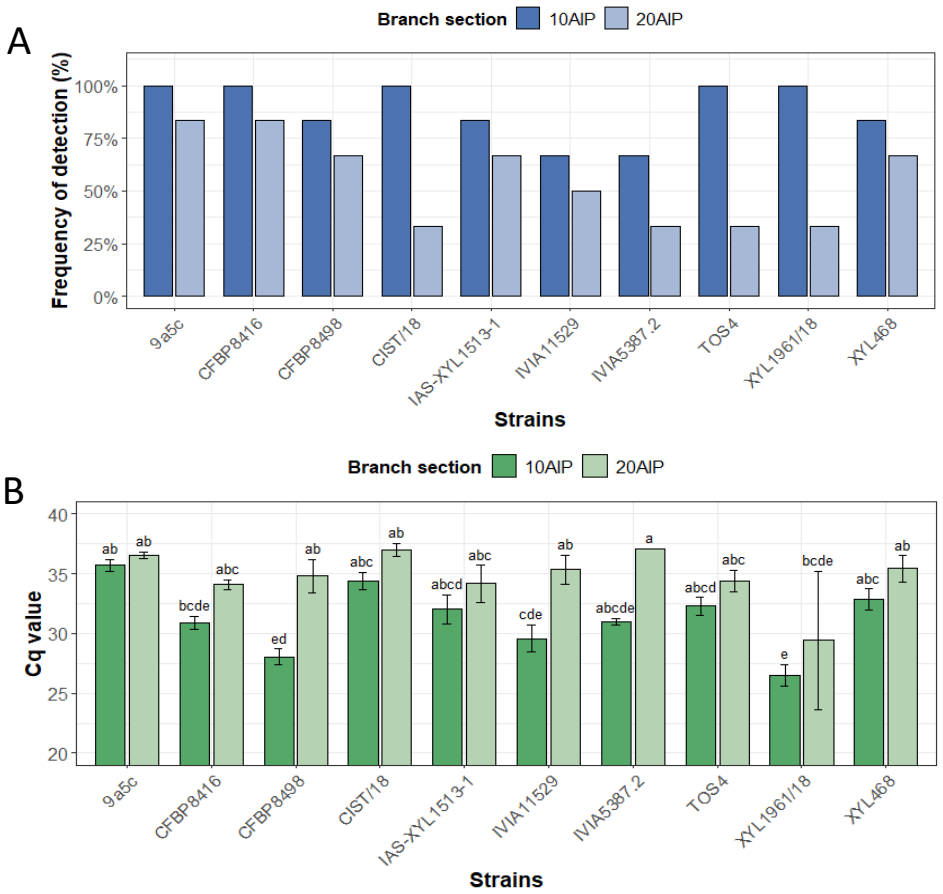


Figure 2.5. Frequency of detection (A) and quantification cycle (Cq) values obtained using the Harper qPCR protocol (B) of positive *Xylella fastidiosa* samples in two stem sections sampled after 4 months post inoculation in Arbequina olive cultivars inoculated with ten *X. fastidiosa* strains. 10AIP: stem section sampled about 7-12 cm above the IP; 20AIP: stem section sampled about 15-20 cm above the IP. Error bars indicate the standard error of the mean. Strains with different letters indicate significant differences (P<0.05) according to Tukey-Kramer test across the interaction between strains and branch section.

In Argentina, the first isolation of *X. fastidiosa* (strain designated OLI17A) from olive cv. Arauco, native to the Tiro Federal II neighborhood in Aimogasta, Arauco Department, La Rioja province was achieved in 2018. According to the Multilocus Sequence Typing (MLST) system, this strain belongs to the ST69 genetic group and the *pauca* subspecies. Inoculum was prepared from colonies of strain OLI17A grown on 12 BCYE plates. The bacterial suspension was adjusted to a concentration of 5.2×10^8 CFU/ml. A total of 11 one-year-old olive trees of the Arauco cultivar (susceptible) and 11 of the Frantoio cultivar (tolerant) were inoculated by using the pinprick technique. In addition, three healthy plants of each cultivar were mock-inoculated with potassium phosphate buffer as controls. The bacterium was inoculated in three consecutive nodes on each of the main stems of the plants. Plants were maintained in a greenhouse at temperatures between 32-38°C, with a 14-hour photoperiod, irrigated to field capacity twice a week, kept insect-free, and monitored for the development of

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symptoms. Plants were evaluated by the qPCR of Harper (Table 2.3). For molecular analyses, leaves were collected from the inoculation point (IP; node a) and from the upper node (node b) at 3 months post-inoculation. At 7 and 12 months, as well as at 1 year and 2 years post-inoculation, samples were collected from the subsequent upper nodes (node b). Koch's postulates were fulfilled by re-isolating the bacterium 2 years after inoculation. The identity of the recovered colonies was confirmed using the general primers RST31 and RST33 specific for *X. fastidiosa*.

Table 2.5. qPCR results and cycle threshold for *Xylella fastidiosa* detection in olive plants of cvs. Arauco and Frantoio sampled at different stem sections from the inoculation point (IP) at different days post-inoculation

Cultivar Plant #	3 months (1st node)	7 months (2nd node)	12 months (3rd node)	16 months (4th node)	24 months (5th node)
Arauco 1	(-)	(-)	(-)	(-)	**(+) S** 30,99
Arauco 2	(-)	**(+) NS** 28,86(b)	(-)	(-)	**(+) S** 24,43
Arauco 5	(-)	(-)	(-)	**(+) S** 32,56	(-)
Arauco 6	(-)	**(+) NS** 32,46(b)	(-)	**(+) S** 29,58	**(+) S** 26,22
Arauco 7	**(+) NS** 28,50(a)/29,19(b)	(-)	(-)	(-)	(-)
Arauco 8	(-)	**(+) NS** 31,00	(-)	**(+) S** 31,38	**(+) S** 30,99
Arauco 10	(-)	(-)	**(+)** 31,26	(-)	**(+)** 26,44
Arauco 12	**(+) NS** 20,09 (a)	**(+) NS** 27,48 (b)	**(+) NS** 31,26(b)	**(+) S** 31,89 (b)	**(+) S** 29,01 (b)
Arauco 13	(-)	**(+)** 30,61	(-)	**(+) S** 31,68	**(+) S** 30,87
Arauco 14	(-)	(-)	(-)	(-)	**(+) S** 28,97
Arauco 19	(-)	(-)	(-)	**(+) S** 30,63	**(+) S** 26,34
Frantoio 11	(-)	(-)	(-)	(-)	**(+) NS** 32,11

(a) Petioles and midribs were extracted for analysis from the leaves at the 3rd IP (approximately 2 to 6 leaves).
(b) Petioles and midribs were extracted from subsequent leaves above the last IP (approximately 1 or 2 leaves) and from the following nodes. S= With symptoms. NS= Asymptomatic.

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First positive qPCR results were obtained 3 months after inoculation in the *Arauco* cultivar, which is considered susceptible, whereas no amplification was detected in the *Frantoio* cultivar. Bacterial translocation through the xylem vessels was confirmed by its detection in nodes located above the inoculation point, indicating that the bacterium was able to move systemically within the plant (Table 2.5).

At 24 months post-inoculation, qPCR detected the bacterium at the fifth node from the inoculation point in 9 of 11 *Arauco* plants (82%). This indicates systemic movement through the xylem across at least five consecutive nodes. Symptom severity increased over time, and by 24 months the infected Arauco plants exhibited pronounced apical dieback, including dry terminal branches. In contrast, only one *Frantoio* plant tested positive two years after inoculation, and it remained asymptomatic. All healthy control plants of both cultivars were consistently negative at all sampling times. These results confirm that *Arauco* is susceptible to *Xylella fastidiosa*, whereas *Frantoio* shows tolerance to strain OLI17A (ST69).

Initial symptoms (leaf chlorosis and apical necrosis) appeared at 16 months and progressed by 24 months to evident shoot desiccation. To complete Koch's postulates, the bacterium was successfully re-isolated from plant No. 19 on BCYE medium. This plant was selected due to its severe symptoms (apical branch dieback) and low Ct value at 24 months (Ct 26.34), consistent with a high bacterial load. Colony identity was confirmed using general *X. fastidiosa* primers (Figure 2.6). Symptom expression remained minimal throughout the experiment. In June 2025, plants were re-assessed by qPCR using samples collected from the sixth node upward, targeting leaves with mild apical or marginal necrosis. Although typical leaf-tip desiccation was absent, slight necrosis was observed in some leaves. The limited symptoms may indicate restricted bacterial proliferation, potentially influenced by environmental or physiological factors. Additional evaluations are planned under warmer conditions to assess temperature effects on bacterial detection and symptom development. Together, these findings demonstrate that *X. fastidiosa* subsp. *pauca* is the causal agent of the observed symptoms under both greenhouse and field conditions and is responsible for Olive Quick Decline Syndrome (OQDS) in affected areas such as Aimogasta (Arauco Department).



Figure 2.6. Olive plant Nº19 of cv. Arauco infected with *Xylella fastidiosa* subsp. *pauca* from which the pathogen was re-isolated.

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2.3.3 Host response in almond

The response of almond plants of cv. Marcona was evaluated against a total of thirty strains of *X. fastidiosa* were inoculated (Table 2.2). Several leaves per plant were sampled at 1, 3 and 6 mpi to assess infection dynamics of each strain, progressively collecting samples from one node higher on the plant at each time point. Both, the frequency of detection and bacterial load, was estimated by qPCR with the qPCR protocol of Bonants (Table 2.3). At 1-month mpi, nearly all strains successfully established in almond plants. Only one strain (B3_SN17.2) showed a slightly lower infection rate, reaching 87.5% of inoculated plants. These results confirm the high efficiency and consistency of the inoculation protocol.

In contrast, marked variability among strains became evident at 3 mpi (Figure 2.7). Infection prevalence ranged from 0% to 100%, with intermediate values distributed across the full range of possible outcomes (12.5% to 87.5%, corresponding to between 1/8 and 7/8 infected plants, respectively). Two strains (XF3348 and XYL1961/18) were no longer detectable (0%), whereas one strain (CCPM1) maintained 100% infection frequency. The remaining strains displayed heterogeneous persistence levels, with no clear clustering pattern based on geographical origin or host of origin. However, when analyzed by subspecies, a differential trend was apparent: a higher proportion of *X. fastidiosa* subsp. *fastidiosa* (*Xff*) strains maintained infection levels above 50% compared with subspecies *multiplex* (*Xfm*) and subspecies *pauca* (*Xfp*).

Within the *X. fastidiosa* subsp. *fastidiosa* group (Figure 2.7A), infection prevalence ranged from low to very high. CCPM1 showed 100% infection, while IVIA5235 also exhibited high persistence (≈85-90%). In contrast, CFPB8073 displayed the lowest infection rate in this group (≈10-15%), and strains such as IVIA5770 and WM1-1 remained at intermediate levels (≈35-40%). Cq values ranged from approximately 22.5 to 35.4, indicating substantial variability in bacterial load among strains. In the *X. fastidiosa* subsp. *multiplex*-group (Figure 2.7B), variability was similarly pronounced. Strains IVIA5901 and XYL1966/18 showed high infection percentages (around 85-90%), and LSV52.20 reached around 75%.

Other strains, such as IVIA6629 and XYL466-19, remained at low levels (approximately 10–25%), and XF3348 was not detected. Cq values ranged from approximately 24 to 34.6. In the Xfp group Figure 2.7C), infection levels were generally moderate to low; however, strain CFPB8498 stood out with a relatively high infection percentage (around 60%), whereas CIST-18 and IAS-XYL1513-1 remained below 25%. Cq values in this group were relatively homogeneous, mostly between 30 and 35. Regarding the other two strains corresponding to subsp. *sandyi*/*morus* (data not shown), CFPB8356 showed a relatively high infection percentage (~60%), while C033 was moderate (~35–40%), with intermediate Cq values (~28–33).

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Figure 2.7. Percentage of positive almond plants inoculated with *Xylella fastidiosa* subsp. *fastidiosa* (A), *X. fastidiosa* subsp. *multiplex* (B) or *X. fastidiosa* subsp. *pauca* strains (C), according to detection by qPCR Harper et al. (2910, *erratum* 2013), and the mean Cq value obtained, three months after the inoculation.

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In the experiment conducted in Italy, given the eradication measures in force in Apulia for both *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex* outbreaks, field isolates were maintained in planta by collecting cuttings in late summer/early autumn and top-graft rootstocks or plants of different almond varieties. Downward bacterial movement, symptoms on the grafted scions and on the plants used as rootstock (both rootstock and cultivars) were monitored.

Briefly, infected almond cuttings (individually tested by qPCR) harbouring either *X. fastidosa* subsp. *fastidiosa*-ST1 or *X. fastidosa* subsp. *multiplex*-ST26 were grafted onto three species: seedlings of *Prunus cerasifera* (myrobolan*)*, and to a limited extent onto almond cv. Avijor and cv. Tuono. Almost 50% of the grafts took. This low percentage was likely a consequence of the poor quality of the plant material (in most cases infected trees were old and abandoned trees). Indeed, those that successfully sprouted produced dwarfed shoots, probably due to the impact of the bacterium on the plant vigour.

For all the grafts that did not succeed, samples were taken from the rootstocks immediately below the bud union, yielding consistently negative results, suggesting that a connection between the vessels must be established for bacterial movement to occur, whereas the solely contact between infected and healthy xylem tissues does not allow infection process to initiate.

On the new sprouts from the grafted scions, typical almond leaf scorch (ALS) symptom, including marginal scorching of leaves, yellow bands between the necrotic tissue and the inner green parts, started to develop between 60-75 days, on both *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex*-infected scions. At the 10th month after grafting, leaves grown on these infected scions tested positive, with a rate of 75% (36 out of 48) and 40.91% (9 out of 22), respectively for *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex*, with mean Cq values of 23.60 and 20.70 respectively.

Tests on the portions below the bud union showed different susceptibility:

(i) leaves and/or young shoots collected from the myrobalan rootstocks, that showed 45.45% (10 out of 22) grafted with *X. fastidiosa* subsp. *multiplex* -scions were positive, indicating that the bacterium successfully moved into the rootstock. Whereas only 3 out of 48 (6.25%) grafted with *X. fastidiosa* subsp. *fastidiosa*-bud sticks gave positive results, suggesting a potential resistance of this rootstock species to *X. fastidiosa* subsp. *fastidiosa* strain. Furthermore, the average values of bacterial titer also corroborate this evidence (31.71 for *X. fastidiosa* subsp. *fastidiosa* vs 18.89 for *X. fastidiosa* subsp. *multiplex*).

(ii) five and two almond plants of cv. Avijor were grafted with *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex*-infected scions respectively, while three almond plants of cv. Tuono with *X. fastidiosa* subsp. *fastidiosa*-infected scions. In all cases, new leaves and sprouts from the infected bud sticks tested *X. fastidiosa*-positive, with mean Cq values of 26.26±3.46, 21.15±1.00 and 20.88±0.062 for *X. fastidiosa* subsp. *fastidiosa*-infected Avijor plants, *X. fastidiosa* subsp. *multiplex*-infected Avijor plants, and *X. fastidiosa* subsp. *fastidiosa*-infected Tuono plants. Leaves and shoots collected from cv. Tuono rootstocks also tested *X. fastidiosa* positive (mean Cq value = 25.52±4.73) suggesting a high susceptibility of this almond cv. Conversely, an opposing outcome was observed testing plant tissues from cv Avijor: *X. fastidiosa* subsp. *multiplex*-grafted plants resulted positive by qPCR (mean Cq value of 28.49±5.94), whereas all *X. fastidiosa* subsp. *fastidiosa*-grafted plants tested negative.

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2.3.4 Host response in blueberry

The susceptibility of blueberry cv Rebel to 27 *X. fastidiosa* strains (Table 2.2) was evaluated by monitoring disease symptom development (Figure 2.8) following the protocol described by Oliver et al. (2015) and Shantharaj et al. (2024) using a 0 to 7 disease severity scale and calculating the area under the disease progress curve (AUDPC) (Campbell and Madden, 1990). Additionally, at the end of the experiment, several petioles were collected from two infected branches above the inoculation point to quantify pathogen load using a calibrated curve for colony-forming units (CFU/ml).

X. fastidiosa symptoms started appearing on plants approximately 9-10 weeks after inoculation. AlmaEm3, the positive *X. fastidiosa* control (highly virulent on blueberry) used in this study, was the treatment in which symptoms appeared first, and it showed the highest disease severity and the greatest disease progression over time (Figure 2.9A). Similarly, IVIA5901 was the European strain to first show symptoms on blueberry plants, but its disease severity and progression did not increase as much as AlmaEm3.

The plants showed typical disease symptoms; leaf discoloration started from the base and moved towards the stem tip (Figure 2.8 and 2.9). Most treatments started developing symptoms no later than 3 weeks after symptoms first appeared in the fastest treatment. Only five treatments (XYL466/19, XYL468/19, IVIA5387.2, CFBP8356, and TOS4) did not show any symptoms (Figure 2.8A,B). Similarly, plants inoculated with PBS buffer treated as negative control, did not show symptoms. XYL1966/18 and CFBP8073 showed greater disease severity than the other treatments (Figure 2.8A,B), although disease severity varied among replicate plants within treatments. AlmaEm3 exhibited a disease severity of around 70%, whereas for the other treatments it ranged between 0-22% (Figures 2.9A,B and 2.10A,B). For AlmaEm3, the disease incidence was 100%, i.e., all plants in that treatment developed symptoms. However, in other treatments, not all plants used as replicates for treatment developed similar symptoms, and in infected plants, not all the branches showed symptoms. Disease incidence in other treatments ranged from 50% to 80%.

Based on the severity of symptoms developed under each treatment, each isolate was classified into three categories: highly symptomatic, mildly symptomatic, and asymptomatic. XYL1966/18 and CFBP8073 were considered highly symptomatic, whereas XYL466/19, XYL468/19, IVIA5387.2, CFBP8356 and TOS4 were considered as asymptomatic strains and remaining strains were classified as mildly symptomatic (Figure 2.8A,B). Symptoms were rated for a total of 9 weeks after first inoculation. The AUDPC calculated using Disease Severity Index (DSI) (%) was significantly (P<0.05) highest only for AlmaEm3 (250-300%), followed by CFBP8073 (approximately 200%) relative to all the other treatments (Figures 2.8A,B and 2.9A,B). For the symptomatic strains, AUDPC values ranged between 20-120% and did not differ significantly among strains. For the five asymptomatic strains mentioned above, AUDPC values were zero. Amplification of bacterial population in all plant tissue samples at the end of experiment indicated that there were detectable bacterial populations in all treatments, irrespective of whether they cause symptoms or not (Figures 2.9C and 2.10C).

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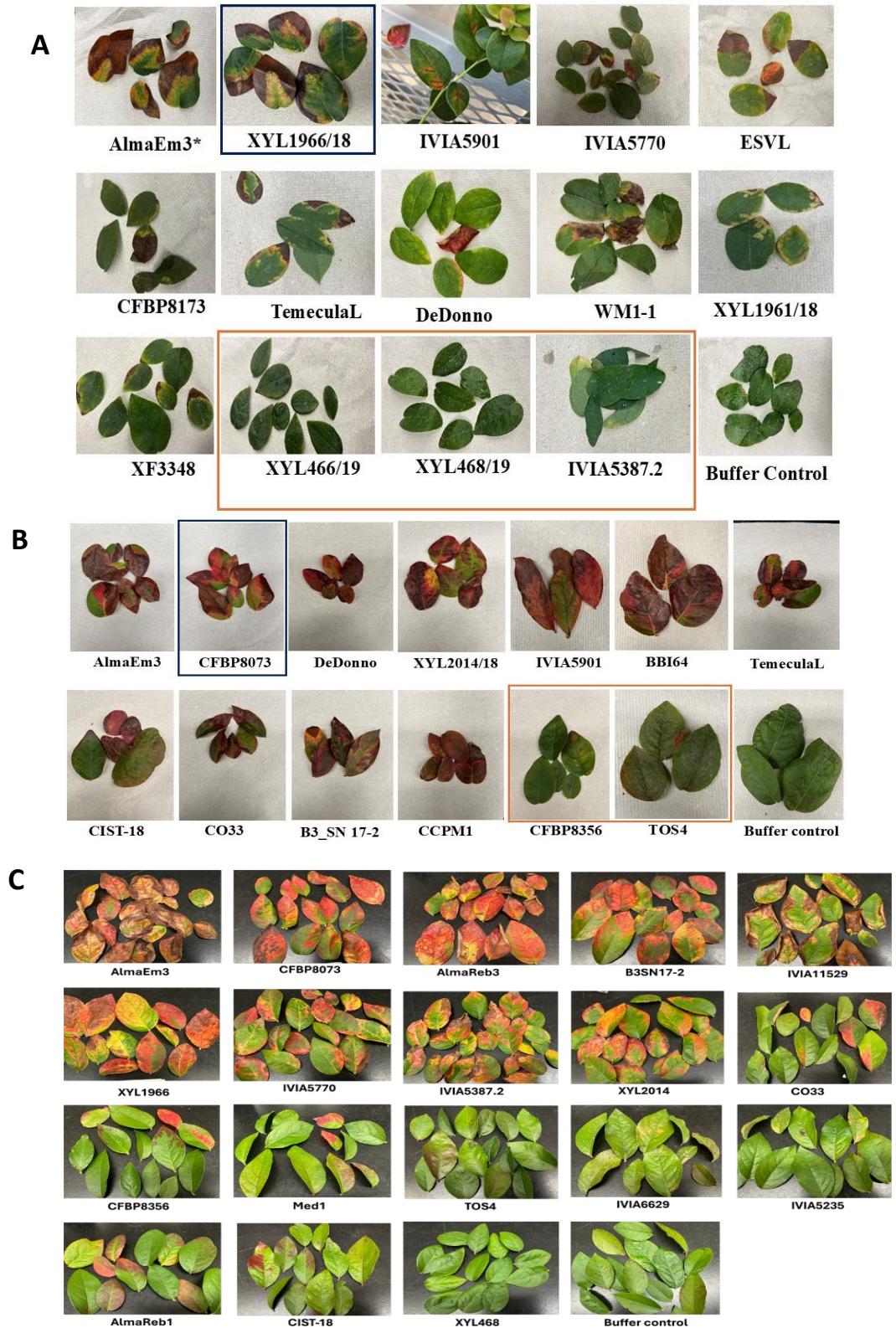


Figure 2.8. Symptoms produced by different *Xylella fastidiosa* strains on *Vaccinium corymbosum* 'Rebel' in a controlled environment in years 2023 (A), 2024 (B) and 2025 (C). AlmaEm3 was isolated from blueberry plants in U.S. and was used as positive control. XYL1966/18 and CFBP8073 strain is highlighted in purple color, showing the similar severity of disease symptoms as AlmaEm3. Three strains at the bottom highlighted in orange color, were all asymptomatic, and the rest of the strains were mildly symptomatic as illustrated in this figure.

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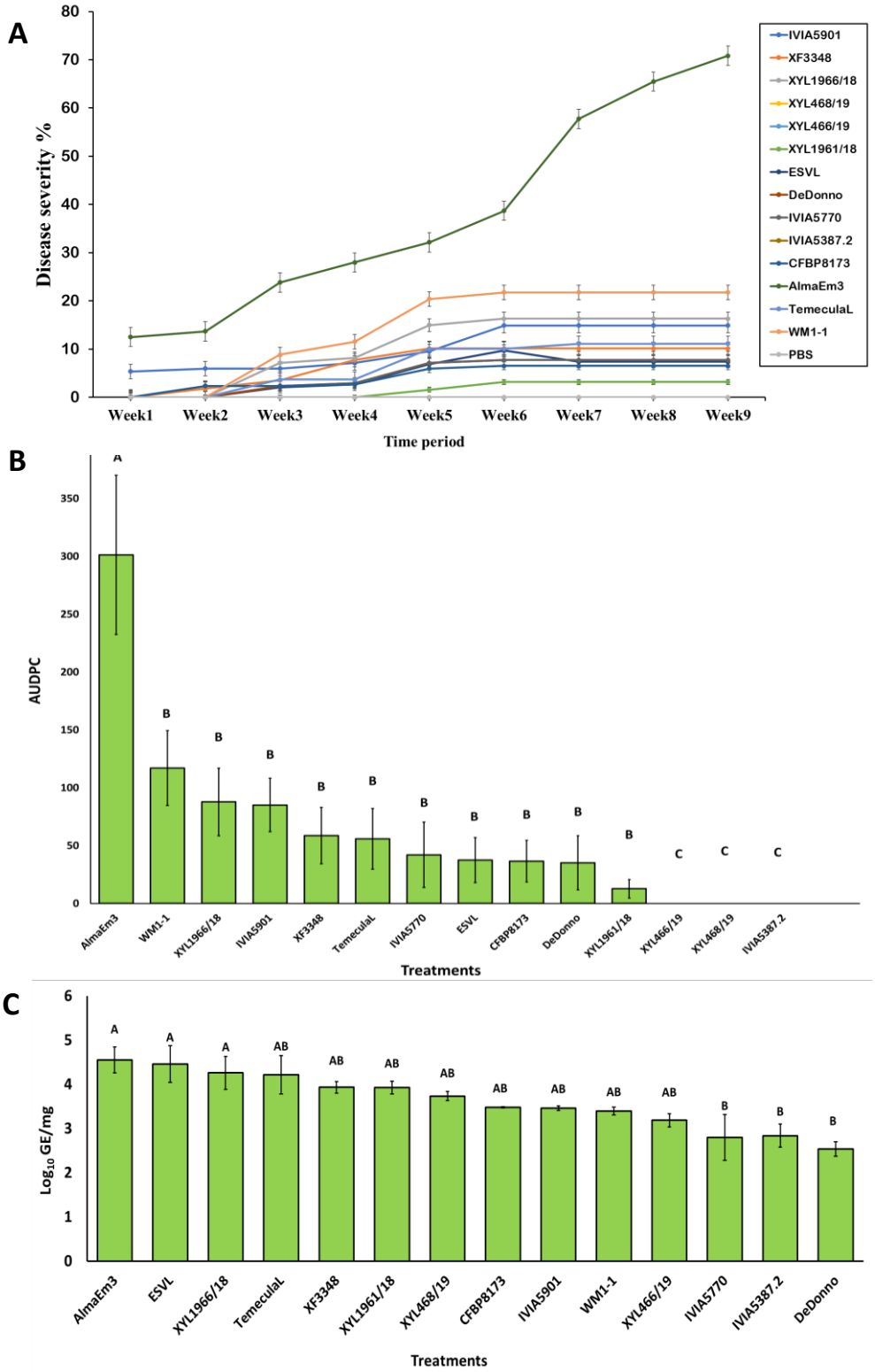


Figure 2.9. Assessment of virulence of different European strains on *Xylella fastidiosa* on blueberry plants tested in 2023. (A) Disease progress curve based on disease severity index percentage. (B) Area under the disease progress curve, (C) Bacterial population levels determined by qPCR.

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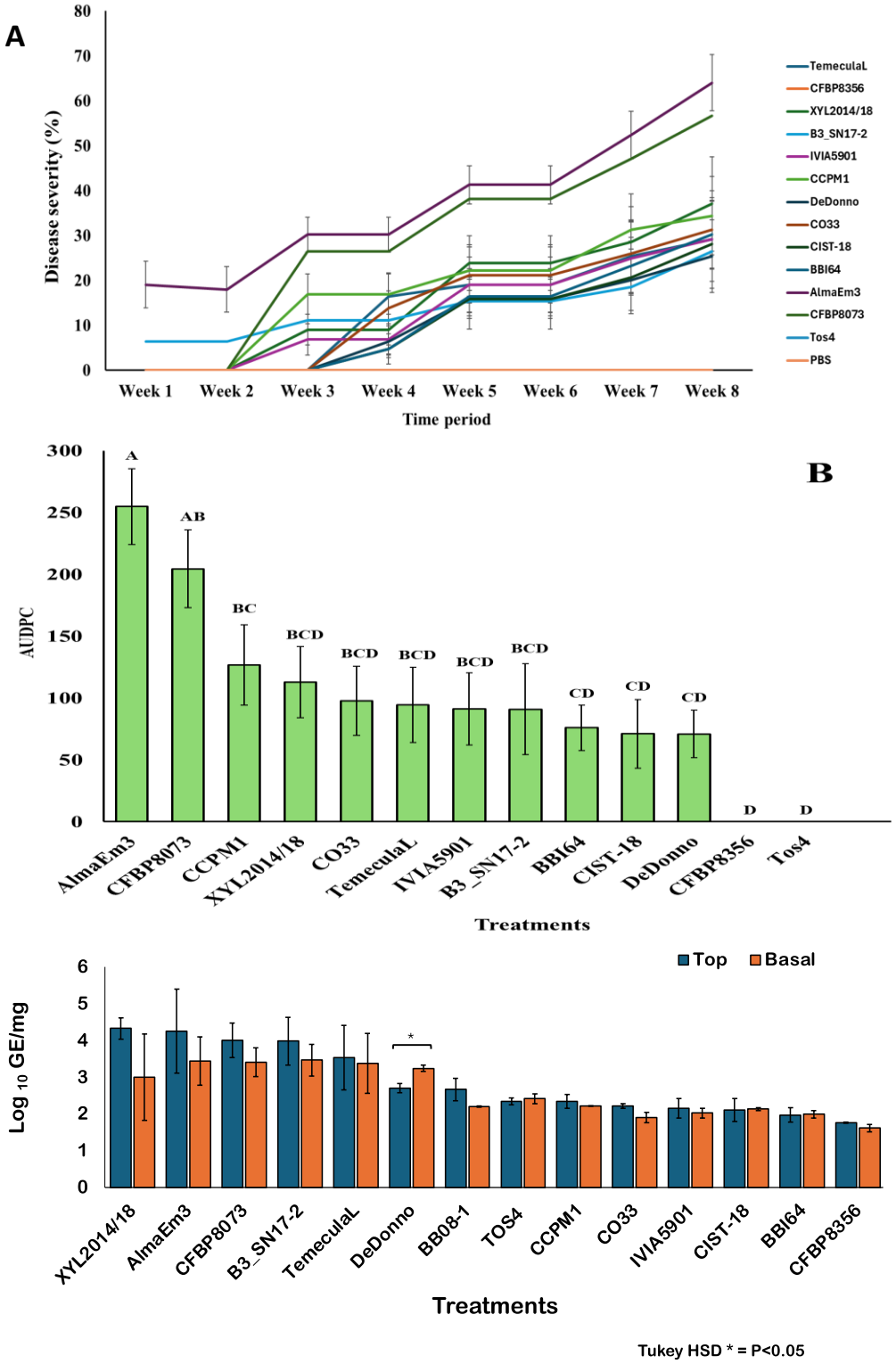


Figure 2.10. Assessment of virulence of different European strains of *Xylella fastidiosa* on blueberry plants tested in 2024. (A) Disease progress curve based on disease severity index percentage. (B) Area under the disease progress curve. (C) Bacterial population levels determined by qPCR.

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In 2023, three European strains ESVL, XYL1966, TemeculaL had similar bacterial populations of ~4.2-4.4 $\log_{10}$ genome equivalents (GE) per milligram of plant tissue. Most strains, i.e., XF3348, XYL1961/18, XYL468/19, CFBP8173, IVIA5901, WM1-1, and XYL466/19 had population levels ranging between 3-4 $\log_{10}$ GE/mg. IVIA5387.2, IVIA5770, and DeDonno had detectable population levels of ~2.5-2.8 $\log_{10}$ GE/mg. IVIA5387.2 and IVIA5770 had significantly (P<0.05) lower population levels as compared to AlmaEm3, whose population levels were highest among all the treatments. In 2024, bacterial populations for top and basal leaves did not show significant differences for all treatments except DeDonno, where the population in basal leaves was higher than that in the top leaves (Figure 2.10C).

To further the assess the virulence of diverse *X. fastidiosa* strains in blueberry plants, another set of strains was inoculated in a third experiment. Disease progression, as measured by the DSI, varied significantly among *X. fastidiosa* strains during the 9-week evaluation period in blueberry plants (Figure 2.11A). The strain AlmaEm3 consistently exhibited the highest DSI values throughout the experiment, reaching approximately 75-80% by week 9, indicating rapid and severe symptom development. In contrast, CFBP8073, AlmaReb3, B3SN17-2, IVIA11529, XYL1966, and IVIA5770 exhibited moderate disease progression, with final DSI values ranging from 40 to 60%. Strains such as IVIA5387.2, XYL2014, and C033 showed only mild disease symptoms that progressed slowly over time, while other strains, such as CFBP8356, Med1, TOS4, IVIA6629, IVIA5235, AlmaReb1, CIST-18, and XYL468, including PBS-inoculated control plants, maintained minimal or no visible symptoms (<10% DSI). Similarly, AUDPC analysis revealed significant differences in disease development among *X. fastidiosa* strains inoculated in blueberry plants (Figure 2.11B). The strain AlmaEm3 exhibited the highest mean AUDPC value (> 550), indicating rapid and severe symptom progression over time. Strains CFBP8073, AlmaReb3, B3SN17-2, and IVIA11529 exhibited moderate disease progression, forming a statistically similar group with AUDPC values ranging from 350 to 400. XYL1966 and IVIA5770 produced intermediate levels of disease, whereas IVIA5387.2, XYL2014, and CO33 induced only mild symptom development. Several other strains, including CFBP8356, Med1, TOS4, IVIA6629, IVIA5235, AlmaReb1, CIST-18, XYL468 exhibited very low AUDPC values.

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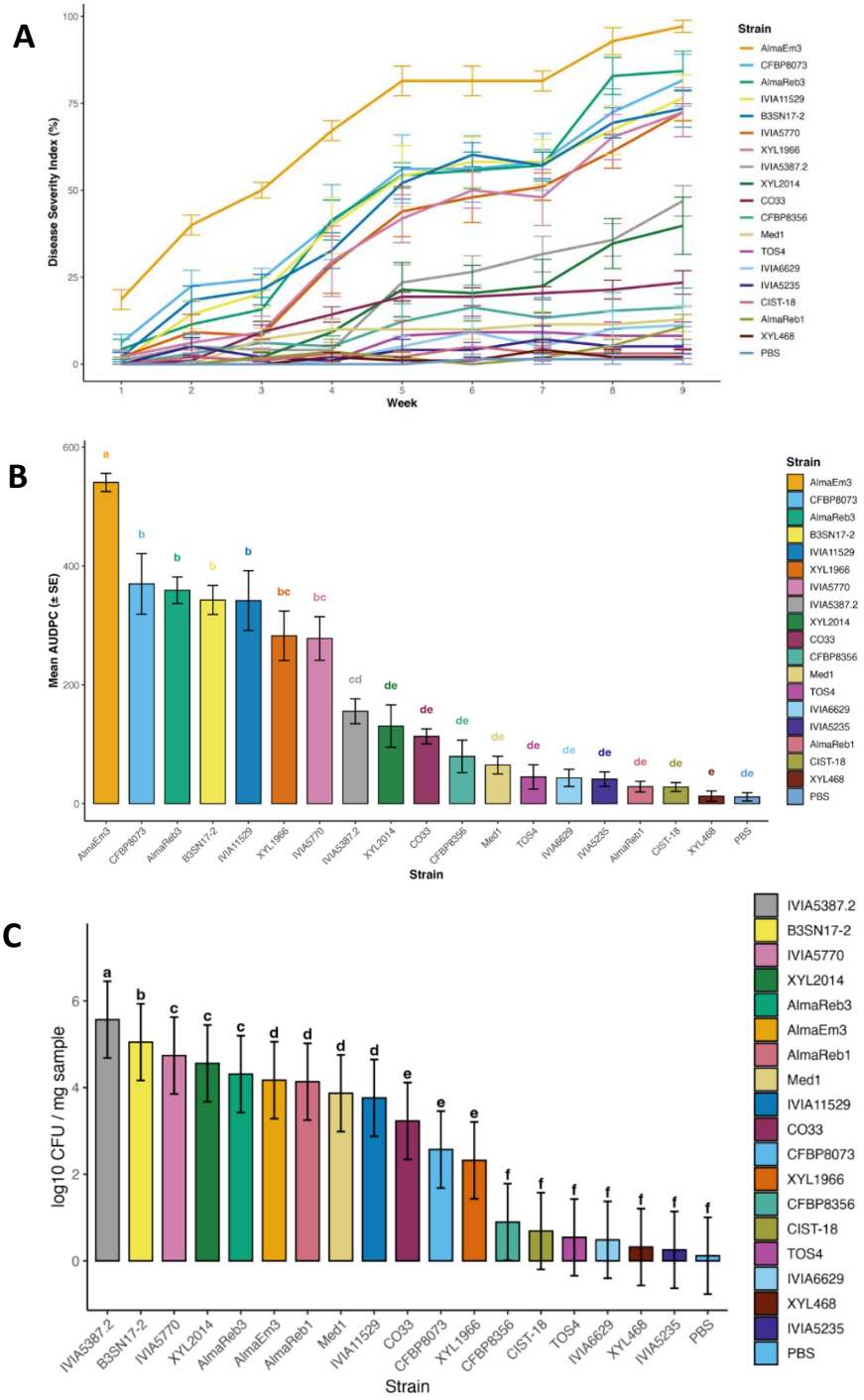


Figure 2.11. Disease progression evaluated through (A) the Disease Severity Index (DSI), (B) Area under the disease progress curve of (AUDPC) and (C) estimated bacterial population levels of multiple *X. fastidiosa* strains (log_{10} CFU mg^{-1} petiole) of several *Xylella fastidiosa* strains inoculated on *Vaccinium corymbosum* 'Rebel'. Bars show estimated marginal means (EMMs) from linear model with strain as a fixed effect (for AUDPC) and from a linear mixed-effects model (fixed: strain; random: plant, leaf nested within plant, and plate) (for (log_{10} CFU mg^{-1}). Distinct lowercase letters indicate Tukey HSD groupings at $\alpha = 0.05$ using compact letter display (CLD). n = 7 plants per treatment. DSI was scored weekly for 9 weeks after symptom onset, was calculated from a 0–7 rating scale as (score / 7) × 100. DSI, %; mean ± SE. AUDPC ± SE. log_{10} CFU mg^{-1} ± 95% CI.

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Quantification of *X. fastidiosa* populations in blueberry midrib samples by qPCR revealed significant variation among bacterial strains (Figure 2.11C). Strain IVIA5387.2 exhibited the highest mean bacterial load (nearly 6 $\log_{10}$ CFU mg^{-1} sample), significantly exceeding that of all other strains. Moderate bacterial loads were detected in strains B3SN17-2, IVIA5770, XYL2014, and AlmaReb3 (~ 5 $\log_{10}$ CFU mg^{-1} sample), whereas AlmaEm3, AlmaReb1, Med1, and IVIA11529 maintained intermediate levels (~ 4 $\log_{10}$ CFU mg^{-1}). In contrast, CO33, CFBP8073, and XYL1966 displayed lower populations (2-3 $\log_{10}$ CFU mg^{-1}), and several strains (CFBP8356, CIST-18, TOS4, IVIA6629, XYL468, and IVIA5235 including the PBS control) remained near background detection levels.

2.3.5 Host response in tobacco

Two independent experiments were conducted using tobacco (*Nicotiana tabacum*), given its importance as a model plant for studying host– *X. fastidiosa* interactions (Baró et al. 2022). Both experiments followed the same methodology for inoculation described in Materials and Methods. The population of *X. fastidiosa* was determined at 7, 14, 21, 28, 35 and 42-days post inoculation (dpi).

In both experiments, disease progression was evaluated by calculating the area under the disease progress curve (AUDPC) using a 0 to 5 disease severity scale (Figure 2.12), and maximum disease level (Dmax) was determined for each strain to estimate their aggressiveness.

Additionally, at each time point, three plants were sampled by collecting stem segments approximately at 5-10 cm above the IP. The area under the growth curve (AUGC), and the maximum population level (Pmax), using Log_{10} CFU/g obtained by interpolating C_q values (Baró et al. 2021) from the corresponding standard curves were evaluated in the first experiment. In the second experiment, bacterial quantification of these stem segments was performed through Cq values obtained using the Harper qPCR protocol (Table 2.3).

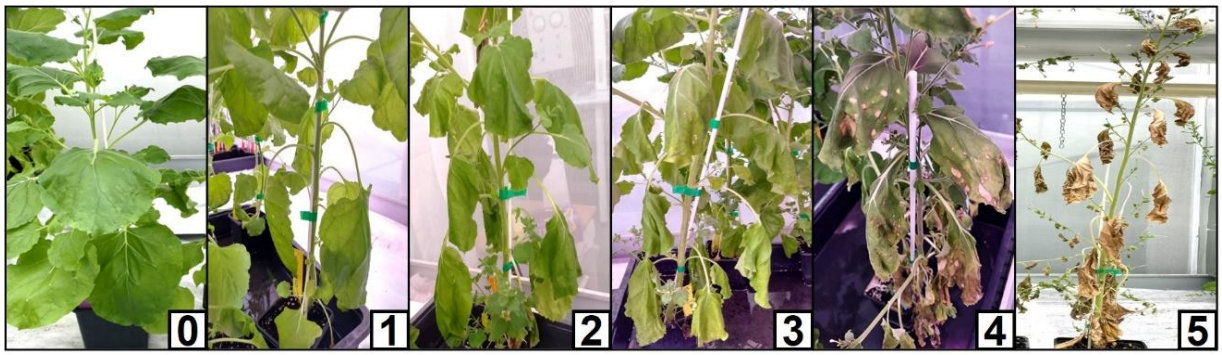


Figure 2.12. Severity scale used to assess disease development in *Nicotiana benthamiana* plants infected *with Xylella fastidiosa*: 0. no symptoms; 1. 1 to 5 leaves just beginning to show decay; 2. 5 to 10 leaves showing significant decay; 3. one half or more of leaves showing decay and few showing necrosis; 4. all leaves showing decay and one-half or more showing necrosis; and 5. dead plant.

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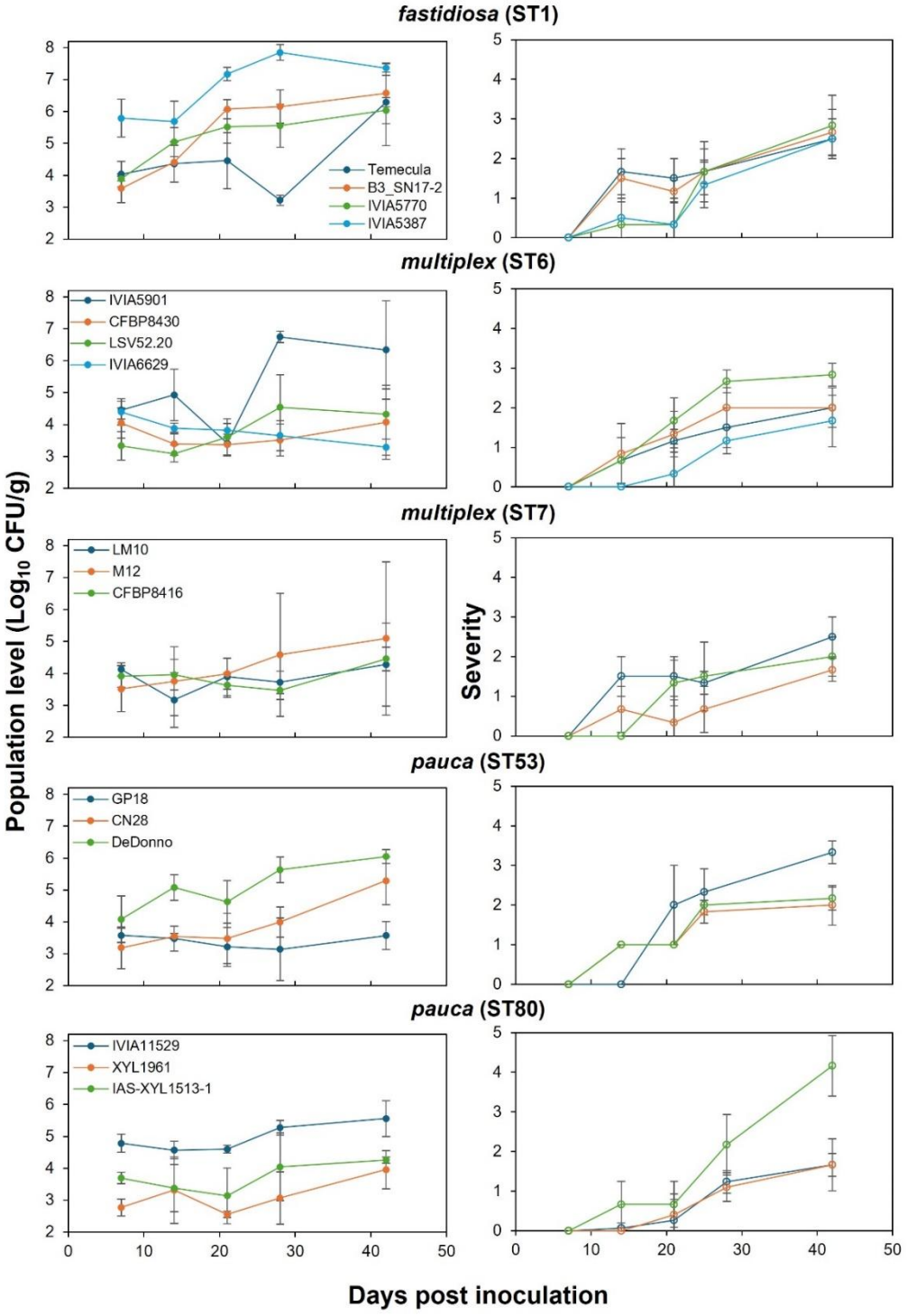


Figure 2.13. Population levels (left) and severity (right) dynamics of *Xylella fastidiosa* strains in *Nicotiana benthamiana* plants. The plants were inoculated with a dose of 3 x 10^6 CFU/plant and sampled at 7, 14, 21, 28 and 42 days post-inoculation (dpi). The sampling zone for qPCR analysis corresponds to 8 cm of stem over the inoculation point. Values are the means of three plants, and error bars represent the standard deviation of the mean.

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Table 2.6. Aggressiveness parameters of the *X. fastidiosa* strains in *Nicotiana benthamiana* at 42 dpi. Values are followed by the standard deviation

Subspecies[a]		Strain	AUGC[b]	AUDPC[c]	P_{max}[d]	D_{max}[e]
fastidiosa	ST 1	Temecula	7.31 ± 0.69	9.42 ± 1.04	6.29 ± 0.14	2.50 ± 0.50
		IVIA 5387	18.25 ± 1.86	6.58 ± 1.53	7.85 ± 0.25	2.50 ± 0.10
		IVIA 5770	10.11 ± 0.75	7.42 ± 1.23	6.04 ± 1.11	2.83 ± 0.76
		B3_SN17-2	14.48 ± 1.65	9.17 ± 1.01	6.57 ± 0.95	2.67 ± 0.58
multiplex	ST 6	IVIA 5901	12.60 ± 1.61	7.08 ± 2.08	6.75 ± 0.18	2.00 ± 0.50
		CFBP 8430	5.79 ± 1.55	8.17 ± 2.50	4.07 ± 1.15	2.00 ± 0.50
		LSV 52.20	4.92 ± 2.01	10.58 ± 0.58	4.54 ± 1.02	2.83 ± 0.29
		IVIA 6629	3.49 ± 0.71	4.58 ± 0.14	4.39 ± 0.41	1.67 ± 0.58
	ST 7	LM10	7.70 ± 2.24	8.75 ± 0.87	4.27 ± 1.30	2.50 ± 0.50
		M12	8.66 ± 3.94	4.50 ± 1.39	5.09 ± 2.40	1.67 ± 0.29
		CFBP 8416	3.45 ± 0.52	6.58 ± 1.61	4.45 ± 0.37	2.00 ± 0.50
pauca	ST 53	DeDonno	9.27 ± 0.24	8.25 ± 0.43	6.05 ± 0.21	2.17 ± 0.29
		GP-18	5.63 ± 1.95	10.50 ± 1.95	3.58 ± 0.22	3.33 ± 0.29
		CN-28	6.63 ± 0.95	7.75 ± 0.87	5.29 ± 0.75	2.00 ± 0.50
	ST 80	IAS-XYL 1513-1	7.06 ± 1.28	10.83 ± 1.94	4.26 ± 0.10	4.17 ± 0.76
		XYL 1961	4.55 ± 1.44	4.58 ± 0.76	3.96 ± 0.60	1.67 ± 0.29
		IVIA 11529	7.83 ± 0.39	4.58 ± 1.63	5.56 ± 0.56	1.67 ± 0.58

[a] ST, sequence type
[b] AUGC, Area Under the Growth Curve
[c] AUDPC, Area Under the Disease Progress Curve
[d] P_{max}, maximum population level (Log_{10} CFU/g)
[e] D_{max}, maximum disease level

Population dynamics showed effective colonization in *N. benthamiana* plants for all strains, confirming the suitability of this host as a model plant. In general, an increase in cell concentration over time was observed, and strains differed in the cell multiplication rates, even among strains of the same subspecies and ST (Figure 2.13 and 2.14). Symptom progression showed a similar pattern among the strains; after the onset of the first symptoms, there was a period during which severity remained stable until it increased again towards the end of the experiment (38-42 dpi). In general, no relationship was observed between symptom and the population concentration of *X. fastidiosa* cells in the plant. Strains that exhibited greater aggressiveness (XYL1513-1, GP18, and LSV 52.20) were not those that reached a high cell concentration (IVIA5387, B3_SN17-2, and IVIA5901). On the contrary, the least pathogenic strains were IVIA6629 (subspecies *multiplex* ST6) and XYL1961 (subspecies *pauca* ST80), and these strains also showed the lowest cell concentration over time, as well as the least developed symptoms.

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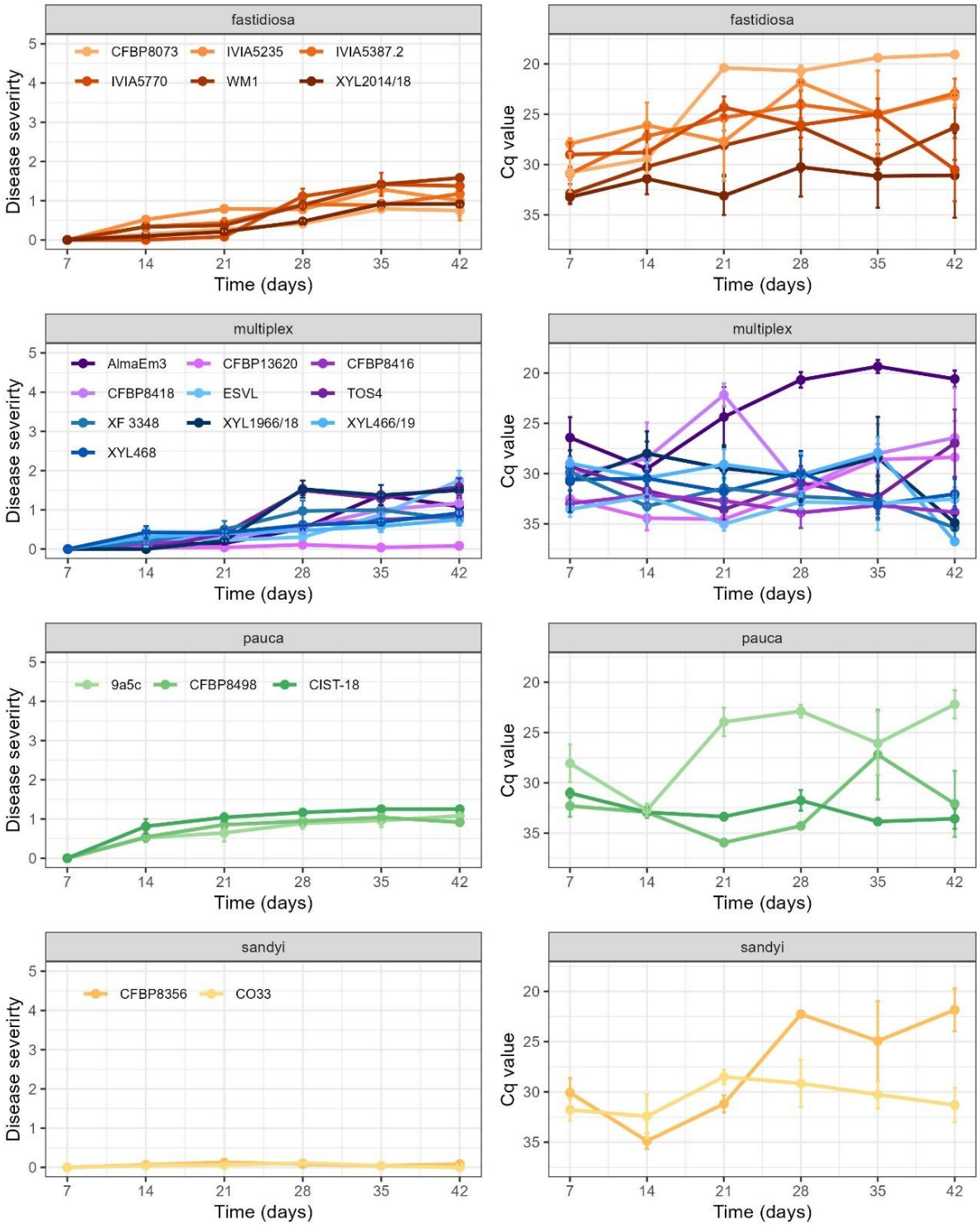


Figure 2.14. Disease severity (left) and population levels (Cq value) (right) dynamics of *Xylella fastidiosa* strains in *Nicotiana benthamiana* plants. Three plants of each treatment were sampled at 7, 14, 28 and 42 days post inoculation (dpi). The sampling zone for qPCR analysis corresponds to 8 cm of stem over the inoculation point. Values are the means of three plants, and error bars represent the standard error of the mean.

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In addition, important differences between *X. fastidiosa* strains from the same subspecies and STs were observed (Table 2.6). For example, the four strains of the *X. fastidiosa* subsp. *fastidiosa* ST1 exhibited similar levels of symptoms and disease progression, but the analysis of population dynamics showed clear differences, where the strain Temecula had an AUGC of 7.31 and the strain IVIA 5387 had an AUGC of 18.25. Similarly, the three strains of the subspecies *pauca* ST80 showed similar population levels (AUGC between 4.55 and 7.83), but disease aggressiveness differed significantly, with both the least and most aggressive strains studied being among them (strain XYL1961/18 and strain IAS-XYL 1513, respectively). The strains that exhibited greater infection capacity (cell concentration in the plant) were the strains of the subspecies *fastidiosa* ST1, as well as IVIA5901 of subspecies *multiplex* ST6 (reaching values between log 6-7 CFU/g at 42 dpi). In contrast, the strains with the lowest colonization levels corresponded to subspecies *pauca* ST80, the strain GP18 (subspecies *pauca* ST53), and IVIA6629 (subspecies *multiplex* ST6), with cell concentrations remaining between log 3-4 CFU/g over time. The strains that caused the highest severity indices were IAS-XYL 1513-1, GP18, both subspecies *pauca* (ST80 and ST53 respectively), and LSV 52.20 (subspecies *multiplex* ST6).

In the second experiment, consistent with the results obtained in the first, effective colonization was observed in all strains inoculated in tobacco and bacterial load generally were increased over time (Figure 2.14). AUDPC was significantly influenced by the inoculated strain (F=5.4, P<0.001). The highest AUDPC values (>17.6) were observed for strains belonging to subspecies *pauca* (9a5c, CFBP8498 and CIST-18: 24.9±0.86, 26.9±0.89 and 34.3±2.02, respectively), and *fastidiosa* (WM1-1: 26.6±0.98), followed by two strains belonging to subspecies *multiplex* AlmaEm3 (19.1±1.34) and CFBP8418 (18.8±2.57), indicating a higher disease development. Intermediate AUDPC values (11.4 to 16.1) were recorded for strains showing moderate symptom development, primarily including strains from subspecies *fastidosa* and *multiplex*. Very low AUDPC (0.4 to 6.6), indicating almost no symptom development were observed for the strains from subspecies *multiplex* (XF3348, ESVL, XYL466/18, CFBP13620) and from *sandyi* (CFBP8356 and CO33).

Based in Cq values at the end of the experiment (42 dpi), the highest bacterial loads were detected for strains of subspecies *fastidiosa* XYL2014/18, IVIA5387.2 and IVIA5770 (19.1±0.26, 22.9±1.44, 23.2±0.81, respectively), AlmaEm3 from *multiplex* (20.6±0.84), CIST-18 from *pauca* (22.2±1.40) and CFBP8356 from *sandyi* (21.8±2.13). The lowest bacterial loads at the end of the experiment were detected for strains with Cq values above 32.5, that in some strains, such as CFBP8498 or 9a5c, low bacterial load did not impair symptom development, reaching the highest disease development (AUDPC > 24.9).

No clear pattern was observed among subspecies or STs suggesting that symptoms expression might be strain-dependent. Strains with higher bacterial load did not necessary induce more severe symptoms, and conversely, strains with lower loads could produce strong symptom development.

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2.3.6 Host response in forest and shrub plant species typical from the Mediterranean

The response to *X. fastidiosa* infection in forest and shrub species typical of the Mediterranean region was primarily assessed by qPCR using the Harper test (Table 2.3) due to the limited or absent symptom development observed after inoculation. In general, for all plant species stem samples were collected at 10-15 cm from inoculation point after 3, 9 and 14 mpi, to detect and quantify *X. fastidiosa*, allowing the evaluation of infection as the proportion of qPCR-positive plants and the estimation of bacterial load.

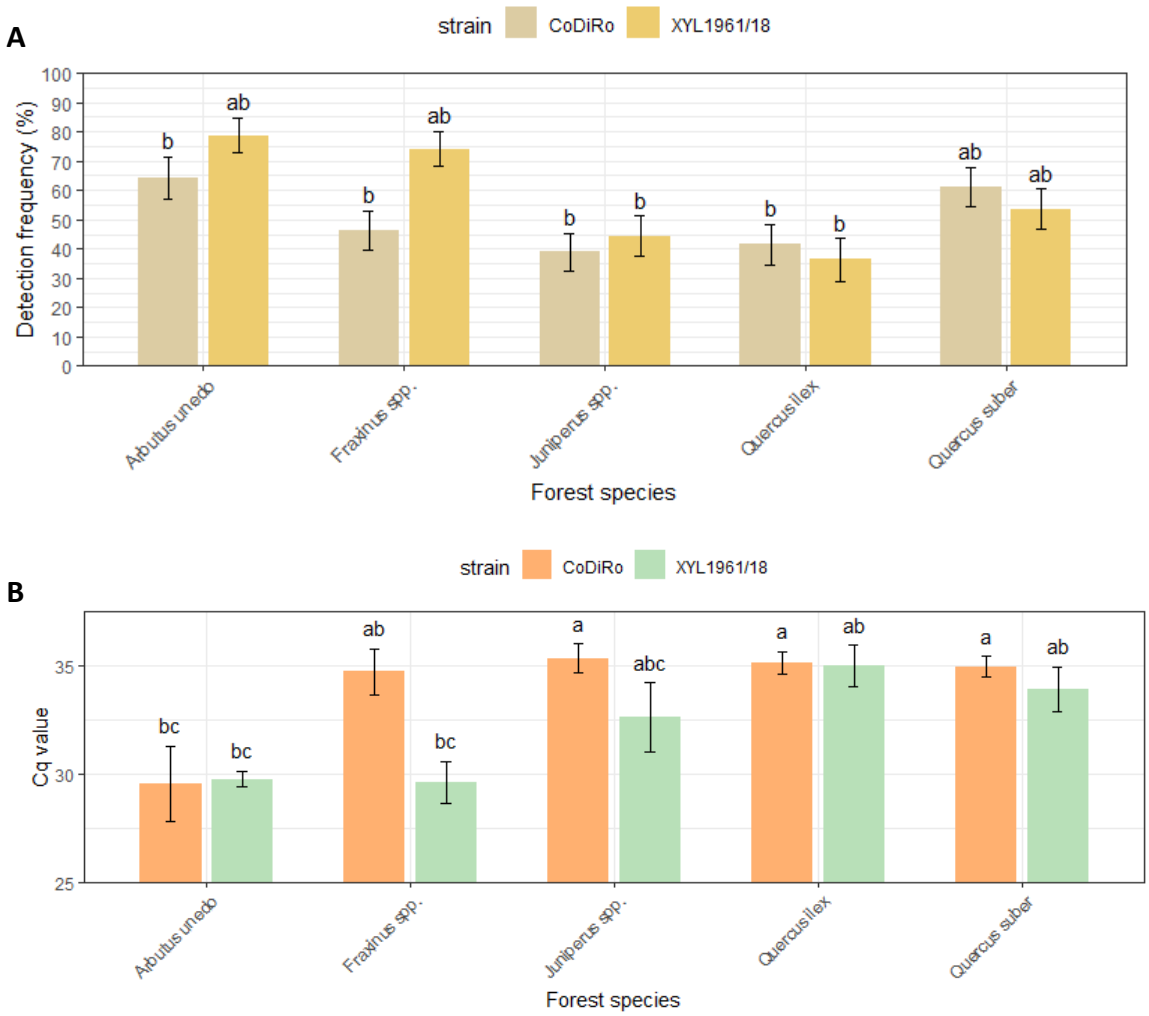


Figure 2.15. Frequency of detection (A) and quantification cycle (Cq values) obtained using the Harper qPCR protocol for the detection (B) of *Xylella fastidiosa*-positive samples in the five forest plant species inoculated with two *X. fastidiosa* strains, across all stem fragments sampled (15AIP and 30AIP) and at 3, 9 and 14 months post the first inoculation. Error bars indicate the standard error of the mean. Strains with different letters indicate significant differences (P<0.05) according to Tukey-Kramer test across the interaction between strains and branch section. mpi: months post inoculation; 15AIP: branch section 15 cm above the inoculation point; 30AIP: branch section 30 cm above the inoculation point.

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None of the forest species (*Arbutus unedo*, *Fraxinus angustifolia*, *Juniperus thurifera*, *Quercus ilex* and *Quercus suber*) inoculated with the two *X. fastidiosa* strains (XYL1961/18 and CoDiRo) developed visible disease symptoms after 14 mpi. The overall results for detection frequency and bacterial load across forest species, strains, sampling time (3, 9, 14 mpi) and stem segments sampled are presented in Figure 2.15. Bacterial detection frequency was strongly influenced by forestry species (F=6.64, P<0.0001), the inoculated *X. fastidiosa* strain (F=6.20, P=0.0132), sampling time (F=27.36, P<0.0001) and the plant section sampled (F=4.14, P<0.0001). The only significant interaction was observed between forest species and strains (F=3.89, P<0.0041).

Pairwise comparisons among forestry species revealed significant differences in bacterial detection frequency. The highest frequencies (P<0.05) were detected in *A. unedo*, *F. angustifolia* and *Q. suber*, reaching 68.3, 54.8 and 53.1%, respectively. In contrast, significantly lower detection frequency was recorded for *Q. ilex* and *J. thurifera*, with values of 36.0 and 34.7%, respectively. However, no significant differences were found between these two species and *Q. suber*. Regarding the inoculated strains, XYL1961/18 showed a significantly higher (P<0.05) frequency of detection (53.6%), compared with CoDiRo (44.3%). Across sampling time, a higher proportion of positives samples (P<0.05) were detected at 9 mpi (3 months after the second inoculation), reaching 76.9%. This was followed by samples collected at 14 mpi (8 months after the second inoculation), with a detection frequency of 41.4%, which remained significantly higher than that sampled at 3 mpi, when the lowest values for frequency detection were recorded (30.6%). Detection frequency was also significantly higher in the section closest to the inoculation point (15AIP), reaching 63.7%, whereas the section of plant located at 30 cm above inoculation point (30AIP) decreased (P<0.05) to 33.0%.

Regarding the interaction between forestry species and *X. fastidosa* strain, the highest frequency of detection (P<0.05) was detected for the strain XYL1961/18 inoculated in *A. unedo* (75.5%) and *F. angustifolia* (72.3%). These values did not differ significantly from the combination *A. unedo* with CoDiRo strain (59.0%) and of both strains inoculated in *Q. suber* (56.3% and 50%). The lowest frequency of detection was significantly recorded was recorded the XYL1961/18 inoculated in *Q. ilex* (31.7%) and for CoDiRO in *J. thurifera* (31.9%), with no significant differences with the combinations of CoDiRO with *Q. ilex* (39.6%) and *F. angustifolia* (37.0%) and XYL1961/18 inoculated in *J. thurifera* (37.5%) (Figure 2.15A).

Regarding bacterial load estimated by qPCR based on Cq values obtained with the Harper qPCR protocol, significant effects were observed for forest species (i=8.39, P<0.0001) and strain (F=8.23, P=0.0049). In contrast, branch section sampled, time of sampling and the interactions among the main factors did not significantly influence bacterial load (F<2.87, P>0.0607). Among the host species evaluated, the highest bacterial loads (P>0.05) were detected in *A. unedo* with Cq of 30.1±0.58, and *F. angustifolia* with Cq of 31.1±0.65. The later did not differ significantly (P>0.05) from the remaining forest species, whose Cq values ranged between 33.7±0.69 and 34.7±0.42, reaching the lowest bacterial load by *Q. ilex*. As found for frequency of detection results, XYL1961/18 exhibited a significantly higher bacterial load (31.4±0.40) compared with CoDiRo (34.0±0.36) (Figure 2.15B).

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In a different study, two strains of *X. fastidiosa* subsp. *multiplex* ST6 (IVIA 5901 and ESVL) from the demarcated outbreak area of Alicante were inoculated in holm oak (*Quercus ilex*) and cork oak (*Quercus suber*). For this experiment, leaf samples were collected periodically from 1 to 24 months post-inoculation (mpi) to monitor bacterial presence over time. As evidenced by the bacterial detection data, with more than 90% of plants testing positive during the first month post-inoculation, the inoculation procedure was considered successful (Figure 2.16). *X. fastidiosa* was detected in *Quercus ilex* (Figure 2.15A) at each sampling time; however, detection frequency of detection declined over time. At 2-years post-inoculation, detection was limited to a single plant and confined to the inoculation point. In *Quercus suber* (Figure 2.16B), *X. fastidiosa* was detected in approximately 20% of the plants, with slightly higher frequencies depending on the strain. A similar detection rate was observed at the final destructive sampling conducted 2-years post-inoculation. Notably, this was the only experiment in which typical disease symptoms developed. Leaf scorching was observed (Figure 2.16C), exclusively in *Q. suber* and only in a subset of plants, indicating restricted symptom expression despite confirmed bacterial presence.

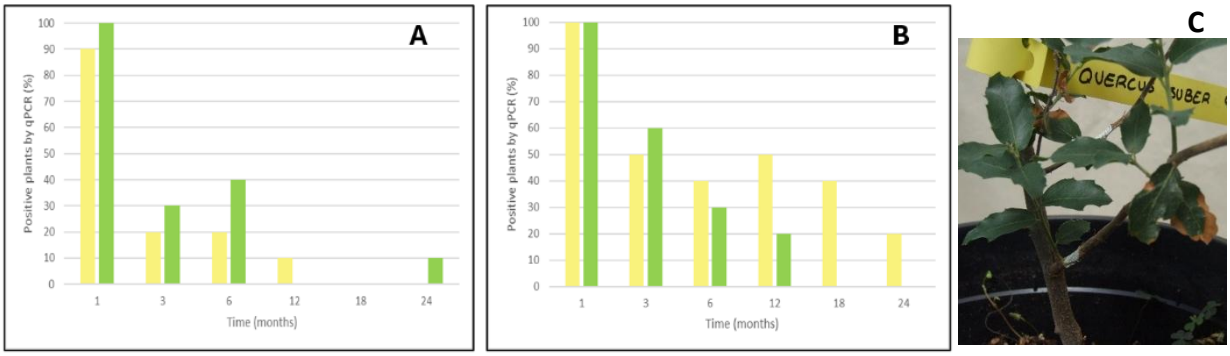


Figure 2.16. Percentage of positive plants of *Quercus ilex* (A) and *Quercus suber* (B) inoculated with strains IVIA5901 (green bars), and ESVL (yellow bars) of *Xylella fastidiosa* subsp. *multiplex*, according to detection by qPCR Harper et al. (2010, erratum 2013). Leaf scorch symptoms (C) in *Quercus suber* inoculated with the *X. fastidiosa* strain IVIA5901, bacterial presence confirmed by qPCR.

Furthermore, an evaluation of the capacity of four *X. fastidiosa* strains belonging to different subspecies to establish infections in *Quercus robur* (English oak), was performed by examining their ability to systemically colonise plant tissues. Oak trees exhibited positive qPCR detection for *X. fastidiosa*, with the majority of leaf samples testing positive by qPCR for IVIA5235 (8/9), Temecula1 (10/10), De Donno (9/10), and CFBP8173 (8/9), indicating more than 90% of positive detection in *Q. robur* at 3-month post-inoculation. At 9 mpi, stem sections were collected at the inoculation point (IP), above the inoculation point (AIP), and below the inoculation point (BIP) to determine whether infection was sustained over time and to assess systemic movement.

At 9 months post-inoculation, *X. fastidiosa* was consistently detected at the inoculation point (IP) in all oak trees across all four strains, indicating sustained infection over the incubation period (Figure 2.17). Detection at the AIP provided evidence of systemic movement for Temecula 1, CFBP8173, and De Donno, with all samples testing positive. In contrast, IVIA5235 showed limited upward movement, with most AIP samples testing negative, suggesting a reduced capacity for systemic colonisation in this direction. At the BIP,

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De Donno (*X. fastidiosa* subsp. *pauca*) and IVIA5235 (*X. fastidiosa* subsp. *multiplex*) were detected in five samples, with two samples testing negative, while CFBP8173 (*X. fastidiosa* subsp. *multiplex*) exhibited the highest proportion of BIP-negative samples, indicating restricted downward distribution. Notably, Temecula 1 (*X. fastidiosa* subsp. *fastidiosa*) demonstrated complete systemic colonisation, with all ten trees testing positive at IP, AIP, and BIP. Taken together, these results show that while all four strains can establish in oak stem tissues, Temecula1 displays the most consistent and extensive systemic colonisation in this host species.

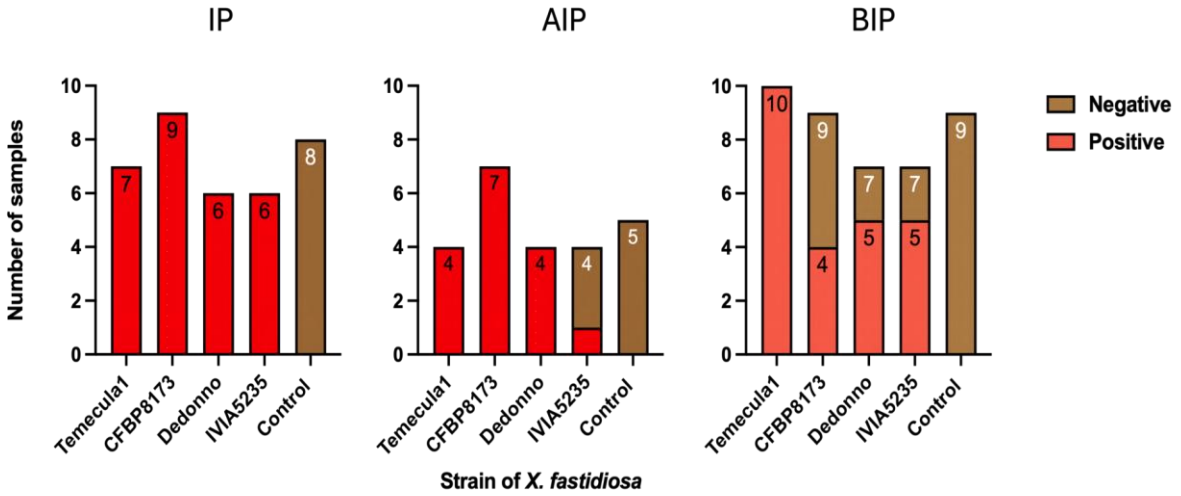


Figure 2.17: Bar plot showing cumulative sample counts for each *Xylella fastidiosa* strain at the point of inoculation (IP) (left), above IP (AIP) (middle) and below IP (BIP) (right) for oak trees at 9 months post inoculation in *Quercus robur*. Each bar represents the total number of samples indicating the cumulative counts of positive (red) and negative (brown) results.

In the experiment conducted on *Cistus monspeliensis*, *Helichrysum italicum*, and *Spartium junceum*, in which several strains representing the three main *X. fastidiosa* subspecies were inoculated, none of the three plant species showed characteristic symptoms during the experimental period. Nonetheless, qPCR analyses revealed that many plants were indeed infected, in some cases with very high *X. fastidiosa* titers (Table 2.7, Figure 2.18). On *C. monspeliensis,* the strains IVIA5770 (subsp. *fastidiosa* ST1) and DeDonno (subsp. *pauca* ST53) had the highest infection rates (75% and 71.4%, respectively) and also reached the highest bacterial titers (Cq 31-24 for IVIA5770 and 29-24 for DeDonno). All other strains had lower infection rates, ranging from 0% for the strain TOS4 (subsp. *multiplex* ST87) to 37.5% for CFBP8416 (subsp. *multiplex* ST7) and lower titers. However, it is important to note that one plant inoculated with TOS4 was just below our detection threshold of Cq 35 and might have reached a higher titer if given more time (Figure 2.18).

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Table 2.7. Infection rates of the eight *Xylella fastidiosa* strains on different plant species

Strain	LSV52.20	CFPB8418	CFBP8416	XYL468	TOS4	IVIA5770	DeDonno	IVIA11529
Subspecies	***multiplex***					***fastidiosa***	***pauca***	
Cistus monspeliensis	25%	16.7%	37.5%	25%	0%	75%	71.4%	25%
Helichrysum italicum	12.5%	100%	25%	100%	87.5 %	87.5	0%	0%
Spartium junceum	37.5%	100%	0%	87.5%	0%	100%	62.5%	0%

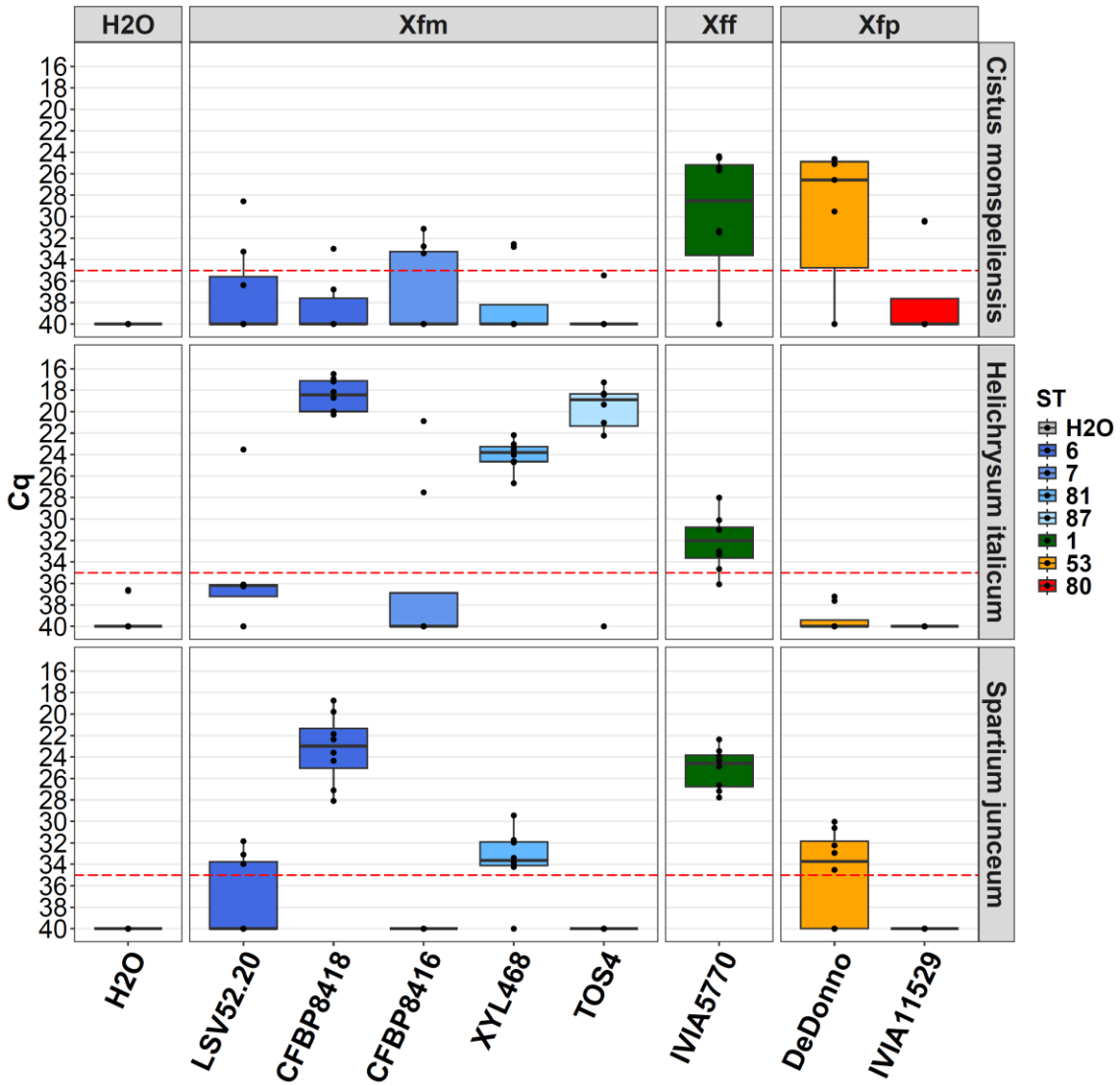


Figure 2.18. Titers (Cq values) of the eight *Xylella fastidiosa* strains depending on the host plant. Boxplots are coloured based on ST. Xfm = *X. fastidiosa* subsp. *multiplex*, Xff = *X. fastidiosa* subsp. *fastidiosa*, Xfp = *X. fastidiosa* subsp. *pauca*. The dashed red line indicates the threshold of Cq=35, below which a plant was considered uninfected.

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On *H. italicum,* three of the five subsp. *multiplex* strains (CFBP8418 (ST6), XYL468 (ST81) and TOS4 (ST87)) had high infection rates (87.5-100%) and very high titers (Cq 20-16 for CFBP8418, 22-17 for TOS4 and 26-22 for XYL468) (Table 2.7, Figure 2.18). For the other two subsp. *multiplex* strains (LSV52.20 (ST6) and CFBP8416 (ST7)), very few plants were infected but those showed very high titers (Cq 27-20). Hence, all five subsp. *multiplex* strains were able to reach high titers in a relatively short time after inoculation of *H. italicum,* but infection success varied among strains. The *X. fastidiosa* subsp. *fastidiosa* strain IVIA5770 also had a high infection rate of 87.5%, but reached lower titers compared to the *multiplex* strains (Cq 34-28). In contrast, both subsp. *pauca* strains were unable to infect *H. italicum* (Table 2.7, Figure 2.18).

On *S. junceum,* the highest infection rate (100%) and titer (Cq 27-18) were observed for the strain CFBP8418 (subsp. *multiplex* ST6), which had been initially isolated from *S. junceum* on the island of Corsica. In contrast, the ST6 strain from Occitanie (LSV52.20) had a much lower infection rate (35%) and titer (Cq 33-31) (Table 2.6, Figure 2.17). This is of interest since *Spartium* sp. are frequently as infected host in the Occitanie region (plateforme ESV 2022, https://doi.org/10.15454/RWBIWD). XYL468 (subsp. *multiplex* ST81) had a high infection rate (87.5%), but lower titers than CFBP8418 (Figure 2.17). The two other subsp. *multiplex* strains (CFBP8416 and TOS4) were unable to infect *S. junceum.* In contrast, the subsp. *fastidiosa* strain IVIA5770 had a 100% infection rate and reached high titers (Cq 27-22). The infection rate of the subsp. *pauca* strain DeDonno was intermediate (62.5%), with titers ogf Cq 34-30. The second subsp. *pauca* strain (IVIA11529, ST80) was unable to infect *S. junceum* (Table 2.7, Figure 2.18).

2.3.7 Host response in cherry and hazel tree

Four *X. fastidiosa* strains, including Temecula1 and IVIA5235 (subsp. *fastidiosa*), CFBP8173 (subsp. *multiplex*) and De Donno (subsp. *pauca*), were evaluated in two tree crops: *Prunus avium* (cherry tree) and *Corylus avellana* (hazel tree). Due to the absence of symptoms during the experiment, bacterial infection was assessed using qPCR. Several samples were collected from different points on the trees, specifically at the inoculation point (IP), above (AIP) and below the inoculation point (BIP) to determine bacterial presence.

Cherry trees exhibited a high overall percentage of successful inoculation, with 100% of samples from all four *X. fastidiosa* strains testing positive by qPCR at 3 mpi. Plant tissue at the inoculation point (IP) consistently tested positive for the presence of *X. fastidiosa*, indicating successful establishment of the bacterium within the host plant.

To establish whether the bacterium was able to systemically colonise the plant, samples taken above (AIP) and below the inoculation point (BIP) showed less consistent detection compared to samples taken at the IP. Table 2.8 shows that detection AIP in cherry plants was inconsistent across all strains. Few samples tested positive for Temecula1 (2/8) and CFBP8173 (3/8), with a small number of inconclusive results (1/8 and 2/8, respectively). In contrast, De Donno and IVIA5235 yielded a higher proportion of positive detections at AIP, although results remained variable, with positive, negative, and inconclusive samples observed. For samples taken BIP, bacterial detection was more consistent across strains:

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CFBP8173 (7/10), De Donno (6/10), Temecula 1 (5/8), and IVIA5235 (4/8), corresponding to detection rates of 70%, 60%, 62.5%, and 50%, respectively (Table 2.8).

Overall, De Donno and IVIA5235 showed the most consistent detection across sampling positions in cherry tissues, indicating greater establishment in this host compared to Temecula1 and CFBP8173. These results indicate that, during early infection, *X. fastidiosa* is more frequently detected in basal tissues than in apical tissues of cherry plants. Taken together, these findings demonstrate systemic movement of *X. fastidiosa* within the host, with a predominance of detection in basal regions at early time points.

Hazel plants also showed a high rate of successful inoculation, with 100% of samples testing positive by qPCR for De Donno, CFBP8173, IVIA5235, and Temecula1. Detection of *X. fastidiosa* in hazel tissues at the assessed time points indicated that the bacterium was able to establish within the host plant *Corylus avellana*.

Table 2.8. Number of cherry and hazel plants analysed by qPCR testing positive for *X. fastidiosa* at below (BIP) and above inoculation point (AIP)

Section plant sampled	Strain Inoculated	Number of tested plants	Number of qPCR positive samples	Number of qPCR inconclusive samples	Frequency of detection (%)
AIP cherry	Temecula 1	8	2	1	25
	CFBP8173	10	3	2	30
	De Donno	10	3	4	30
	IVIA5235	8	2	3	25
BIP cherry	Temecula 1	8	5	2	62.5
	CFBP8173	10	7	3	70
	De Donno	10	6	3	60
	IVIA5235	8	4	3	50
BIP hazel	Temecula 1	5	5	0	100
	CFBP8173	6	5	0	83.3
	De Donno	6	5	0	83.3
	IVIA5235	5	5	0	100

2.3.8 Host response of endemic *Xylella fastidiosa* strains in economically important crops

The specific evaluation of two *X. fastidiosa* subsp. *multiplex* ST6 strains IVIA 5901 and ESVL, present in the demarcated area of Alicante and several strains belonging to subspecies *fastidiosa, multiplex* and *pauca* strains endemic to Costa Rica and Brazil was performed in several crops of regional importance (Table 2.2).

For IVIA 5901 and ESVL, petiole sampling was performed to assess bacterial presence and symptom development was monitored for 24 months and varied with the plant species inoculated, the strain, the sampling time and the experiment. In general, inoculation was

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successful, except in dragon fruit and pistachio, where re-inoculation was necessary to achieve detection of *X. fastidiosa* in at least 50% of the plants at the inoculation point 1 month after inoculation. Overall, there was a trend towards a reduction in the population of both strains over time, even in re-inoculated plants.

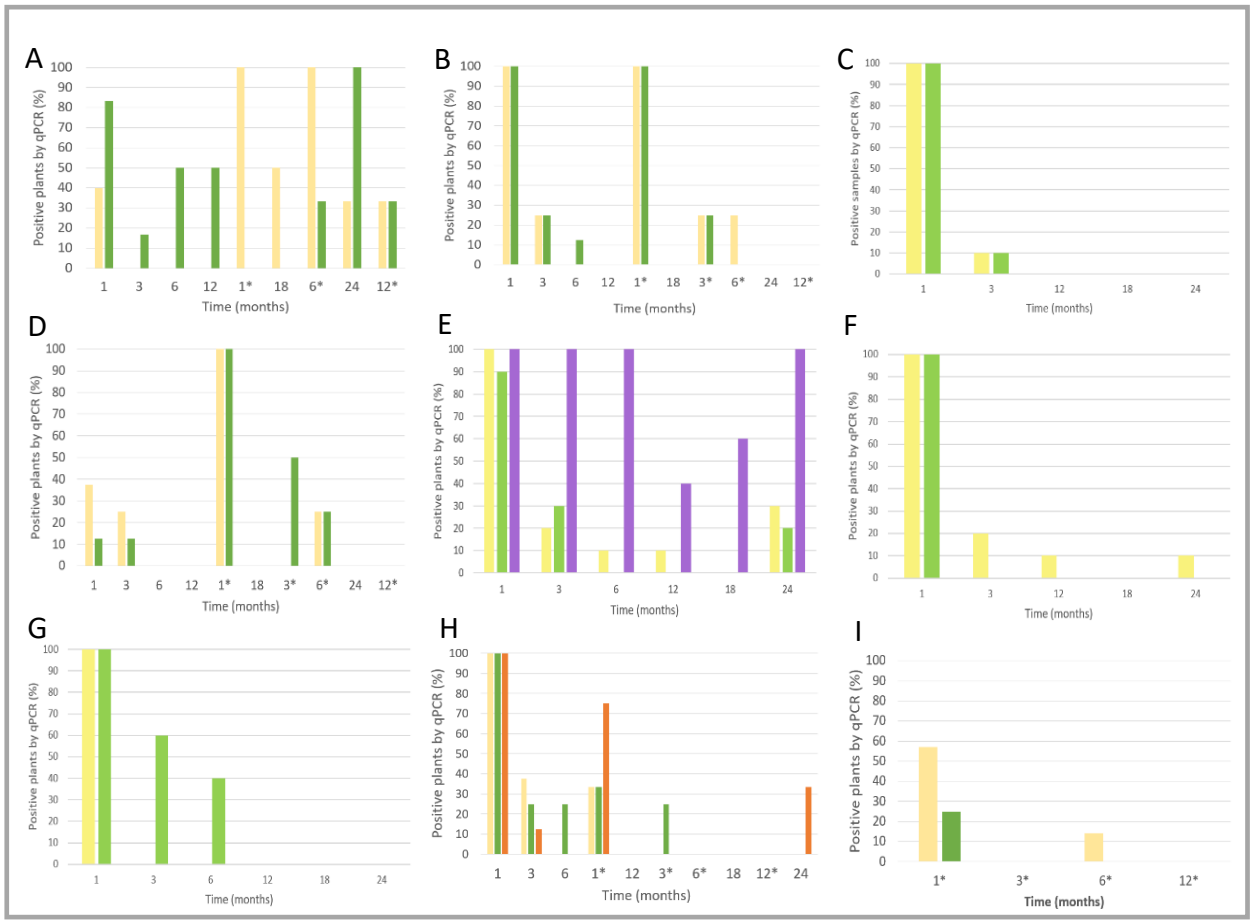


Figure 2.19. Percentage of positive plants inoculated with strains IVIA 5901 (green bars), ESVL (yellow bars) of *Xylella fastidiosa* subsp. *multiplex*, IVIA 5770 (purple bars, only in grapevine) of *X. fastidiosa* subsp. *fastidiosa*, and De Donno (orange bars, only on olive) of *X. fastidiosa* subsp. *pauca*, according to detection by qPCR Harper et al. (2010, erratum 2013). The asterisk indicates the result in re-inoculated plants. A, Almond cv. Marcona; B, carob; C, orange; D, dragon fruit; E, grapevine cv. Garnacha; F, loquat; G, oleander; H, olive cv. Arbequina; I, pistachio.

The most abundant host in the Alicante outbreak, almond, showed the highest populations of *X. fastidiosa* after 1 and even 2 years, but with an overall tendency towards reduced systemic movement over time, as a few plants were positive and Cq values increased with distance from the inoculation point. Figure 2.19A shows the qPCR results for the most susceptible almond cultivar, Marcona. Figure 2.19) shows the results of qPCR by Harper protocol only, but same results were obtained using Ouyang et al. (2013) with slightly higher Cq values. After 1 year, IVIA 5901 was detected in half of the inoculated plants, at about 15 cm above the inoculation point. In 30% of the re-inoculated plants, both IVIA 5901 and ESVL were detected after 1 year at the same distance from the inoculation point. In carob (Figure

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2.19B), the percentage of positive plants dropped to 20%, 3 months after inoculation. After 1 year, the bacterium was not detected in any of the inoculated or re-inoculated plants. In the three citrus species tested, orange (Figure 2.19C), lemon and mandarin, *X. fastidiosa* was detected in only 10% of the plants after 3 months, and after 1 year it was no longer detected in any plants.

Similar results were observed in dragon fruit (Figure 2.19D), since after 6 months *X. fastidiosa* was not detected in either the inoculated or re-inoculated plants. In grapevine (Figure 2.18E), *X. fastidiosa* was detected in less than 30% of plants as early as 3 mpi, and in none after 1 year. However, in the destructive sampling after 2 years, *X. fastidiosa* was detected in 3 plants, but only at the inoculation point. In loquat (Figure 2.19F), *X. fastidiosa* was not detected after 1 year, and in the destructive analysis at the inoculation point it was detected only one challenged plants and at Cq > 30. In oleander (Figure 2.19G), IVIA 5901 was detected only up to 6 months after inoculation, in less than 50% of plants, and after 1 year it was no longer detected in any plant. In olive (Figure 2.19H), *X. fastidiosa* subsp. *multiplex* was not detected after 1 year, either in the re-inoculated plants or in the destructive sampling after 2 years at the inoculation point; the De Donno strain was detected in only 10% of plants after 3 months, and it was detected in almost 40% of plants in the destructive sampling after 2 years. In pistachio (Figure 2.19I), *X. fastidiosa* was detected in only a small percentage of the plants as early as 1 month, and after 1 year it was not detected in any of them; this occurred even when the plants were re-inoculated.

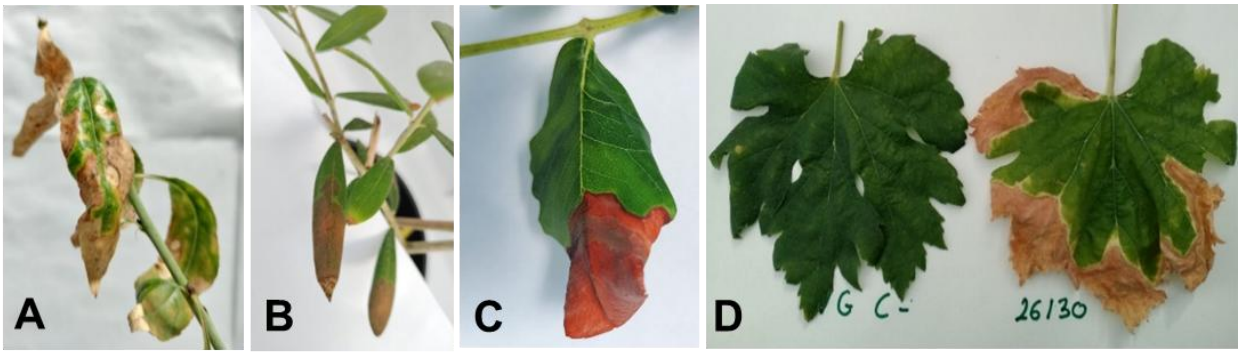


Figure 2.20. Photographs of plants inoculated with *Xylella fastidiosa* strains. (A), sample of almonds inoculated with strain IVIA 5901 of *X. fastidiosa* subsp. *multiplex*, with typical symptoms of almond leaf scorch, that gave positive result by PCR (Harper et al., 2010, erratum 1013); (B), sample of olive tree inoculated with strain IVIA 5901, that gave negative result by PCR; (C), sample of carob tree inoculated with strain IVIA 5901, that gave negative result by PCR; (D), in the right side, a leaf of grapevine inoculated with strain IVIA 5770 of *X. fastidiosa* subsp. *fastidiosa*, with typical symptoms of Pierce's disease, that gave positive result by PCR (in the left, a leave inoculated with water as negative control).

Some of the inoculated plants showed symptoms compatible with desiccation caused by *X. fastidiosa*. However, only in some cases were the samples qPCR-positive, for example in some almond plants (Figure 2.20A) inoculated with *X. fastidiosa* subsp. *multiplex* strains. In other cases, leaf scorch symptoms in challenged plants did not correspond to qPCR-positive (Figure 2.20B and C). In grapevines inoculated with IVIA 5770 of *X. fastidiosa* subsp. *fastidiosa*,

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symptoms were typical of Pierce's disease and samples were qPCR-positive (Figure 2.20D); in grapevines inoculated with *X. fastidiosa* subsp. *multiplex* strains no symptoms were observed.

Host plants inoculated with strains of *X. fastidiosa* from Costa Rica and Brazil were monitored for 2 years for symptom development, except for basil and *Spartium* spp., which were evaluated for 6 and 14 months respectively, to assess bacterial infection and disease development. At the end of the monitoring period, mature leaves and bark chips were collected from the inoculation site. Samples were also taken from root and aerial tissues to assess whether *X. fastidiosa* strains were able to disseminate throughout the plant. Plants inoculated with strains belonging to subspecies *fastidiosa* from Costa Rica did not develop any symptoms consistent with bacterial infection.

Table 2.9. Host range and pathogenicity for endemic *Xyella fastidiosa* subsp. *fastidiosa*

Plant species	*X. fastidiosa* strains				
	XF-68 (*Guava*)	**XF-1094 (*Vinca*)**	**XF-1090 (*Coffee*)**	**XF-1093 (*Coffee*)**	**XF-1105 (*Coffee*)**
***C. Arabica* (Coffee)**	5/5 (100%)	5/5 (100%)	4/5 (80%)	5/5 (100%)	1/5 (20%)
***P. Americana* (Avocado)**	3/5 (60%)	4/5 (80%)	3/5 (60%)	3/5 (60%)	3/5 (60%)
***C. Sinensis* (Sweet orange)**	3/5 (60%)	2/5 (40%)	3/5 (60%)	2/5 (40%)	4/5 (80%)
***P. guajava* (Guava)**	5/5 (100%)	2/5 (40%)	0/5 (0%)	2/5 (40%)	4/5 (80%)
***P. friedrichsthalium* (Sour guava)**	0/3 (0%)	0/3 (0%)	1/3 (33%)	2/3 (66%)	2/3 (66%)
***O. basilicum L.* (Basil)**	12/12 (100%)	12/12 (100%)	12/12 (100%)	12/12 (100%)	9/12 (75%)

Table 2.9 shows the overall results at 2 years post-inoculation. We also tested systemic movement by sampling tissues above the inoculation point as well as in the root tissues to distinguish systemic infection from persistence restricted to the inoculation site. In almost all plants tested, we detected *X. fastidiosa* dispersal. Basil plants showed the highest infection rates, approaching 100% for all strains except for XF-1105 (75%). Basil provided an optimal experimental model for evaluating our *X. fastidiosa* subsp. *fastidiosa* strains, since plants were infected in a relatively short time and we were able to recover several isolates. Strain XF-1105 isolated from coffee showed a different overall profile from the other two strains from coffee. This pattern may reflect strain-level differences in host compatibility rather host of isolation alone. XF-68 isolated from guava showed 100% infection in guava plants, but none in sour guava. In contrast, coffee isolates were the only isolates able to infect sour guava at detectable rates. In avocado and orange, there was no evident preference for a specific strain according to our results.

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In the experiment using several *X. fastidosa* strains from Brazil inoculated on *S. junceum*, after 14 mpi this host was confirmed as host for the subspecies *multiplex* and subspecies *pauca*, independently of whether the strains were isolated from citrus, coffee or olive plants (Table 2.10). In general, *X. fastidiosa* subsp. *pauca*, regardless of ST, caused more severe symptoms in *S. junceum* than subspecies *multiplex*. The addiction of 0.01% Tween 20 increased the percentage of infection and the number of dead plants compared with treatments Tween 20 (Table 2.10). Although the pin-prick inoculation method ensures direct access to xylem tissues, these results may reflect improved dispersion of bacterial cells in the inoculum suspension, improving inoculation efficiency.

Table 2.10. Infection and phenotype observed in *Spartium junceum* inoculated with *Xylella fastidiosa* strains diluted in phosphate buffer saline (PBS) and in PBS plus tween 20 solution.

	Control		*Xf* pauca ST16/coffee (Copl4p5)		*Xf pauca* ST13/citrus (9a5c)		*Xf pauca* ST16/olive (XF361)		*Xf pauca* ST11/citrus (J1a12)		*Xf multiplex* ST6/plum (Am327)	
	PBS +TW	**PBS**	**PBS +TW**	**PBS**	**PBS +TW**	**PBS**	**PBS +TW**	**PBS**	**PBS +TW**	**PBS**	**PBS +TW**	**PBS**
No. of tested plants	7	7	6	10	6	10	6	10	6	10	5	10
No. qPCR positive samples	0	0	5	7	4	6	5	8	5	6	3	4
No. dead plants	0	0	4	0	2	0	5	2	4	0	1	1
% dead plants	0	0	80.0	0.0	50.0	0.0	100	25.0	80.0	0.0	33.0	25

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3. SURVEYS IN COUNTRIES PARTICIPATING IN THE PROJECT

3.1 Background

A primary objective of this deliverable was to characterize host plants associated with both established and newly identified outbreaks, expand knowledge of the host range of epidemiologically relevant strains, and apply genomic tools to investigate the population structure and diversity of circulating *X. fastidiosa* genotypes.

Results were obtained across geographically diverse systems, including southwestern and central eastern Spain, southern Italy, France, Israel, the United States, and Costa Rica. In addition, a complementary global in silico analysis was conducted, enabling the identification of potential new host plants and revealing evidence consistent with a putative novel *Xylella* species. This section provides an integrated overview of new host identifications, outbreak characterization, pathogenicity assessments, and genomic analyses of novel isolates.

3.2 Material and methods

3.2.1 Field Surveillance and sample processing

Field surveys were conducted at the different sites according to the specific scope of the survey (i.e. to delimiting surveys in the case of the new outbreaks; etc…) . Symptomatic and asymptomatic plant material was collected from a range of host and non-host plant species in and around known infected areas, consisting primarily of leaf petioles or stem sections containing vascular tissue. Samples were processed using different DNA extraction protocols, i.e. homogenised in PBS (Valencia, Spain) or ground directly in CTAB extraction buffer (Israel, Balearic Islands, Costa Rica, Italy). In Costa Rica, molecular detection (see below) was complemented by immunofluorescence to assess bacterial loads.

3.2.2 DNA extraction and bacterial isolation

Genomic DNA was extracted either using a CTAB-based procedure following the EPPO protocol PM7/24 (5) (EPPO 2023) or using commercial kits for DNA extraction, according to commonly used procedures adopted in each laboratory.

In the case of bacterial isolation, plant samples were surface sterilised prior to processing by sequential immersion in 70% ethanol and 20% bleach, followed by three rinses with sterile water with the attempt of isolating the bacterium. Whenever possible, bacterial isolation was performed on BCYE or PD3 medium (EPPO, 2023) with incubation at 28 °C for 12–20 days.

3.2.3 Molecular detection and typing

Detection of *X. fastidiosa* was performed by real-time qPCR using TaqMan-based assays of Harper and Ouyang, following EPPO (2023) (Table 2.3). Subspecies identification was performed using the tetraplex real-time PCR developed by Dupas et al. (2019), which simultaneously identifies the three main *X. fastidiosa* subspecies in a single reaction. The

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subspecies-specific assays of Hodgetts et al. (2021) were additionally implemented as a routine diagnostic procedure in the Balearic Islands surveys (Table 2.3). Multilocus sequence typing (MLST) was performed following the schemes of Yuan et al. (2010), targeting seven housekeeping genes (*leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, *gltT*). Allelic profiles were queried against the publicly available pubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa). In samples with low bacterial concentrations, a nested-MLST approach (Cesbron et al. 2020) was applied.

3.2.4 Whole-Genome Sequencing and comparative genomics

In Spain, a hybrid assembly strategy combining Oxford Nanopore Technology (ONT) long-read sequencing and Illumina short-read sequencing was applied to achieve complete, closed genomes (Román-Écija et al. 2023). Hybrid genome assembly was performed with Hybracter (Bouras et al. 2024), and annotation was performed using Prokka v1.14.6 (Seemann, 2014). In Alabama, Illumina short-read data only were generated (SeqCenter, Pittsburgh, PA), with *de novo* assembly using SPAdes and annotation through Prokka via the KBase platform. Subspecies assignment and phylogenetic relationships were assessed using OrthoFinder (protein ortholog clustering) and fastANI (whole-genome Average Nucleotide Identity, ANI).

An in silico genomic surveillance study was conducted by mining raw sequence data in the NCBI Sequence Read Archive (SRA). *Xylella*-like reads were identified in public datasets from diverse plant and insect samples globally, and draft genomes were assembled from these reads for strain characterisation. Phylogenomic analyses were based on 146 single-copy protein-coding genes shared across 89 *Xylella* spp. strains. Average Nucleotide Identity (ANI) analyses were used to evaluate potential new species boundaries. Full methodological details are published in Briand et al. (2025).

In Apulia (Italy), the complete genome assembly of two *X. fastidiosa* subsp. *fastidiosa* strains, “BA_GR31” isolated from grape and “BA_AL21” isolated from almond were obtained and published, by combining the libraries sequenced by NovaSeq 6000 Illumina platform (150 bp paired-end) and with PacBio Sequel IIe system (Amoia et al. 2025). In addition, the draft genomes of additional 16 strains (see Deliverable 2.1) isolated from grapes and almond have been produced, with the aim to retrieve information on the divergence date from the American clade of *X. fastidiosa* subsp. *fastidiosa*-ST1 and to infer phylogenetic relationships with the recent *X. fastidiosa* subsp. *fastidiosa* outbreak in Portugal. Similarly, for the outbreak associated to the spread of the isolates belonging to the subspecies multiplex ST26, for two strains the integrated approaches isolated from almond were obtained and published, by combining the libraries sequenced by NovaSeq 6000 Illumina platform (150 bp paired-end) and with PacBio Sequel IIe system, allowed to reconstruct the complete genome of two strains: BA_AL92_2 (isolated from almond and BA_CH92_4 isolated from cherry (manuscript under review). Five additional strains have been sequenced through the Illumina platform to obtain the draft genome. DNA recovered from infected plums (infected with the same genotype, ST26) from Brazil has been obtained from Partner P15-APTA-IAC in order to perform phylogenomic studies to gather genetic-based information on the putative date of introduction of this genotype in Apulia, give its rare occurrence worldwide.

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3.3 Results

Table 3.1 provides a cross-system summary of the main findings of this section.

Table 3.1. Integrated overview of new host findings, outbreak characterizations, and key genomic contributions, by geographic system

Location / system	Key host(s) / new finding	Subspecies / ST	Detection method	Key outcome
Cáceres, Spain (wild shrubs, grapevine)	*Lavandula, Cistus, Cytisus, Calicotome, Spartium, Pterospartum, Ulex, Vitis vinifera*	*fastidiosa* ST1	qPCR, MLST, WGS (hybrid ONT + Illumina)	*X. fastidiosa* subsp. *fastidiosa* ST1 isolated from 9 hosts; 3 complete genomes assembled; pathogen locally established in wild scrubland
Apulia, Italy (vineyards, almond orchards)	*Vitis vinifera* (table grape), *Prunus dulcis*; other *Prunus* spp.	*fastidiosa* ST1 *multiplex* ST26	qPCR, MLST; artificial inoculation; grafting experiments	Pierce's disease confirmed in grapevine; Grape and almond are the two predominant host species in the X. fastidiosa subsp. fastidiosa outbreak; Almond is the predominant host in the Xfm outbreak
Alabama, USA (blueberry, grapevine)	*Vaccinium* sp. (blueberry), *Vitis vinifera* (grapevine)	*multiplex* (AL-BB1, AL-BB2) *fastidiosa* (AL-GR1)	Isolation, 16S rDNA, WGS (Illumina), fastANI, OrthoFinder	3 complete genomes deposited; blueberry strains phylogenetically close to Georgia *X. fastidiosa* subsp. *multiplex* strains
Different Provinces in Argentina	Almond, olive, citrus	*pauca* ST69, ST78, ST90	qPCR and MLST	New hosts-ST associations. Description of a new ST (ST90)
Upper Galilee, Israel (vineyards, natural habitats)	*Cercis siliquastrum* (Judas tree; new host), wild *Prunus dulcis*, wild *Vitis vinifera*	*fastidiosa* ST1	qPCR (Harper 2010); bacterial isolation; MLST	Judas tree confirmed as new reservoir host in natural and urban habitats; same ST1 as crops
Costa Rica (Puriscal, Heredia)	*Persea americana* (avocado), +8 alternative hosts (see Section 4)	*fastidiosa* / *pauca*	PCR (RST31/33), qPCR (HL5/6, SYBR), immunofluorescence	Avocado positive (10/50 trees); new alternative hosts detected; matrix inhibitory — protocol ongoing
Balearic Islands, Spain (Mallorca, Menorca, Eivissa, Formentera)	38 plant species across 399 surveyed; key: *Prunus dulcis*, *Vitis vinifera*, *Olea europaea*, *Spartium junceum*	*fastidosa* ST1, *multiplex* ST81, *pauca* ST80, *pauca* ST53 (new outbreak 2024)	qPCR (Harper 2010); nested-MLST (Cesbron 2020); Hodgetts 2021	38 host species confirmed; ST53 outbreak confined to ~2 km; mixed infections documented; wild olive and ash as landscape connectors
Global (in silico SRA data mining)	*Lavandula stoechas, Populus trichocarpa, Coffea* spp., *Oryza rufipogon*	*fastidiosa*, *multiplex*, *pauca*, new sp. *Xylella rufipogonis*	SRA mining; genome assembly; phylogenomics	New Xylella species proposed; new hosts and countries (Nicaragua, Namibia, Zambia, Japan)

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3.3.1 Surveys and new outbreak in Cáceres, Spain

A surveillance campaign conducted in the *X. fastidiosa* subsp. *fastidiosa* outbreak area in Cáceres, Spain (DOE, 2024; 2025), analysed 48 plant samples from 11 forestry and agricultural host genera, together with xylem-feeding insects from families Cicadellidae, Aphrophoridae, and Issidae. *X. fastidiosa* was detected in 23 plant samples spanning eight genera: *Lavandula*, *Cistus*, *Cytisus*, *Calicotome*, *Spartium*, *Pterospartum*, *Ulex*, and *Vitis* (grapevine). No *X. fastidiosa* was detected in *Ficus carica* samples or in Cicadellidae and Issidae. Among insect samples, only a single *Philaenus spumarius* specimen (Aphrophoridae) tested positive. MLST typing assigned all detected strains to ST1 of subsp. *fastidiosa*, indicating genetic homogeneity within the outbreak and confirming that the pathogen is locally established.

X. fastidiosa was successfully isolated from nine plant samples: IVIA-906/24 (*Cistus* sp.), IVIA-1116/24 (*Cistus ladanifer*), IVIA-1118/24, IVIA-124/25, IVIA-125/25, and IVIA-314/25 (*Cytisus* sp.), IVIA-1119/24 (*Cistus salviifolius*), IVIA-126/25 (*Ulex* sp.), and IVIA-313/25 (*Vitis* sp.). Colony morphology was consistent with *X. fastidiosa* subsp. *fastidiosa*, and all isolates were confirmed by PCR and MLST-assigned to *X. fastidiosa* subsp. *fastidiosa* ST1. Hybrid ONT–Illumina WGS yielded complete genome assemblies for three isolates (IVIA-906/24, IVIA-1116/24, IVIA-1118/24) with genome sizes of 2.53 Mb, 51.7% GC content, 99.64% completeness, and sequencing depth exceeding 1,150×. No plasmids were detected in any assembly. Annotation predicted 2,419–2,422 CDS, 46–49 tRNAs, 6 rRNAs, and 1 tmRNA per genome. A fourth isolate (IVIA-1119/24) yielded only a partial assembly of 10 contigs. All genome data are deposited under NCBI BioProject PRJNA1357838 (Table 3.2).

Table 3.2. Sequencing statistics, genome assembly metrics, and annotation summary for *Xylella fastidiosa* subsp. *fastidiosa* ST1 isolates from the Cáceres, Spain outbreak. Complete assemblies are deposited under NCBI BioProject PRJNA1357838.

Feature	IVIA-906/24	IVIA-1116/24	IVIA-1118/24	IVIA-1119/25
Host	*Cistus* sp.	*Cistus ladanifer*	*Cytisus* sp.	*Cistus salviifolius*
ONT reads (after trimming)	592,862	373,219	373,494	269,617
Illumina paired reads	3,583,588	6,090,778	8,231,502	7,674,339
Genome size (bp)	2,534,294	2,532,475	2,533,848	Partial (10 contigs)
GC content (%)	51.7	51.7	51.7	—
Completeness (%)	99.64	99.64	99.64	—
Genome coverage	1,161×	1,188×	1,218×	—
Plasmid detected	No	No	No	No
CDS	2,419	2,422	2,422	—
Genes	2,475	2,478	2,478	—
rRNA	6	6	6	—
tRNA	46	49	49	—
tmRNA	1	1	1	—
GenBank BioSample	SAMN53088846	SAMN53088847	SAMN53088848	Pending

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The exclusive detection of ST1 across all infected plants and the single positive vector, combined with the broad representation of Mediterranean scrubland species as infected hosts, confirm that *X. fastidiosa* subsp. *fastidiosa* ST1 is well established in the Cáceres area, cycling through wild vegetation and potentially spreading to susceptible crops.

3.3.2 Surveys in the new outbreaks in Apulia (Italy)

3.3.2.1 Outbreak of Xylella fastidiosa subsp. *fastidiosa*

The occurrence and epidemic spread in Apulia region (southern Italy) of highly pathogenic *X. fastidiosa* subsp. *pauca* ST53, decimating olive trees has prompted the region to implement and adopt an intensive integrated monitoring program, targeting both the insect vectors and the host plants (the so called "specified plants", i.e. those known to be susceptible to the bacterial isolates causing the infections). The regional monitoring program is managed under the supervision of the regional plant protection service, with the active collaboration of the Partner P5-CNR[1], being the official and accredited laboratory for the confirmation of new findings, the typing of the recovered isolates and the assessment of the susceptible host plant species. In the framework of these annual monitoring campaign, for two consecutive years (2022-2023) *X. fastidiosa*-positive specimens of *Philaenus spumarius* were detected in the *Xylella*-free area in the province of Bari, raising major concerns. As such, the area was intensely inspected to search for host plants serving as bacterial reservoir for the spittlebugs, with olive trees being the main species targeted in the field inspections. However, none of the olive samples collected tested positive for the bacterium, but when sampling was extended to other plant species the presence of infected almond trees was recorded. The DNA recovered from these infected almond trees was used for multi-locus sequence typing (MLST) analysis to define the genetics of the infecting isolates, unravelling the occurrence of DNA sequences belonging to the subspecies *fastidiosa*, genotype ST1 (Cornara et al. 2024), i.e. clustering with isolates of the subspecies fastidiosa, notoriously detected as causal agents of the Pierce's Disease of grapevine. Thus, in early 2024, in the Apulia region a new outbreak caused by isolates genetically and biologically distinct from the notorious Xfp-ST53 was reported. Following the identification of this additional subspecies in the region, the surveillance carried out in the newly demarcated area took into account the list of plants known to be susceptible to this subspecies, as indicated in Annex II of the EU Regulation 1201/2020, with grapes being the primary inspected species.

In depth surveys in the area demarcated area for *X. fastidiosa* susbp. *fastidiosa* unravelled that in the epidemiology of the infections almond and grape represent the main host plants, although it remains to be investigated if almond represents the reservoir for grapes, and if grape to grape infections occurs.

Beside the official sampling, P5-CNR extended the inspections and sampling in vineyards where through the official monitoring infected vines were recorded. It should be remarked that due to the eradication measures in place in this outbreak, surveys and sampling were limited in time due to the removal of the plants, and symptoms assessment

[1] This allows the Beneficiary P5-CNR to have full access to the newly identified infected materials for carrying out genetic, molecular and biological studies.

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limited to the period when the infections were first identified. In this timeframe (i.e. before rouging the infected vineyards), four vineyards were sampled to assess the occurrence of symptoms and the incidence and pathway of the infections.

3.3.2.2 Natural host range in the Xylella fastidiosa *subsp.* fastidiosa *demarcated area*

Analyses of the data made available from the official monitoring campaign carried out by the Regional Phytosanitary Service of Apulia, allowed to retrieve information on the host range under the local epidemiological conditions of this outbreak (Table 3.3).

Table 3.3. Summary of results of the official survey in the demarcated area for *Xylella fastidiosa* subsp. *fastidiosa* in Apulia, Italy, in 2024. Results (negative and positive) refer to molecular analysis (qPCR). In grey are marked the plant species for which naturally infected plants were found in the area.

Species	Negative	Positive	Samples tested
Ambrosia artemisiifolia L.	1		1
Calicotome spinosa	1		1
Cercis occidentalis	32		32
Citrus spp.	1948		1948
Erysimum L.	1		1
Juglans regia L.	74		73
Lupinus aridorum	1		1
Magnolia grandiflora L.	14		14
Morus L.	221		221
Nerium oleander	582		582
Polygala myrtifolia	69	1	70
Prunus armeniaca L.	941		941
Prunus avium	859	8	867
Prunus domestica	440		440
Prunus dulcis	6331	209	6331
Prunus persica	368		368
Prunus spp	23		23
Rhamnus alaternus	166		166
Rubus spp.	1		1
Salvia rosmarinus	299		299
Sambucus L.	3		3
Spartium junceum	76		76
Vinca L.	19		19
Vitis L.	12667	119	12786
Total	**25138**	**337**	**25265**

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Over a total of more than 25.000 samples tested as showed in the table above among the 26 species/genera addressed by the official inspections and sampling, four were found to support bacterial infections, with the prevalence of infected almond and grapes, perhaps playing a relevant role in the epidemiology of the outbreak (Table 3.3).

3.3.2.3 In depth surveys in vineyards

Vineyard 1, consisted of a small non-irrigated plantation for hobby wine production, with mixed cultivars (Montepulciano, Falanghina, and others). In this 20-year-old vineyard, several plants were already died at the time (2024) when the infections were first recorded. The vineyard was surrounded by several olive and almond trees (Figure 3.1), with the latter found infected. The incidence of the symptomatic and infected vines is reported in Figure 3.3.



Figure 3.1. Vineyard 1 consisting of 6 rows by approximately 30 plants, surrounded by olives and almond trees.

Symptoms assessment was carried out in September after grape harvest. Besides recording the number of dead vines or those with severe withering and desiccation of the branches, plants were scored for the presence of green islands in the mature shoots, matchstick leaves and shrivelled fruits (those left on the plants by the farmer because already damaged) (Figure 3.2).

Dead vines accounted for 13% of the total plants, while 11% of the plants consisted of young vines most likely replanted during the past years to replace dead vines. Whereas approx. 80% of the plants were annotated as symptomatic, i.e. with some putative Pierce's Disease (PD) alterations, although the overall status of the vineyard was affected by the low input management practices, and thus suffering water stress and other biotic stresses (i.e. wood decay diseases). The laboratory tests indicated that almost 50% of the vines were infected (74 out of 160). Among these, 63 vines (85%) were scored as symptomatic, indicating a strong correlation between infections and occurrence of putative PD symptoms. On the

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other hand, some of the symptoms reported in literature as "specific symptoms" of PD, were found not associated with infections, i.e. matchstick and occurrence of green islands were recorded in 40% of the vines testing negative for Xf. On the contrary the phenomena of desiccations and branch withering occurred exclusively in the infected vines.

In the graphical map below (Figure 3.3) the dead vines (black box) and infected vines (red block) are indicated, with the distribution not following any specific pathway, most likely due to the small size of the plot.



Figure 3.2. Symptoms recorded in vineyard 1. From the left to the right: shoots with matchsticks, green islands, branch withering and desiccation and shriveled grape.

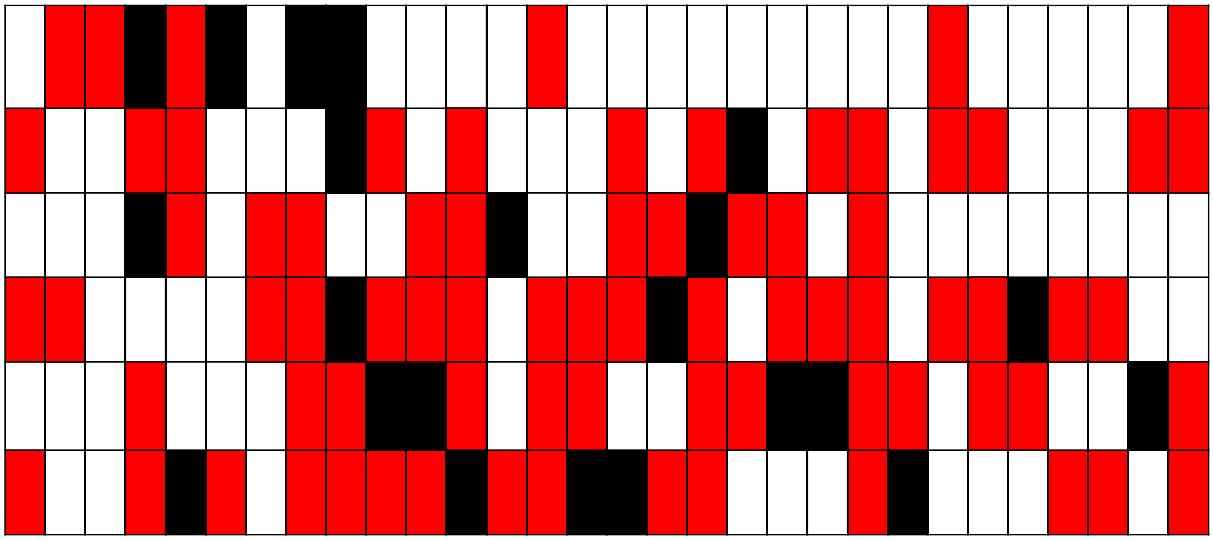


Figure 3.3. Graphical representation of the infected (red) and dead vines (black), white box indicates negative plants. The map has the same orientation as Figure 3.1

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Vineyard 2, consisted of 6-year-old commercial table grape vineyard of the cultivar "Sugraone" (Superior®). This was a well-managed vineyard with intensive cultivation practices to ensure high quality and production levels. A total of 788 vines were inspected and sampled, with 3,7% being dead in the previous years and thus already removed. Visual inspections on the remaining 760 vines showed the occurrence of putative symptoms (as those indicated above) on approx. 60%, with approx. 9% affected by extensive desiccation phenomena (Figure 3.4), 17% showing partial desiccation. Laboratory tests carried out by qPCR (Harper et al. 2010) showed an incidence of the infections reaching 50%. Among those showing the desiccation phenomena almost 90% tested positive, whereas this % dropped to 24% among the symptomless plants.



Figure 3.4. Infected vines on the left which exhibit premature leaf fall and non-infected vines on the right.

Vineyard 3, of the cultivar ARRA30 was in the same farm and adjacent to vineyard 2 (Figure 3.5), thus under the same management program. A total of 390 vines were inspected and sampled, by selecting those located in the rows neighbouring vineyard 2. In this case, visual inspections showed the occurrence of a lower incidence of symptomatic vines (i.e. 4,5% showing partial desiccation of the branches), 1% of the vines were previously uprooted, while none of the vines exhibited severe desiccation. Consistent with the visual assessments, diagnostic tests indicated a significant lower incidence (9% vs 50%) of the infections compared to vineyard 2. Most of the positives (78%) corresponded to the symptomatic vines, while the remaining positives were asymptomatic.

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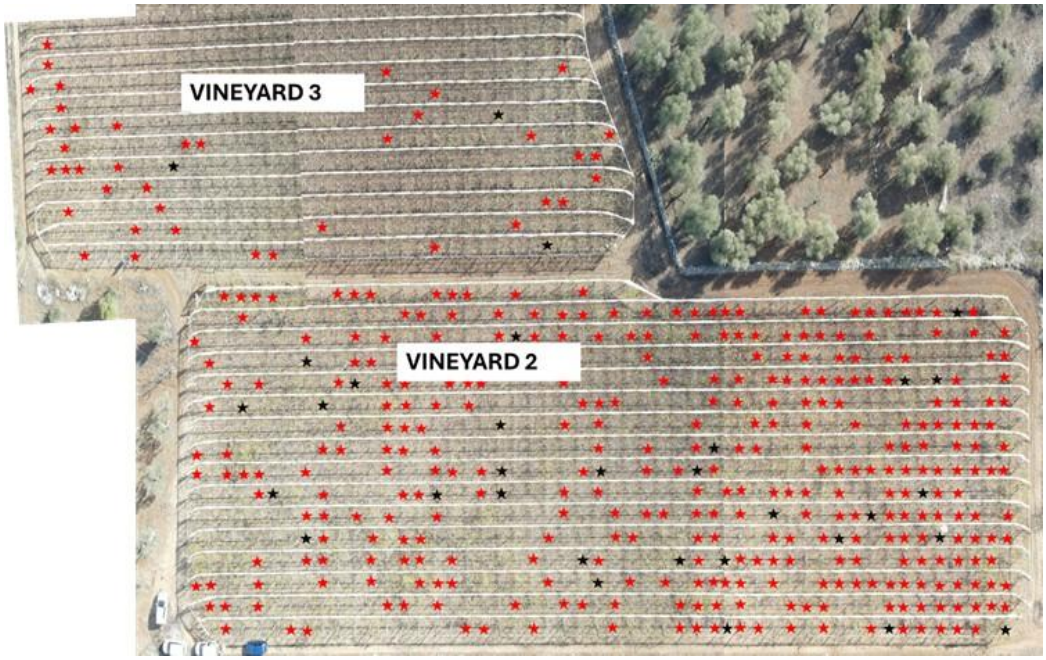


Figure 3.5. Map of vineyard 2 and 3, with red stars indicating positive vines and black stars the dead plants.

Vineyard 4, consisted of a 7-year-old commercial table grape vineyard of the cultivar "Melanie". A total of 170 vines were surveyed and sampled. Sixteen percent of the plants consisted of young replacements, suggesting that plant dead and failure occurred in the previous years. Visual inspections in this vineyard revealed clear phenomena of leaf-scorching and severe phenomena of shrivelled grape along with drying phenomena affecting 40% of the vines (Figure 3.6). Laboratory tests confirmed the presence of the infections in 99% of the symptomatic vines, whereas 14% of the positives were symptomless vines.



Figure 3.6. Symptoms recorded on infected vines of the cultivar Melanie in vineyard 4. On the left vine with severe leaf scorching, on the right a shriveled grape on a symptomatic vine.

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3.3.2.4 *Outbreak of* Xylella fastidiosa *subsp.* multiplex *in Apulia, Italy*

Concomitantly to the finding of the *X. fastidiosa* subsp. *fastidiosa* outbreak, infected almonds were interceptedin the *X. fastidiosa*-free area, in the municipality of Santeramo in Colle (Bari province). Infections were recorded on almond trees not showing any specific alteration. Multilocus sequence typing analysis revealed that the isolates were associated with sequence type ST26 (Montilon et al. 2024). Although subspecies *multiplex* is the most predominant in Europe, this was the first report of this ST, whose occurrence is also very limited in the American continent.

Surveys to delimit the outbreak targeted the plant species known to be susceptible to subsp. *multiplex* as indicated in the EU Regulation 1201/2020 (Table 3.4). Results of the monitoring campaign, showed that the outbreak is quite extended, encompassing several municipalities in the province of Bari and Taranto.

Table 3.4. Summary of results of the official survey in the demarcated area for *Xylella fastidiosa* subsp. *multiplex* in Apulia, Italy, in 2024. Results (negative and positive) refer to molecular analysis (qPCR). In grey are marked the plant species for which naturally infected plants were found in the area.

Species	Negative	Positive	Samples tested
Prunus armeniaca	74		74
Prunus avium	1755	8	1763
Morus	5		5
Spartium junceum	34	1	35
Prunus dulcis	14102	546	14648
Juglans regia	75		75
Nerium Oleander	43		43
Prunus persica	31		31
Prunus domestica	137	3	140
Salvia rosmarinus	57		57
Total	16313	558	16871

It should be reported that in this area 840 samples of *Vitis* spp. were also tested although not reported among the official *X. fastidiosa* subsp. *fastidiosa* host species in the EU regulation. Results did not show any infections, whereas as indicated in the table above almond trees appear to be the main host species. In this area almond is widespread as marginal crop mainly with few trees in mixed cultivations with olives or grapes or pastures. So far, no severe impact has been reported, nor there are reports of the occurrence of infections in intensive and specialized almond plantations.

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3.3.3 Surveys and new outbreak in Balearic Islands, Spain

X. fastidiosa was first detected in the Balearic Islands in 2016. Official surveys conducted in accordance with Commission Implementing Regulation (EU) 2020/1201 and EPPO standard PM 7/24 have revealed widespread presence across three of the four main islands: subsp. *fastidiosa* ST1 and subsp. *multiplex* ST81 in Mallorca; subsp. *multiplex* ST81 exclusively in Menorca; and subsp. *pauca* ST80 in Eivissa (Ibiza). Formentera has remained free of detection to date. The aggregate survey dataset comprises 25,708 samples collected across the archipelago, of which 2,085 (8.1%) tested positive for *X. fastidiosa* (Table 3.5).

Table 3.5. Summary of official *Xylella fastidiosa* survey results across the Balearic Islands. *Xf*-positive refers to samples testing positive by molecular analysis (qPCR).

Island	Samples collected	Samples analyzsed	*Xf*-positive samples	*Xf*-positive (%)
Mallorca	14,726	14,610	1,359	9.3
Menorca	2,986	2,986	268	9.0
Eivissa (Ibiza)	5,874	5,874	458	7.8
Formentera	2,122	2,122	0	0.0
Total	**25,708**	**25,592**	**2,085**	**8.1**

3.3.3.1 Host range in the Balearic Islands

X. fastidiosa was detected in 38 of the 399 plant species sampled (Table 3.6), spanning cultivated, ornamental, and wild Mediterranean taxa.

Although detections spanned a wide taxonomic range, positivity rates varied considerably among species. Almond (*Prunus dulcis*, 21.6%), grapevine (*Vitis vinifera*, 14.3%), lavender (*Lavandula angustifolia*, 14.0%) and olive (*Olea europaea* var. *europaea*, 9.4%) among cultivated species. Wild olive (*Olea europaea* var. *sylvestris*, 10.5%), Mediterranean buckthorn (*Rhamnus alaternus*, 10.4%), and ash (*Fraxinus angustifolia*, 9.8%) are prominent among semi-natural taxa.

The high positivity rates in wild olive, ash, and Mediterranean buckthorn, all widely distributed in forests and semi-natural habitats across the islands, suggest that these species function as ecological connectors between cultivated areas and the surrounding landscape, facilitating spatial dissemination of *X. fastidiosa*. This landscape-level bridge effect likely contributes to the exceptionally widespread distribution of the pathogen across the archipelago.

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Table 3.6. Complete list of plant species in which *Xylella fastidiosa* was detected during official surveys in the Balearic Islands (38 out of 399 species tested).

Plant species	Plant species	Plant species
Acacia saligna	*Laurus nobilis*	*Rhamnus alaternus*
Calicotome spinosa	*Lavandula angustifolia*	*Ruta chalepensis*
Cistus albidus	*Lavandula dentata*	*Salvia officinalis*
Cistus monspeliensis	*Nerium oleander*	*Salvia rosmarinus*
Clematis cirrhosa	*Olea europaea var. europaea*	*Santolina chamaecyparissus*
Eleagnus angustifolia	*Olea europaea var. sylvestris*	*Santolina magonica*
Ficus carica	*Phagnalon saxatile*	*Spartium junceum*
Fraxinus angustifolia	*Phillyrea angustifolia*	*Teucrium capitatum*
Genista hirsuta	*Phlomis italica*	*Thymus vulgaris*
Genista lucida	*Polygala myrtifolia*	*Ulex parviflorus*
Genista valdes-bermejoi	*Prunus avium*	*Vitex agnus-castus*
Helichrysum stoechas	*Prunus domestica*	*Vitis vinifera*
Juglans regia	*Prunus dulcis*	

3.3.3.2 The new outbreak of ST53 in Sencelles municipality

In January 2024, a geographically restricted outbreak of *X. fastidiosa* subsp. *pauca* ST53 was identified in the municipality of Sencelles, central Mallorca Island, the first confirmed detection of this strain in Mallorca. As of the preparation of this deliverable, all detections remain confined within approximately 2 km of the initial focus, with no evidence of further spread.

A total of 1,845 samples from 32 plant species were analysed within the outbreak area. Of these, 349 (18.9%) tested positive for *X. fastidiosa*. subsp. *pauca* was the dominant detected subspecies (211 samples), with cultivated olive showing the highest positivity rate (61.9%), followed by *Nerium oleander* (26.3%) and lavender (25.0%). Notably, 30 samples showed mixed infections involving both subsp. *pauca* and subsp. *multiplex*, a pattern not observed for other subspecies elsewhere in the Balearic Islands and of considerable epidemiological interest. The full subspecies breakdown by plant species is presented in Table 3.7.

The occurrence of mixed infections involving *X. fastidiosa* subsp. *pauca* and subsp. *multiplex* within individual plants adds epidemiological interest to this outbreak. This contrasts with other *X. fastidiosa* subspecies in Mallorca Island, which show wider spatial distribution but rarely present mixed infections. Official surveys under EU regulatory requirements will continue beyond the project's completion.

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Table 3.7. *Xylella fastidiosa* (*Xf*) subspecies-level detection breakdown in the Sencelles (Mallorca) *Xf* subsp. *pauca* ST53 outbreak area. Percentages are calculated per species relative to the total number of samples analysed for that species within the outbreak area.

Category / plant species	No. positive samples	Total samples	Positive (%)
TOTAL SAMPLES (32 plant species)	**349**	**1,845**	**18.9**
Xf* subsp. *pauca	**211**		
Lavandula angustifolia	2	8	25.0
Nerium oleander	5	19	26.3
Olea europaea var. europaea	52	84	61.9
Olea europaea var. sylvestris	142	1,270	11.2
Rhamnus alaternus	10	270	3.7
***Xf* subsp. *pauca* + *multiplex* (mixed)**	**30**		
Lavandula angustifolia	1	8	12.5
Olea europaea var. europaea	3	84	3.6
Olea europaea var. sylvestris	20	1,270	1.6
Polygala myrtifolia	1	1	100
Rhamnus alaternus	5	270	1.9
Xf* subsp. *fastidiosa	**9**		
Prunus dulcis	1	23	4.4
Rhamnus alaternus	7	270	2.6
Xf* subsp. *multiplex	**91**		
Ficus carica	2	16	12.5
Laurus nobilis	1	7	14.3
Lavandula angustifolia	6	8	75.0
Lavandula dentata	4	6	66.7
Olea europaea var. sylvestris	49	1,270	3.9
Prunus dulcis	19	23	82.6
Rhamnus alaternus	9	270	3.3
Salvia rosmarinus	1	23	4.4
***Xf* subsp. *fastidiosa+multiplex* (mixed)**	**1**		
Rhamnus alaternus	1	270	0.4
***Xf* subspecies not yet determined**	**37**		
Olea europaea var. sylvestris	15	1,270	1.2
Rhamnus alaternus	6	270	2.2
Olea europaea var. europaea	4	84	4.8
Nerium oleander	1	19	5.3
Prunus dulcis	6	23	26.1
Vitis vinifera	9	34	26.5

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3.3.4 Surveys in Israel

X. fastidiosa was first detected in Israel in 2016 in almond orchards of the Hula Valley, and in commercial vineyards of the Upper Galilee in 2021. To assess whether non-crop plant species in and around infected vineyards function as inoculum reservoirs, a two-year survey (2023–2024) targeted nine plant species across natural habitats in the demarcated area.

In 2023, three species tested positive: Judas tree (*Cercis siliquastrum*), wild almond (*Prunus dulcis*), and wild grapevine (*Vitis vinifera*). All three showed leaf scorch symptoms compatible with *X. fastidiosa* infection. The remaining six surveyed species tested negative (Table 3.8).

In 2024, the survey was expanded to include *Cercis siliquastrum* trees in both natural habitats and private and municipal gardens across the Upper Galilee. All symptomatic trees sampled tested positive by qPCR. MLST analysis confirmed that all positive *C. siliquastrum* trees are infected by *X. fastidiosa* subsp. *fastidiosa* ST1, the same sequence type as in commercial vineyards and almond orchards, demonstrating active inoculum flow between wild and cultivated environments.

Table 3.8. Results of *Xylella fastidiosa* surveillance in non-crop plant species sampled in and around infected vineyards in the Upper Galilee, Israel (2023–2024).

Plant species	Common name	Habitat	*Xf* detected	Subspecies / ST
Cercis siliquastrum	Judas tree	Natural habitat, parks, gardens	Yes	*fastidiosa* ST1
Prunus dulcis (wild)	Wild almond	Natural habitat	Yes	*fastidiosa* ST1
Vitis vinifera (wild)	Wild grapevine	Natural habitat	Yes	*fastidiosa* ST1
Quercus calliprinos	Palestine oak	Natural habitat	No	—
Quercus boissieri	Boissier oak	Natural habitat	No	—
Dittrichia viscosa	False yellowhead	Natural habitat	No	—
Capparis spinosa	Caper bush	Natural habitat	No	—
Crataegus aronia	Hawthorn	Natural habitat	No	—
Pistacia palaestina	Palestinian pistachio	Natural habitat	No	—

Cercis siliquastrum is a common ornamental and forestry tree deeply established in natural habitats, roadsides, urban gardens, and private properties across northern Israel. Its role as an infected reservoir host of *X. fastidiosa* subsp. *fastidiosa* ST1, sharing the same sequence type as simultaneously infected commercial crops, has important management implications: eradication measures confined to agricultural settings will not eliminate inoculum sources, as the pathogen persists in abundant wild and urban hosts that are logistically impractical or legally impossible to remove. Quantifying the relative contribution of these landscape reservoirs to crop infection rates remains a priority for epidemiological modelling.

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3.3.5 Surveys in Alabama, USA

Annual field surveys targeting blueberry (*Vaccinium* sp.) and grapevine (*Vitis vinifera*) plantations showing leaf scorch symptoms in Tallapoosa County, Alabama, yielded three new *X. fastidiosa* isolates: AL-BB1 and AL-BB2 (subsp. *multiplex* from blueberry) and AL-GR1 (subsp. *fastidiosa* from grapevine). Blueberry Leaf Scorch, caused by subsp. *multiplex*, was first described in Georgia in 2009 and has since spread throughout most southeastern US states; Pierce's Disease of grapevine, caused by subsp. *fastidiosa*, is endemic in grape-growing regions across the southern United States.

Bacterial isolation used surface-sterilised stem segments (blueberry) or petiole segments (grapevine) incubated in PD2 medium at 28°C. Colony identity was confirmed by 16S rDNA amplification and Sanger sequencing. Genome sequencing identified genome sizes ranging from 2.42 to 2.43 Mb, with GC content of 51.52–51.66%, 99.64% completeness, and 0% contamination (Table 3.9).

FastANI-based comparative genomics revealed that AL-GR1 (*X. fastidiosa* subsp. *fastidiosa*) clusters with grapevine-associated strains 16B5, 15B3, NOB1, and CFBP8083 from Georgia, Mississippi, and North Carolina, consistent with the population structure of southeastern US Pierce's Disease strains. Isolates AL-BB1 and AL-BB2 (*X. fastidiosa* subsp. *multiplex*) cluster with blueberry-associated strains AlmaEM3 and SRBB from Georgia, confirming host-associated population structuring within subsp. *multiplex* in this region.

Table 3.9. Genome assembly statistics and features of *Xylella fastidiosa* isolates from blueberry (AL-BB1, AL-BB2) and grapevine (AL-GR1) in Tallapoosa County, Alabama, USA. Genomes deposited in NCBI GenBank under the listed BioSample accession numbers.

Feature	AL-BB1	AL-BB2	AL-GR1
Subspecies	*multiplex*	*multiplex*	*fastidiosa*
Host of isolation	Blueberry (Vaccinium sp.)	Blueberry (Vaccinium sp.)	Grapevine (Vitis vinifera)
Assembly software	SPAdes / KBase	SPAdes / KBase	SPAdes / KBase
No. of contigs	82	73	103
N50 (bp)	115,464	96,852	104,401
Genome size (bp)	2,421,995	2,431,504	2,434,313
Contamination (%)	0.0	0.0	0.0
Completeness (%)	99.64	99.64	99.64
GC content (%)	51.66	51.66	51.52
No. of CDS	2,265	2,271	2,281
GenBank BioSample	SAMN49108562	SAMN49108563	SAMN49108564
Closest reference strains (ANI)	AlmaEM3, SRBB (Georgia, USA)	AlmaEM3, SRBB (Georgia, USA)	16B5, 15B3, NOB1, CFBP8083 (Georgia / Mississippi / North Carolina, USA)

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3.3.6 Surveys in Costa Rica

Monitoring in Costa Rica has been expanded to include avocado (*Persea americana*) as a potential host, based on previously reported symptoms including chlorosis and leaf deformation consistent with those associated with *X. fastidiosa* infection in this crop (Montero-Astúa et al. 2008). A plantation of approximately 100 avocado trees in the northern central valley (Heredia province), situated in a former coffee-growing area where *X. fastidiosa* is known to circulate and insect vectors are present, was selected for investigation.

Testing of 50 trees using petioles, twigs, and root material yielded positive results by PCR and immunofluorescence in 10 trees (20%), predominantly from petiole and root samples. Signal intensities were consistently low across positive samples, reflecting high inhibitory activity of the avocado matrix, a recognised challenge for *X. fastidiosa* molecular detection in this host. Protocol optimisation to reduce matrix effects is ongoing. Despite the preliminary nature of these results, the detection rate and symptom pattern support avocado as a potential *X. fastidiosa* host in Costa Rica, with implications for a rapidly expanding avocado production sector. Continued dedicated surveillance is warranted.

Additional screening of plant species co-occurring within the Puriscal sentinel plot (detailed in Section 4) detected *X. fastidiosa* in 8 of 23 tested species, including *Hibiscus rosa-sinensis*, *Acnistus arborescens*, *Psidium guineense*, *Eriobotrya japonica*, *Catharanthus roseus*, *Spondias purpurea*, and *Thymus vulgaris* (Mediterranean thyme). The detection of *X. fastidiosa* in thyme is of particular note given its Mediterranean origin and its potential relevance to trade in ornamental and medicinal plants.

3.3.7 Surveys in Argentina

In Argentina, official monitoring (in conjunction with SENASA of La Rioja) has been conducted since 2013 in the country's main olive-growing regions. In 2024, an ornamental oleander plant (*Nerium oleander* L.) located in the garden of a residence in Córdoba, Argentina, exhibited symptoms suggestive of the bacterium, such as chlorosis and foliar necrosis, followed by desiccation along the leaf blade, causing wilting and leaf drop (Figure 3.7). In 2015, sampling was intensified in search of new infected hosts on farms in Aimogasta and Cruz del Eje.

Several strains of *X. fastidiosa* were isolated from the plants found infected and characterized using MLST analysis: one from almond (Sumalao, Catamarca Province), four from olive (Aimogasta, La Rioja; Cruz del Eje, Córdoba; and Catamarca), and one from citrus (Bella Vista, Corrientes). Additionally, when isolation was not possible DNA samples extracted from plants infected with the bacteria were also included in the MLST characterization (Table 3.10).

MLST analysis of *X. fastidiosa* isolates from different hosts in Argentina, determined that strains OLI17A, ALM4, AM2-Angelina, MF4P1, Ais_citrus_Nelly, and Ais_oli_Pl60, as well as eight additional isolates obtained from infected plant samples, belong to the subspecies *pauca*. Two different STs (ST69 and ST78) were obtained in olive trees found on the same farm in the Cruz del Eje Department (Córdoba) and in Aimogasta, (La Rioja) (Table 3.10). The ST78 genetic group was also found in almond trees from Catamarca, olive trees from Cruz del

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Eje, Córdoba, and olive trees from Aimogasta, La Rioja. It should be noted that ST69 was previously detected by Coletta-Filho *et al*. (2017) in Argentine in citrus.

Interestingly, a new sequence type, i.e., ST90 was identified in an olive tree from Cruz del Eje, Córdoba, but its subspecies is currently unknown, since based in te seven alleles of the MLST analysis it seems to be an outgroup close to subspecies *pauca* (see Figure 5.2). These results are considered relevant due to their potential impact on the dissemination of the bacteria in different hosts (Tolocka *et al*., 2017a, b).

Table 3.10. Molecular characterization of strains and plant materials infected with *Xylella fastidiosa* in Argentina analyzed using the MLST Typing system

Sample code	Host/Culture system	Province/Department/ Location/Neighborhood	subspecies	Sequence Type-ST
OLI 17A	olive cv. Arauco/tradicional	La Rioja/Arauco/Aimogasta/ Tiro Federal II	***pauca***	**69**
ALM4	almond var. Catamarca/traditional	Catamarca/EEA-INTA	*pauca*	**78**
CITRI I	citrus orange cv. Valencia /traditional	Corrientes/Bella Vista	*pauca*	**69**
CE1	olive cv. Frantoio/tradicional	Córdoba/Cruz del Eje/San Isidro	*pauca*	**69**
CATA1	olive cv. Arauco/tradicional	Catamarca/Pomán	*pauca*	**69**
AM2-Angelina	olive cv. Arauco/tradicional	La Rioja/Arauco/Aimogasta/La Merced	*pauca*	**69**
MF4P1	olive cv. Nevadillo/tradicional	Córdoba/Cruz del Eje/ Cno. Paso Viejo	*pauca*	**78**
Oli V	olive. cv. unidentified	Córdoba/Capital	*pauca*	**69**
MF9P4	olive cv. Arauco/tradicional	Córdoba/Cruz del Eje/ Cno. Paso Viejo	?	**90**
M9_EX3	olive cv. Arauco/tradicional	La Rioja/Aimogasta	*pauca*	**78**
Ais_citrus_Nelly	citrus orange cv. Valencia	Corrientes/Bella Vista	*pauca*	**69**
Oli_M7	olive. cv. unidentified	Catamarca	*pauca*	**69**
Ais_oli_Pl60	olive cv. Arauco/tradicional	La Rioja/Aimogasta	*pauca*	**69**
Citrus_1_Nelly_M39	citrus orange cv. Valencia	Corrientes/Bella Vista	*pauca*	**69**
Oleander	oleander	Córdoba, Argentina	*pauca*	**New**

qPCR analysis confirmed that the oleander plant was infected by *X. fastidiosa*. This is the first time *X. fastidiosa* has been detected in an ornamental plant in the country (SINAVIMO code 1295). MLST analysis from the oleander plant revealed the following known alleles leuA = 7, petC = 6, malF = 7, nuoL = 8, and gltT = 8. However, analysis of the cysG locus, performed by the curator of the MLST *Xylella fastidiosa* Database, identified an allele distinct from those previously reported in other host species in Argentina, including almond, olive, and citrus. This novel allele has been designated cysG = 37. The leuA allele (7) is consistent with that reported in olive trees from Aimogasta (La Rioja), almond trees from Catamarca, and citrus

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trees from Bella Vista (Corrientes), but differs from the allele identified in olive trees from Cruz del Eje (Córdoba). Based on the allelic combination obtained, the resulting sequence type (ST) appears to be novel and distinct from those previously described in the country. A new ST assignation is still pending from the curator of the MLST database.



Figure 3.7. A. Oleander plant infected with *X. fastidiosa* sampled in Córdoba, Argentina. B. Detail of leaf scorch symptoms on leaves.

3.3.8 Global *in silico* surveillance

An in silico genomic surveillance approach was used to mine the NCBI Sequence Read Archive (SRA) for *Xylella*-like sequence reads embedded in publicly available datasets from diverse plant, insect, and environmental samples worldwide (Briand et al. 2025). Reads consistent with *Xylella* were identified in 62 SRA datasets. Eight draft genomes and one complete (circularised) genome were assembled to support taxonomic and phylogenomic analyses. Key findings are summarised in Table 3.11.

New hosts and strains in the USA: A new *X. fastidiosa* subsp. *multiplex* strain was identified in *Lavandula stoechas*; a second strain representing a new sequence type of subsp. *multiplex* was detected in *Populus trichocarpa* (black cottonwood), a species not previously reported as a *Xylella* host. New subsp. *pauca* strains in Latin America: Two new strains were identified from *Coffea* spp. in Brazil and Colombia. The Colombian strain from *Coffea eugenioides* is most closely related to a clade of ST53 strains known to infect coffee, oleander,

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and olive in Costa Rica and Italy, directly relevant to the epidemiological risk associated with this lineage. New country and continental records: *Xylella*-consistent reads were detected in banana rhizosphere samples from Nicaragua (first putative country record) and in a fly transcriptome from Namibia (first putative African record). *Xylella*-like reads were also identified in the perennial African grass *Alloteropsis angusta* from Miombo woodland in Zambia.

A proposed new species, *Xylella rufipogonis*: Four highly similar genomes (one circularised) assembled from wild rice (*Oryza rufipogon*) samples from China and Myanmar were shown by ANI and phylogenomics to represent a distinct new *Xylella* species, for which the name *X. rufipogonis* is proposed. Whether this species is pathogenic or commensal in wild rice, and whether it can colonise other hosts, remains unknown. Additionally, a strain most closely related to *X. taiwanensis*, previously considered endemic to Taiwan, was identified in a Japanese dataset, suggesting a broader geographic range for this species than currently recognised.

Table 3.11. Summary of key findings from in silico SRA data mining for *Xylella* spp. worldwide (Briand et al. 2025).

Finding	Taxon / strain	Host plant	Geographic area	Significance
New strain	*X. fastidiosa* subsp. *multiplex*	*Lavandula stoechas*	USA	New host for Xfm in USA
New strain (new ST)	*X. fastidiosa* subsp. *multiplex*	*Populus trichocarpa* (black cottonwood)	USA	Previously unreported host species
New strain	*X. fastidiosa* subsp. *pauca*	*Coffea* sp.	Brazil	New genetic lineage in coffee
New strain	*X. fastidiosa* subsp. *pauca*	*Coffea eugenioides*	Colombia	Related to ST53 clade (Costa Rica / Italy)
First country record	*X. fastidiosa*	Banana rhizosphere	Nicaragua	First putative country record
First continental record	*X. fastidiosa* subsp. *multiplex*	Fly transcriptome	Namibia, Africa	First record from Africa
New biome record	*Xylella* sp.	*Alloteropsis angusta* (grass)	Zambia, Africa	Miombo woodland / African savannah
Proposed new species	*X. rufipogonis* sp. nov.	*Oryza rufipogon* (wild rice)	China, Myanmar	4 genomes (1 circularised); distinct new species
Potential *X. taiwanensis*	*Xylella* sp.	Unknown	Japan	Geographic range extension beyond Taiwan

Collectively, these findings substantially expand the known distribution, host range, and taxonomic diversity of the genus *Xylella*. The discovery of a proposed new species in wild rice, and the identification of *Xylella*-associated sequences across tropical, sub-Saharan, and East Asian environments, indicate that current surveillance infrastructure is insufficient to capture the full global diversity of this genus. Ecological interactions between *Xylella* species, their plant hosts, and insect vectors are considerably more complex than currently understood.

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4. SENTINEL PLOTS

4.1 Background

The BeXyl sentinel plot network was designed as a coordinated early-warning system for the detection of *X. fastidiosa* in areas where the pathogen is not yet established or its full distribution is unknown. Sentinel plots consist of groups of known host plants strategically placed in high-risk environments, either adjacent to existing infected areas, along suspected pathways of spread, or in regions where climatic and agronomic conditions are favourable for establishment. By exposing susceptible plant species to natural inoculation pressure, sentinel plots provide a sensitive and ecologically realistic tool to monitor the emergence and dispersal of *X. fastidiosa* in diverse geographic and climatic contexts.

The strategic value of sentinel plots lies in their capacity to detect low-level or incipient infections before the pathogen reaches detectable densities in commercially or ecologically sensitive host populations. They also serve as long-term observatories to assess infection dynamics, the role of local vector communities in disease spread, and the relative susceptibility of economically important or ecologically relevant plant species. Within the BeXyl project framework, the sentinel plot network was additionally designed to be globally comparative: by applying standardised protocols across geographically distinct sites, spanning Europe and the Americas, the network enables the detection of multiple *X. fastidiosa* subspecies and the characterisation of infection risk under contrasting climatic regimes spanning a remarkable climatic gradient from Mediterranean and semi-arid European conditions through humid subtropical North American environments to tropical Central American systems and arid South American regions, providing a platform for comparative *X. fastidiosa* surveillance and ecological research across diverse environmental contexts.

The BeXyl sentinel plot network comprises 12 active sites distributed across 8 countries spanning Europe, North America, Central America, and South America. Additionally, specialized monitoring efforts are deployed across coffee plantations in Costa Rica using sentinel plants to evaluate *X. fastidiosa* prevalence within this agricultural system. Standardized protocols ensure methodological consistency across sites, particularly for botanical gardens and arboreta participating in sentinel activities.

4.2 Material and methods

4.2.1 Site selection and plant species composition

The network of sentinel plots includes installations in Apulia (Italy), Valencia, Córdoba, Girona, and Son Real and Sencelles (Spain), Corsica (France), Ferreira do Alentejo (Portugal), Alabama (USA), Puriscal (Costa Rica), Maria da Fé (Brazil), and Aimogasta (Argentina). All sentinel plots were established in locations free of confirmed *X. fastidiosa* infection, with the deliberate exception of the Son Real and Sencelles plots in Mallorca (Spain), Apulia (Italy), Corsica (France), Alabama (USA), Maria da Fé (Brazil), and Puriscal (Costa Rica), which were placed within or adjacent to known infected areas to maximise inoculation pressure (Figure 4.1). Site selection took into account: (i) proximity to areas with confirmed or suspected *X. fastidiosa* activity; (ii) accessibility and logistical feasibility for long-term monitoring; (iii) the

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presence of appropriate agronomic infrastructure; and (iv) the availability of collaborative partners with capacity for field management and laboratory analysis. The plots established in Argentina and Costa Rica were coordinated with local agricultural agencies and private landowners through formal technical cooperation agreements.

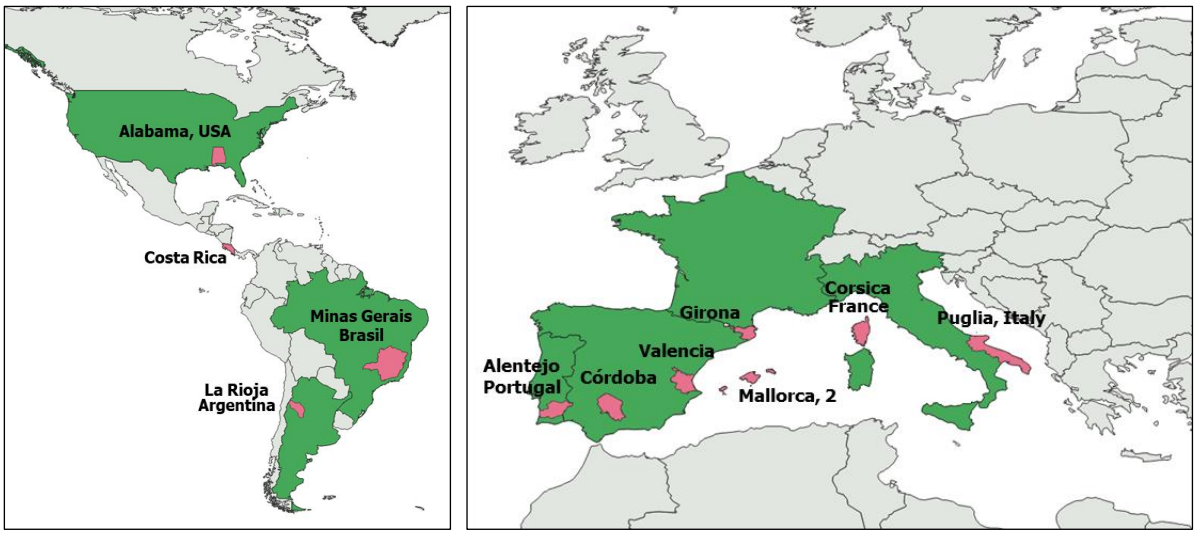


Figure 4.1. Geographic distribution of BeXyl sentinel plots

The plant species composition at each site was designed to include a core set of species common to the majority of sentinel plots that included *Olea europaea* cv. Koroneiki, *Citrus sinensis*, *Spartium junceum*, and *Prunus dulcis*, to enable cross-site comparisons. Local partners additionally selected species of particular agronomic or ecological relevance to their respective regions. For example, *Fraxinus excelsior* and *Quercus* spp. were included in Mallorca; *Carya illinoinensis* (pecan) was included in Alabama; *Ceratonia siliqua* was added in Valencia; *Cistus monspeliensis* and *Helichrysum italicum* were included in Corsica; and *Polygala myrtifolia* and *Duranta erecta* were incorporated in Brazil. In Costa Rica, the existing citrus plantation was complemented with introduced *Nerium oleander* as a sentinel for *X. fastidiosa* subsp. *pauca* ST53, and a broad diversity of native and ornamental plant species present on the farm were screened opportunistically (Table 4.1).

The experimental design was standardised across sites insofar as logistically feasible. The majority of plots adopted a randomised block design with a minimum of two to four blocks and four to five replicates per species or cultivar. Spacing between plants varied by site (ranging from 1 × 2.5 m to 3 × 6 m) but was generally chosen to allow access for regular monitoring and sampling. Plant material was obtained from certified nurseries with official plant passports wherever legally required (European sites). Field preparation included soil conditioning and the installation of drip irrigation systems tailored to the agronomic requirements of the planted species.

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Table 4.1. Overview of all BeXyl sentinel plots, including location, plant species composition and establishment date

Responsible entity	Location	Plant species	Date
IAS-CSIC	Córdoba, Spain (IAS-CSIC facilities)	Almond, olive (4 cvs.), sweet orange, grapevine (2 cvs.), Spanish broom, lavender, rosemary	Feb 2025
IAS-CSIC Plantas Continental	Ferreira do Alentejo (private farm), Portugal	Almond, olive, sweet orange, grapevine, Spanish broom, lavender, rosemary	Jun 2025
IVIA	Valencia, Spain (IVIA facilities)	Almond, olive, sweet orange, carob, lavender	Jul 2024
University of Girona	Girona, Spain (UdG campus)	Almond, olive, Spanish broom, lavender, grapevine	2024
CNR	Apulia, Italy (regional station)	Olive, sweet orange, *Acacia saligna*, Spanish broom, lavender, oleander	May 2023
INRAE	Corsica, France (INRAE San Giuliano)	Olive, sweet orange, Spanish broom, *Helichrysum italicum*, *Cistus monspeliensis*	Jun 2024
Auburn University	Fairhope, Alabama, USA, Auburn University	Olive, grapevine, olive, pecan, oleander	Feb 2024
APTA	Maria da Fé, Minas Gerais, Brazil (EPAMIG farm)	Olive, sweet orange, *Polygala myrtifolia*, *Catharanthus roseus*, oleander, *Duranta erecta*	Mar 2025
University of Costa Rica	Puriscal, Costa Rica (private farm)	Sweet orange, oleander, mango, loquat, *Spondias purpurea*, timber trees, avocado plus native and ornamental species	2023–2024
IRFAP	Son Real, Mallorca, Spain (public farm)	Olive, almond, sweet orange, *Spartium junceum, Quercus ilex, Q. suber, Arbutus unedo, Ficus carica, Fraxinus excelsior, Juniperus thurifera*	Spring 2024
IRFAP	Sencelles, Mallorca (private farm), Spain	Same 10 species as Son Real	Sep 2025
INTA	Aimogasta, La Rioja, Argentina (CAC S.A. farm)	Olive (5 cvs.), almond (3 cvs.), Spanish broom	Oct 2024

Individual site descriptions are provided below.

4.2.1.1 Córdoba, Spain

A sentinel plot was established in February 2025 at the experimental farm of the Institute for Sustainable Agriculture (IAS-CSIC) in Córdoba, Spain. The plot was established in close proximity to a quarantine greenhouse facility where artificial inoculation experiments with *X. fastidiosa* are conducted. The plot consists of two randomised blocks, each containing four replicates per species or cultivar. Eleven host plants representing five species were included: almond (*Prunus dulcis* cv. Marcona), olive (*Olea europaea* cvs. Koroneiki, Ogliarola, Leccino, FS17), sweet orange (*Citrus sinensis* cv. Lane Late), grapevine (*Vitis vinifera* cvs. Sauvignon Blanc and Sirah), and Spanish broom (*Spartium junceum*). *Lavandula angustifolia* and *Rosmarinus officinalis* were planted along the plot margins as supplementary species.

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4.2.1.2 Ferreira do Alentejo, Portugal

A plot was established in June 2025 in the municipality of Ferreira do Alentejo, Portugal, on the farm of Plantas Continental S.A., an olive mother-plant producer and BeXyl stakeholder. The plant species composition is identical to that of the Córdoba plot. Both plots received drip irrigation and plants were sourced from certified nurseries with accompanying plant passports.

4.2.1.3 Valencia, Spain

A sentinel plot was established on the experimental grounds of the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia (39°35'N, 0°23'W), a site previously cultivated with sweet orange trees. The soil is classified as loam-clay (clay loam), with a Papadakis bioclimatic classification of subtropical semi-warm (thermal regime) and dry Mediterranean (moisture regime). Five host plant species were planted, with 20 individuals per species for a total of 100 plants: almond (*Prunus dulcis*), carob (*Ceratonia siliqua*), lavender (*Lavandula angustifolia*), olive (*Olea europaea* cv. Koroneiki), and sweet orange (*Citrus sinensis*). A randomised block design with four blocks and five replicates per species was applied. Drip irrigation was provided at 60 minutes per day in summer and every 48–72 hours in winter, with a compound fertiliser (8-4-10) applied periodically.

4.2.1.4 Girona, Spain

A sentinel plot was established at the Astrit experimental area of the Universitat de Girona (UdG) campus in Montilivi, Girona. Plants were grown in containers to facilitate management and were maintained under standard irrigation and fertilisation practices. Six host species were included, each with 10 to 15 individuals: olive (*Olea europaea* cv. Koreneiki), almond (*Prunus dulcis* cv. Avijor), holm oak (*Quercus ilex*), Spanish broom (*Spartium junceum*), lavender (*Lavandula angustifolia*), and grapevine (*Vitis vinifera* cvs. Cabernet Sauvignon and Chardonnay). A meteorological station installed within the experimental area provides continuous climatic data for the site.

4.2.1.5 Son Real, Mallorca, Spain

A sentinel plot was established in spring 2024 at Son Real, a publicly owned agroforestry farm in Santa Margalida, Mallorca (39°44'N, 3°10'E). This site was deliberately placed within a demarcated zone where *X. fastidiosa* subsp. *multiplex* ST81 is the predominant circulating strain. The plot comprises 20 replicates of 10 plant species arranged in four randomised blocks, planted at 1 m × 2.5 m spacing under drip irrigation. Species included: *Citrus sinensis*, *Olea europaea*, *Spartium junceum*, *Arbutus unedo*, *Fraxinus excelsior*, *Ficus carica*, *Juniperus thurifera*, *Prunus dulcis*, *Quercus ilex*, and *Quercus suber*. Plants are exposed to natural inoculation from surrounding infected olive and almond trees.

4.2.1.6 Sencelles, Mallorca, Spain

A second sentinel plot was established in Sencelles, Mallorca (39°39'N, 2°54'E) in September 2025, within the area of a recent outbreak of *X. fastidiosa* subsp. *pauca* ST53. The plot design and species composition are identical to that of the Son Real plot. Sampling and qPCR analysis will begin in summer 2026.

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4.2.1.7 Apulia, Italy

A sentinel plot was established in May 2023 at a regional agricultural station in Apulia, Italy, located within the demarcated zone for *X. fastidiosa* subsp. *pauca*. Twelve replicates of six species were planted in three randomised blocks of four replicates each: olive (*Olea europaea* cv. Koroneiki), citrus (*Citrus sinensis* cv. Valencia Late / C35 rootstock), *Acacia saligna*, *Spartium junceum*, *Lavandula angustifolia*, and *Nerium oleander*. A total of 80 plants were included (with 5 lavender and 1 broom plant desiccated at the time of sampling in June 2025).

4.2.1.8 Corsica, France

A sentinel plot was established in June 2024 at the INRAE research centre in San Giuliano, Corsica (42°17'N, 9°31'E). This location was selected due to the presence of the Citrus Biological Resource Centre (CRB-Citrus), one of the world's largest citrus collections, making surveillance of potential citrus-infecting *X. fastidiosa* strains of particular importance. Five plant species were included to maximise coverage of major *X. fastidiosa* subspecies: *Olea europaea*, *Citrus sinensis*, *Cistus monspeliensis*, *Helichrysum italicum*, and *Spartium junceum*. Plants were grown in a greenhouse for one year prior to outdoor planting. The plot consists of four randomised blocks with 20 plants per species, totalling 100 plants. *S. junceum* was grown from seed; all other species were sourced from nurseries. Daily and monthly meteorological data are obtained from the nearby Alistro station (meteo.data.gouv.fr).

4.2.1.9 Fairhope, Alabama, USA

A sentinel plot was established in February 2024 at the Gulf Coast Research and Extension Center of Auburn University in Fairhope, Alabama (30°33'N, 87°52'W). Four plant species were planted (20 individuals each) in a completely randomised block design with four blocks and five replicates: grapevine (*Vitis vinifera* cv. Chardonnay), olive (*Olea europaea* cv. Koroneiki), pecan (*Carya illinoinensis* cv. Caddo/Cape Fear), and oleander (*Nerium oleander*). Row spacing was approximately 6.0 m, with 3.0 m between plants within rows. Climatic data are obtained from the nearby NOAA Climate Reference Network weather station (AL Fairhope 3 NE).

4.2.1.10 Maria da Fé, Brazil

A sentinel plot was established in March 2025 at the experimental farm of the Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) in the municipality of Maria da Fé, Minas Gerais State, Brazil. The site was selected due to high inoculum pressure and documented incidence of Olive Quick Decline Syndrome (OQDS). Five plots were established on the farm, each containing five replicates of the following species in a randomised design: *Olea europaea* cv. Koroneiki, *Citrus sinensis* cv. Valencia, *Catharanthus roseus*, *Polygala myrtifolia*, *Nerium oleander*, and *Duranta erecta*. By May 2025 (two months after planting), sharpshooter (Cicadellidae) specimens had already been observed on *P. myrtifolia*, *C. sinensis*, and *N. oleander*. Insects are preserved in 70% ethanol for *X. fastidiosa* infectivity determination.

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4.2.1.11 Puriscal, Costa Rica

A sentinel plot was established in Santiago, Puriscal, San José, Costa Rica (9°51'N, 84°18'W), at an elevation of 1,105 m a.s.l. on a privately owned small-scale orange orchard (ca. ~3,000 m²). The site has a bimodal annual climate with a dry season from December to April (~14 mm precipitation, ~40% humidity) and a rainy season from May to November (~166 mm, ~70% humidity). Mean temperature ranges from 19°C (minimum) to 30°C (maximum), with approximately 12 hours of daylight throughout the year. The location was selected based on a prior isolation of *X. fastidiosa* subsp. *fastidiosa* from a diseased *Vinca* plant, confirming local *X. fastidiosa* circulation. The plot includes 25 sweet orange (*Citrus sinensis*) trees and 20 introduced *Nerium oleander* plants screened before introduction to ensure *X. fastidiosa*-free status. A broad diversity of productive, ornamental, and native plant species co-occurring on the farm were also screened. Leafhoppers (Cicadellidae) are abundant and diverse at this site.

4.2.1.12 Aimogasta, La Rioja, Argentina

A sentinel plot was established at the CAC S.A. (NUCETE) farm in Aimogasta, La Rioja, Argentina (28°33'S, 66°48'W) through a formal technical cooperation agreement between INTA, SENASA La Rioja, and the CAC S.A. company. The company donated 0.44 hectares and provides management support (irrigation, fertilisation, weeding). Planting took place in October 2024 and included 184 plants distributed across four rows: olive (cvs. Koroneiki, Arbosana, Oliana, Hojibanca, Arbequina), almond (*Prunus dulcis* cvs. Soleta, Isabelona, Avijor), and Spanish broom (*Spartium junceum*). A 5 × 4 m planting framework was applied. Prior to field planting, seedlings of olive cvs. Koroneiki and Arbequina were tested by DAS-ELISA and PCR, and all plants were negative for *X. fastidiosa*. All plant material for the sentinel plot was confirmed free of *X. fastidiosa* before field establishment.

4.2.2 Climatic characterization

The sentinel plot network a wide range of climate regimes across four continents, representing a comprehensive gradient from Mediterranean to subtropical, and arid to tropical conditions. The European sites are predominantly characterized by Mediterranean climates (Csa classification), including Apulia, Valencia, Corsica, Mallorca, Córdoba, and Ferreira do Alentejo, which share the fundamental pattern of hot, dry summers and mild, wet winters. However, these Mediterranean locations exhibit significant variation in aridity levels, with Valencia and Mallorca showing semi-arid tendencies (BSk elements) characterized by lower annual precipitation (400-500 mm) compared to more mesic Mediterranean sites like Corsica (650-995 mm). Córdoba represents the most extreme Mediterranean conditions with intense summer heat and pronounced drought stress, while Girona introduces continental influences due to its inland position and proximity to the Pyrenees.

The American locations provide a stark climatic contrast, with Alabama representing a humid subtropical regime (Cfa) fundamentally different from the Mediterranean pattern. Alabama's climate features year-round precipitation (1,200-1,680 mm annually), high humidity levels, and pronounced seasonal temperature variations with hot, humid summers and mild winters. Costa Rica introduces tropical highland conditions with distinct wet and dry seasons, representing yet another precipitation regime where seasonal distribution rather

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than annual totals defines water availability. The South American sites span the broadest climatic extremes within the network: Argentina's La Rioja represents true arid desert conditions (BWk/BSk) with minimal precipitation (200-400 mm) and extreme water stress, while Brazil's Maria da Fé exhibits subtropical highland characteristics (Cwb/Cfb) with moderate temperatures and seasonal precipitation patterns influenced by elevation.

Climatic monitoring is conducted at all sentinel plot sites, either through on-site meteorological stations or through nearby reference stations. In Girona, a meteorological station was installed directly within the experimental area of the UdG campus. In Corsica, daily and monthly data are provided by the Alistro meteorological station (meteo.data.gouv.fr). In Alabama, climatic data were obtained from the NOAA Climate Reference Network station AL Fairhope 3 NE. The climatic data were supplied by the Meteorological Station located in Aimogasta with georeferences 28º34`44.97``S 66º43`15.36``W, using the iMetos model (3.3). At other sites, regional meteorological services and existing agricultural weather networks provide the relevant climatic information.

4.2.3 Monitoring activities: Plants and insect vectors

All sentinel plots were subject to regular visual inspections for the presence of symptoms potentially associated with *X. fastidiosa* infection, including leaf scorch, marginal necrosis, chlorosis, and early defoliation. In addition to symptom assessment, plant material was collected periodically for molecular testing.

Plant sampling protocols varied slightly among partners, but all followed the EPPO-recommended approach: leaf or branch samples (including tissue containing vascular elements) were collected from multiple positions per plant. At IVIA (Valencia), three leaves per plant were collected from the lower, middle, and apical portions; petioles were excised with a flame-sterilised scalpel and freeze-dried prior to homogenisation using a TissueLyser II (Qiagen). At INRAE (Corsica), branches were debarked and 0.5-2 g of underlying vascular tissue was scraped and processed through sonication and maceration to maximise bacterial cell release. In Apulia, and at the Mallorca plots, sampling followed published protocols consistent with official survey guidelines. All sites applied *X. fastidiosa*-specific real-time qPCR for molecular detection, with the majority using the TaqMan-based assay of Harper et al. (2010) on calibrated thermocyclers. The Valencia site additionally used a duplex qPCR (Bonants et al. 2019), and the Costa Rica site used PCR combined with immunofluorescence. DNA was extracted using either CTAB-based protocols (EPPO, 2023) or commercial kits (GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific) depending on the laboratory.

Insect vector monitoring was conducted at several sites. In Apulia, insects were collected in 2023, 2024, and 2025 using sweep nets and yellow sticky traps at pre-fixed sampling units in the area surrounding the plot, covering multiple vegetation compartments (ground cover, shrubs, trees). In Corsica, sweep net captures of sap-feeding hemipterans were performed at each plant sampling visit. Captured insects were visually identified to species level and sexed, and individual specimens were subjected to molecular detection of *X. fastidiosa*. In Costa Rica, leafhoppers (Cicadellidae) are routinely observed and collected from

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multiple host plants within the plot. In Brazil, sharpshooter and spittlebug populations are under active surveillance and specimens are preserved in 70% ethanol for subsequent infectivity analyses. The main sampling dates by site are summarised in Table 4.2.

Table 4.2. Summary of plant and insect vector sampling activities carried out across BeXyl sentinel plot network

Site	Sampling dates (plants / insects)
Córdoba, Spain	First sampling April 2025
Valencia, Spain	Three samplings, Sept and Dic 2024, Feb 2025
Girona, Spain	First sampling: February 2026 (qPCR results pending)
Son Real, Mallorca	2 Oct 2024; 28 Jul 2025; 16 Sep 2025
Sencelles, Mallorca	First sampling: summer 2026
Ferreira do Alentejo, Portugal	First sampling planned Spring 2026
Apulia, Italy	Vectors: summer–Oct 2023, 2024, 2025; Plants: June 2025
Corsica, France	Oct 2024; May 2025; Oct 2025; (May 2026 planned)
Alabama, USA	Visual inspection: end of summer 2024 and 2025; molecular testing pending
Maria da Fé, Brazil	First sampling: February 2026 (~12 months post-planting)
Puriscal, Costa Rica	2023 and 2024 (multiple rounds, citrus and oleander)
Aimogasta, Argentina	Visual inspection: March 2025; molecular: Sep–Nov 2025

4.3 Results

4.3.1 Detection of *Xylella fastidiosa* over time: Plants and Vectors

At the time of preparation of this deliverable, the majority of sentinel plots have tested negative for *X. fastidiosa* across all plant species and sampling events. Positive detections have been confirmed at two sites: Son Real (Mallorca, Spain) and Puriscal (Costa Rica). The following subsections present site-specific results in detail.

4.3.1.1 Córdoba, Spain

The plot was established in February 2025 and plant establishment was satisfactory at both sites, with no major agronomic issues reported. A first sampling was performed on April 2025, briefly, for each plant, branch sections were collected for woody plants and leaves from herbaceous species present in the sentinel plot and bacterial detection was performed following Bonants et al. (2019) protocol. No characteristic symptoms typical of *X. fastidiosa* were observed and all samples were negative for *X. fastidiosa* for all plant species evaluated.

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4.3.1.2 Valencia, Spain

Three sampling rounds were conducted at the IVIA sentinel plot at approximately 2 to 3-month intervals. For each plant, leaves from the lower, middle, and apical portions were collected and subjected to duplex real-time qPCR (Bonants et al. 2019). All samples from all three rounds were negative for *X. fastidiosa* across all five plant species. No symptoms potentially associated with *X. fastidiosa* infection were observed throughout the monitoring period, and no potential insect vectors were detected in or around the plot during periodic surveys.

4.3.1.3 Girona, Spain

The Girona sentinel plot was established during 2024–2025 and has been under regular visual inspection. No symptoms compatible with X. fastidiosa infection have been observed in any of the planted species. The first qPCR sampling was performed in February 2026; results are pending at the time of writing but are expected to be negative based on the absence of symptoms and the site's disease-free status.

4.3.1.4 Apulia, Italy

The Apulia sentinel plot is located within the demarcated zone for *X. fastidiosa* subsp. *pauca*. Vector monitoring using sweep nets and sticky traps was conducted during the summers of 2023, 2024, and 2025. Populations of the primary vector *Philaenus spumarius* were consistently low throughout the monitoring period, with approximately 0.1 specimens per sweep on ground vegetation and 0.35 specimens per sweep or per trap on shrubs and trees, predominantly on *Cupressus*. The first molecular plant sampling was conducted in June 2025 (80 plants total, including 5 desiccated lavender and 1 desiccated broom plant). All samples tested negative for *X. fastidiosa* by qPCR (Harper et al. 2010), including the six dead plants. The mortality of lavender and broom individuals was attributed to biotic or abiotic stresses unrelated to *X. fastidiosa* given the negative molecular results.

4.3.1.5 Corsica, France

The Corsica sentinel plot was visited and sampled three times since its establishment in June 2024: in October 2024, May 2025, and October 2025. All 89 remaining plants (11 lost due to mortality and accidental removal) have consistently tested negative for *X. fastidiosa* across all three sampling events. Nine *H. italicum* individuals died shortly after planting, likely due to heavy autumn rainfall; these were confirmed negative and are being replaced. Two *S. junceum* plants were accidentally removed during site maintenance.

Insect vector captures revealed that *P. spumarius* is rare at this site, with only 1 to 3 individuals captured per visit. In contrast, *Neophilaenus* sp. was the dominant spittlebug taxon in October 2024 and May 2025. A diverse community of leafhoppers was captured at each visit (n = 4–27 individuals), and molecular species identification based on the mitochondrial COI gene is underway for the October 2025 collection. All captured insects have tested negative for *X. fastidiosa* to date. The apparent absence of *X. fastidiosa* detections is consistent with the historically low number of positive plant samples reported by official surveillance authorities in this area and may also reflect limited vector efficiency given the low *P. spumarius* abundance.

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4.3.1.6 Alabama, USA

The Alabama sentinel plot was established in February 2024 and has been under visual inspection at the end of the 2024 and 2025 growing seasons. No symptoms potentially associated with *X. fastidiosa* were observed in any of the four planted species. Molecular testing has not yet been conducted; this is planned for an upcoming visit. *X. fastidiosa* strains causing Pierce's Disease of grapevine are known to be present in the broader region.

4.3.1.7 Maria da Fé, Brazil

The sentinel plot at the EPAMIG farm in Maria da Fé was established in March 2025 in an area with documented high inoculum pressure of *X. fastidiosa* and high OQDS incidence. Plants showed good growth by January 2026. Sharpshooter specimens (Cicadellidae) were first observed in May 2025, just two months after planting, on *P. myrtifolia*, *C. sinensis*, and *N. oleander*, and have continued to be observed since. This early and consistent vector activity at this site, combined with the high background inoculum, suggests favourable conditions for natural *X. fastidiosa* transmission. Leaf sampling for *X. fastidiosa* detection by qPCR was scheduled for February 2026, approximately 12 months after planting. Results are pending at the time of writing.

4.3.1.8 Puriscal, Costa Rica

The Puriscal sentinel plot produced the most extensive detection dataset of all sites to date. Positive detections of *X. fastidiosa* were confirmed across multiple plant species and sample types, confirming active pathogen circulation at this location. Screening of 25 sweet orange (*C. sinensis*) trees revealed that 7 (28%) tested positive for *X. fastidiosa* by PCR and immunofluorescence. Eight trees (32%) exhibited symptoms potentially compatible with *Xf* infection, including variegated chlorosis. However, no clear association between symptom expression, tree age, and *Xf* detection status was observed, several positive trees were asymptomatic and several symptomatic trees tested negative, indicating that symptom-based surveillance alone would be insufficient at this site.

In addition, 10 *N. oleander* plants introduced as sentinels for *X. fastidiosa* subsp. *pauca* were screened after one year of exposure. Four (40%) tested positive for *X. fastidiosa*; one plant was heavily infected, with more than 20 bacterial cells per microscopic field detectable by immunofluorescence (ca. 10^6 bacteria). Leaf scorch symptoms were observed in some of the plants, though the correlation between symptom expression and *X. fastidiosa* detection was consistent.

Screening of 23 additional plant species co-occurring on the property, spanning tropical, ornamental, Mediterranean, and native taxa, showed positive *X. fastidiosa* detections in eight species (35%): *Artocarpus altilis* (breadfruit), *Hibiscus rosa-sinensis*, *Acnistus arborescens*, *Psidium guineense*, *Eriobotrya japonica*, *Catharanthus roseus*, *Spondias purpurea*, and *Thymus vulgaris*. *Nerium oleander* plants already present in the plot (i.e., not introduced as sentinels) were highly positive, as was *Hibiscus rosa-sinensis*, which is used as a live fence on the property. Notably, thyme (*Thymus vulgaris*), a Mediterranean herb, was found positive, a finding of potential relevance for ornamental and aromatic plant trade.

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Cicadellid populations are abundant and diverse at this site, and a full leafhopper species inventory is planned for the next project phase.

4.3.1.9 Aimogasta, Argentina

Plants at the Aimogasta sentinel plot were established in October 2024. A visual monitoring event conducted on 19 March 2025 found no suspicious symptoms attributable to *X. fastidiosa*. Some plant establishment failures were recorded in olive, almond, and *S. junceum.* Monitoring and the first collection of plant material were carried out in October 2025, considering the west-to-east sampling direction in each planted row. The samples were analyzed in December 2025 and January 2026 using the qPCR of Harper. Pronounced symptoms consistent with frost damage were recorded, despite the use of frost-protection netting. Low temperatures were documented during the week preceding sample collection. In this initial survey, 122 samples were analyzed. Samples considered suspicious or doubtful (Ct > 33) included olive cv. Oliana (F1 P28), olive cv. Koroneiki (F3 P7), olive cv. Arbosana (F3 P18), and almond cv. Soleta (F3 P13). Due to the high Ct values it was not possible to confirm the subspecies of the positive samples.

4.3.1.10 Son Real, Mallorca, Spain

Three sampling events were conducted at the Son Real sentinel plot on 2 October 2024, 28 July 2025, and 16 September 2025. Samples were analysed by real-time qPCR following Harper et al. (2010). Positive detections were recorded in three plant species: *Ficus carica*, *Fraxinus excelsior*, and *Spartium junceum*. Notably, the positive *F. carica* detection in September 2025 corresponds to a different individual plant from the one detected positive in October 2024, suggesting either multiple independent infection events or complex within-plot transmission dynamics. The two consecutive *Fraxinus excelsior* detections (July and September 2025) also correspond to different individual plants. All other species, including *Olea europaea*, *Citrus sinensis*, *Prunus dulcis*, and both *Quercus* species, remained negative throughout. Although the number of detections is limited, their occurrence in three distinct host species over the 2-year monitoring period is significant.

4.3.1.11 Sencelles, Mallorca, Spain

The Sencelles plot was established in September 2025 within the outbreak area of *X. fastidiosa* subsp. *pauca* ST53. No sampling data are available yet; the first sampling is scheduled for summer 2026. Based on observations from Son Real, rapid infection dynamics are not anticipated.

4.3.1.12 Ferreira do Alentejo, Portugal

The plot was established in June 2025, and plant establishment has been satisfactory, with no major agronomic issues reported with the exception of a high mortality of *L. angustifolia* plants probably associated with environmental stress related to insufficient adaptation to local conditions. No *X. fastidiosa* testing results are yet available; however, the first sampling of this plot is planned for spring 2026, but no symptoms potentially associated to *X. fastidiosa* have been observed.

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4.3.2 Long-term Surveillance and monitoring

Given the long-term nature of sentinel surveillance and the accumulating investment in plant establishment, monitoring infrastructure, and accumulated baseline data, a number of partners have developed or are actively pursuing plans to maintain their sentinel plots beyond the end of the BeXyl project. The continuity of these plots is considered scientifically valuable, as infection dynamics in sentinel systems typically develop over multiple years and long-term datasets are necessary to draw robust conclusions about *X. fastidiosa* spread rates, host susceptibility, and the effectiveness of natural barriers.

Post-project continuity has been secured for several key sites across the network. Corsica benefits from INRAE's explicit commitment to indefinite maintenance with biannual monitoring visits (May and October) supported through internal funding mechanisms, representing particular strategic value given its proximity to the CRB-Citrus biological resource centre. The Puriscal, Costa Rica site will continue long-term surveillance operations due to confirmed *X. fastidiosa* presence and ongoing regional monitoring needs, while Maria da Fé, Brazil has secured funding through March 2027 for continued temporal infection analyses via qPCR and vector monitoring activities.

Additional sites have provisional continuation plans dependent on funding acquisition. The Mallorca sentinel plots at Son Real and Sencelles are planned for several additional years of operation, contingent on securing appropriate resources for plot maintenance, sampling, and laboratory analyses. The Alabama site faces greater uncertainty, with the responsible team preparing extension requests to the Alabama Agricultural Experiment Station, though funding confirmation remains pending. Continuity arrangements for the remaining European sites, Córdoba, Portugal, Valencia, Girona, and Apulia, have not yet been formalized and will require future planning to maintain the network's comprehensive geographic and climatic coverage.

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5. BOTANIC GARDENS AND ARBORETA

5.1 Background

Within the BeXyl framework and the scope of the work package 1, botanic gardens and arboreta are recognised as sentinel collections, as they host taxonomically diverse, well-documented plant collections, often including species cultivated outside their native ranges that can be used to investigate host range and vulnerability studies as the one being presently investigated in this group.

This report describes the engagement of botanic gardens in South and Central America, the implementation of harmonised monitoring and sampling protocols, interim surveillance outputs, and the documentation and communication materials developed during the reporting period.

Additionally, the botanical garden "Parc de Saleccia" in Corsica (https://www.parc-saleccia.fr/) was included in the study due to its diversity of plant species, including local Mediterranean plants but also species originating from South Africa, Australia, New Zealand and the Americas.

5.2 Material and methods

5.2.1 Host prioritisation strategy

Host selection followed a structured, risk-based prioritisation approach designed to maximise the probability of detecting *X. fastidiosa* and identifying both current strain present in the region as well as novel host–pathogen associations. Criteria included:

1. Listings from the EFSA *X. fastidiosa* host plant database.
2. Relevance for Europe and the Mediterranean basin, prioritising crops, forest species and ornamentals of agronomic, ecological or trade importance (e.g. *Coffea* spp., *Citrus* spp., *Olea europaea*, *Prunus* spp.).
3. Symptom-based prioritisation, targeting plants showing leaf scorch, chlorosis, dieback, stunting or reduced vigour compatible with *X. fastidiosa* infection.
4. Where multiple individuals of the same species were available, the oldest accessions and/or symptomatic plants were prioritised. Asymptomatic plants were included where necessary to ensure taxonomic coverage, acknowledging the frequent occurrence of latent infections.

5.2.2 Study sites and coordination framework

Sampling was implemented through a network of botanic gardens participating in the International Plant Sentinel Network (IPSN). Sites were located in South and Central America, regions deliberately selected because *X. fastidiosa* is widely thought to have emerged from

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this region, where the pathogen has co-evolved with a broad diversity of cultivated, ornamental and wild host species.

Engagement was facilitated through initial introductory online meetings during 2023-24 with representatives of botanic gardens in the Central American and South American regions, alongside participation in regional congresses aimed at introducing the project and its aims. Organisations interested in participating and contributing to this work (see table 5.1) later registered with the IPSN coordinators, who managed next steps during the current reporting period over two formal coordination meetings (8 May and 14 July 2025), complemented by continuous technical support and written guidance.

Table 5.1. Participating botanic gardens, geographic context, and national *Xylella fastidiosa* status.

Country	Botanic garden	City	Region	National *Xylella* status
Brazil	Instituto Inhotim	Brumadinho	South America	Endemic; widely reported in citrus, coffee, olive and ornamentals
Colombia	Jardín Botánico de Cali	Cali	South America	Presence reported, particularly in coffee and citrus
Colombia	Jardín Botánico de Medellín	Medellín	South America	Presence confirmed; pathogen considered endemic
Ecuador	Jardín Botánico Padre Julio Marrero	Guayaquil	South America	No established outbreaks; historical detections in exported plant material
Guatemala	Jardín Botánico CECON-USAC	Guatemala City	Central America	Regional presence documented; endemic in Central America
Guatemala	Orquidario y Arboreto, Universidad Rafael Landívar	Guatemala City	Central America	Regional presence documented; endemic in Central America
Corsica, France	Parc de Saleccia	Ile-Rousse	Europe	Regional presence documented

Additionally, the botanical garden “Parc de Saleccia” in Corsica, a region in Europe with presence of *X. fastidiosa* and where no eradication measures are implemented due to the widespread of the bacterium in the island, was chosen for its diversity of plant species, including local Mediterranean plants but also species originating from South Africa, Australia, New Zealand and the Americas. Furthermore, to date no plants have been confirmed infected with *X. fastidiosa* and no vector surveillance has been carried out in this garden.

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5.2.3 Sampling design and field procedures

5.2.3.1 Sampling in the botanic gardens of America

Sampling followed the harmonised BeXyl protocol for *X. fastidiosa* detection (provided in Spanish and Portuguese to participating gardens), aligned with EPPO PM 7/24 guidelines and adapted for botanic garden conditions.

Within each garden, at least one individual per prioritised host species was sampled; where feasible, 4–5 individuals per species were included to capture intra-specific variability.

Sampling focused on xylem-rich tissues, reflecting the vascular localisation of *X. fastidiosa*. Large-leaved species were sampled using basal leaf portions including the petiole and central vein, while small-leaved species required multiple leaves to reach a target mass of 0.5–1.0 g. In species with reduced petioles or during dormancy, xylem-containing stem or woody tissue was collected.

Samples were gently shaken to remove potential insect vectors, placed in sealed containers, kept cool, and transported promptly to on-site preparation areas to be processed. Metadata was recorded using a standardised sampling form (see protocol), including species identity, accession number (or garden-assigned identifier), symptom description, sampling date and photographic references.

5.2.3.2 Sampling in the botanic garden of Corsica

Twenty-three plant species were sampled (1 plant/species) (Table 5.2). While almost all of them were already known as host plants for different *X. fastidiosa* subspecies in different parts of the world, most of them have not yet been found infected in France – with the exception of *Cistus* sp., *Lavandula* sp., *Myrtus* sp. and *Polygala myrtifolia* (1).

5.2.4 Sample processing, traceability and shipment

Sample processing was carried out on-site using BeXyl sampling kits. Xylem-rich tissue (0.5–1.0 g) was homogenised in BIOREBA extraction bags using phosphate-buffered saline (PBS).

For each plant sample, two technical replicates were prepared:

- replicate A for shipment to the reference laboratory and
- replicate B retained locally as backup.

Extracts were sealed, labelled with a unique alphanumeric code, and stored at −20 °C (or −18 °C) for a minimum of 15 days prior to shipment to ensure bacterial inactivation and nucleic acid preservation.

Shipment to the reference laboratory (IAS-CSIC, Córdoba, Spain) was centrally coordinated by the BeXyl project team and accompanied by standardised documentation (letter of use and destination, proforma invoice and explanatory note).

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5.2.5 DNA extraction and *X. fastidiosa* detection

In the samples from the botanic gardens of America, DNA was extracted using the Maxwell RSC PureFood GMO and Authentication Kit and the Maxwell DNA extraction system (Promega, Wisconsin, USA), following the manufacturer's instructions for DNA plant extraction.

In the samples from Corsica, DNA was extracted from 1-1.5 g of plant tissue using our standard CTAB-based extraction protocol, including a sonication step at 42 kHz for one minute and maceration at room temperature for 45 minutes to increase the release of bacterial cells from biofilms.

In the samples from the botanic gardens of America, detection of X. fastidiosa was performed by real-time qPCR using TaqMan-based assays of Bonants et al. (2019) that combines the protocols of Harper and Ouyang, following EPPO (2023) (Table 2.3). Subspecies identification was performed using the tetraplex real-time PCR developed by Dupas et al. (2019) in quantitative PCR (qPCR) and digital PCR (dPCR), which simultaneously identifies the three main X. fastidiosa subspecies in a single reaction. A nested- Multilocus sequence typing (MLST) approach (Cesbron et al. 2020) was appliedto amplify seven housekeeping genes (leuA, petC, malF, cysG, holC, nuoL, gltT). Allelic profiles were queried against the publicly available pubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa).

In the samples from Corsica, presence and titer of X. fastidiosa was analysed in triplicates using Harper's qPCR. The subspecies of positive samples was determined using Dupas' tetraplex qPCR and the sequence type through PCR amplification of the seven MLST genes as previously described.

To construct individual phylogenetic trees for the seven MLST genes, the obtained sequences for each gene were aligned using MAFFT, which automatically selects the appropriate alignment strategy, with the reference sequences of each allele described up to date from the pubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa). Maximum-likelihood (ML) phylogenetic trees were inferred from the aligned sequences using FastTree with the nucleotide model. This two-step process was repeated for all seven genes. Additionally, a ML phylogenetic tree was constructed following the same methodology using the concatenated alignment of all seven MLST genes, including the 90 sequence types (STs) described for *X. fastidiosa* as reference.

5.3 Results

5.3.1 Sampling implementation and coverage

All participating botanic gardens successfully implemented the harmonised sampling protocol following training and guidance. Sampling encompassed a broad taxonomic range of woody and perennial plant species, including crops, ornamentals and forest taxa of relevance to Europe and the Mediterranean basin.

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A total of 146 plant samples were collected by the six botanical gardens in America (Table 5.2). The representative species sampled in each garden are indicated in Table 5.2. Sampling also included some *X. fastidiosa* host plants listed in the EFSA database as well as additional taxa of sentinel value (Table 5.3). Full species-level inventories with accession identifiers are maintained in the BeXyl project database. Tropical forest species were included to broaden the sentinel scope and to capture potential host–pathogen associations that may not yet be documented in formal host databases.

Table 5.2. Sampling implementation overview by botanic garden (interim).

Botanic garden	No. of plants sampled	Scientific names of sampled host species*	Symptomatic plants observed
Instituto Inhotim (Brazil)	11	*Ginkgo biloba*, *Jacaranda mimosifolia, Lavandula dentata, Morus alba, Nerium oleander*, *Olea europaea, Persea americana, Phoenix reclinata, Salvia rosmarinus,* other European and native plants/trees,	Yes
Padre Julio Marrero (Ecuador)	17	*Coffea* spp., *Persea americana*, *Citrus* spp., *Solanum americanum*, *Hibiscus rosa-sinensis, Phoenix reclinata*, other Brassicaceae, Poaceae and Cyperaceae species.	Yes
CECON-USAC (Guatemala)	40	*Coffea arabica*, *Citrus spp.*, *Nerium oleander*, *Ginkgo biloba*, *Prunus persica, Persea americana, Hisbiscus* spp., *Rosa* spp., Salvia rosmarinus, *Thymus vulgaris*, other European and native plants/trees,	Yes
Orquidario y Arboreto, Universidad Rafael Landívar (Guatemala)	11	*Acer sacharum, Acer negundo*	Yes
Cali (Colombia)	47	*Coffea arabica*, *Citrus spp.*, *Strelitzia reginae*, Rosa sp,, other orgamental plants and trees.	Yes
Medellín (Colombia)	20	*Coffea arabica*, *Phoenix* spp., *Olea europaea, Nerium oleander, Magnolia* spp., *Persea americana, Strelitzia reginae, Syzygium paniculatum*, *Hevea brasiliensis, Nicotiana tabacum*, other ornamental and native	Yes
Parc de Saleccia (Corsica)	23	*Arbutus unedo, Callistemon citrinus* (syn: Melaleuca citrina), *Cistus albidus, Cistus* sp., *Citrus* sp., *Echium fastuosum, Euphorbia dendroides, Gazania rigens, Juniperus sp., Grevillea* sp., *Hibiscus* sp., *Jacaranda* sp., *Lavandula* sp., *Myrtus communis* subsp. *Tarantina, Myrtus* sp., *Pistacia* sp., *Polygala* sp., *Polygala myrifolia, Salvia microphylla Sambucus* sp., *Tamarix* sp., *Vinca major, Vitex* sp.	Yes

*Scientific names listed are representative examples of sampled taxa per garden and do not constitute a complete species inventory.

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Table 5.3. Representative host species sampled through the botanic garden network (interim).

Host species / group	Plant type	EFSA-listed host	EU / Mediterranean relevance
Coffea arabica	Crop (woody perennial)	Yes	High
Citrus spp.	Crop tree	Yes	Very high
Olea europaea	Crop tree	Yes	Very high
Prunus persica	Crop tree	Yes	High
Nerium oleander	Ornamental shrub	Yes	High
Salva rosmarinus	Ornamental shrub	Yes	High
Strelitzia reginae	Ornamental shrub	Yes	Moderate
Persea americana	Fruit tree	Yes	Moderate
Other native tropical forest species (multiple genera)	Forest trees	Variable	Sentinel value

5.3.2 Detection of *X. fastidiosa* in the samples

5.3.2.1. Results obtained in the botanic gardens of America

All submitted samples were successfully received and processed within the BeXyl diagnostic workflow. Results indicate that DNA belonging to *X. fastidiosa* has been detected in a total of 18 samples from a subset of samples in four botanical gardens with frequencies ranging from 9.1 to 19.1% of positives (Table 5.4).

A total of 18 plant samples tested positive for *X. fastidiosa* and belonged to 10 plant species that included *Catharanthus roseus, Coffea arabica, Cyperus* sp., *Cyperus alternifolius, Citrus* sp., *Hibiscus × rosa-sinensis, Olea europaea, Rhipidocladum racemiflorum, Salvia Rosmarinus, Streliztia reginae* (Table 5.5).

Additionally, indicative signals for subsp. *fastidiosa* and subsp. *pauca* and subsp. *sandyi* in some cases were obtained when performing the test of Dupas et al. 2019 by qPCR or dPCR, although it was not possible to characterize the subspecies in all the *X. fastidiosa* positive samples by this approach, due to the lower sensitivity of the Dupas protocol as compared to Harper qPCR protocol (Table 5.4 and Table 5.5).

Phylogenetic analysis of the allelic variants identified for each locus of the MLSTr panel revealed the presence of previously described alleles at certain genes, including *leuA* and *gltT*. In addition, for these two loci and the remaining five genes of the panel, multiple allelic profiles were detected that did not match any alleles currently reported in reference databases. These results indicate the occurrence of novel allelic variants across several MLSTr loci (Figure 5.2).

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Table 5.4. Summary of preliminary diagnostic outcomes by garden code (interim).

Garden code	*Xylella* detected	Detection frequency	subspecies signal[a]	MLST Alleles[b]		Sequence type (ST)[c]
				Known	New	
J1	Yes	9.1%	*sandyi*	Yes	Yes	New
J2	No	0%	–	–	–	New
J3	Yes	12.5%	*fastidiosa/sandyi*	Yes	Yes	New, 72 & 76
J4	Yes	19.1%	*pauca*	Yes	Yes	New, 74
J5	No	0%	–	–	–	New
J6	Yes	15.0%	*fastidiosa*	Yes	Yes	New, 1,2,3 & 4

[a] Indicative subspecies signals are based on qPCR of Dupas and sequencing of some amplified alleles from the MLST panel.
[b] The presence on known or new alleles is reported when compared to the reference sequences of each allele on the MLST panel from the pubMLST database.
[c] The closest ST is indicated when 6 or 7 alleles were amplified from the MLST panel.

Table 5.5. Plant species infected by *X. fastidiosa* in the botanical gardens

Host plant	*X. fastidiosa* subspecies[a]
Catharanthus roseus	*fastidiosa*
***Citrus* sp.**	NA
***Citrus* sp.**	NA
Coffea arabica	*pauca*
Coffea arabica	*pauca*
Coffea arabica	*pauca*
Coffea arabica	*pauca*
Coffea arabica	NA
***Cyperus* sp.**	*pauca**
Cyperus alternifolius	*fastidiosa/sandyi**
Hibiscus × rosa-sinensis	*fastidiosa*
Hibiscus × rosa-sinensis	NA
Olea europaea	NA
Rhipidocladum racemiflorum	NA
Salvia rosmarinus (=Rosmarinus officinalis)	*pauca**
Salvia rosmarinus(=Rosmarinus officinalis)	*fastidiosa/sandyi**
Salvia rosmarinus(=Rosmarinus officinalis)	*fastidiosa/sandyi*
Streliztia reginae	NA

[a] Indicative subspecies signals are based on qPCR of Dupas, or (*) by sequencing some amplified alleles from the MLST panel. NA= No amplification signal by both protocols.

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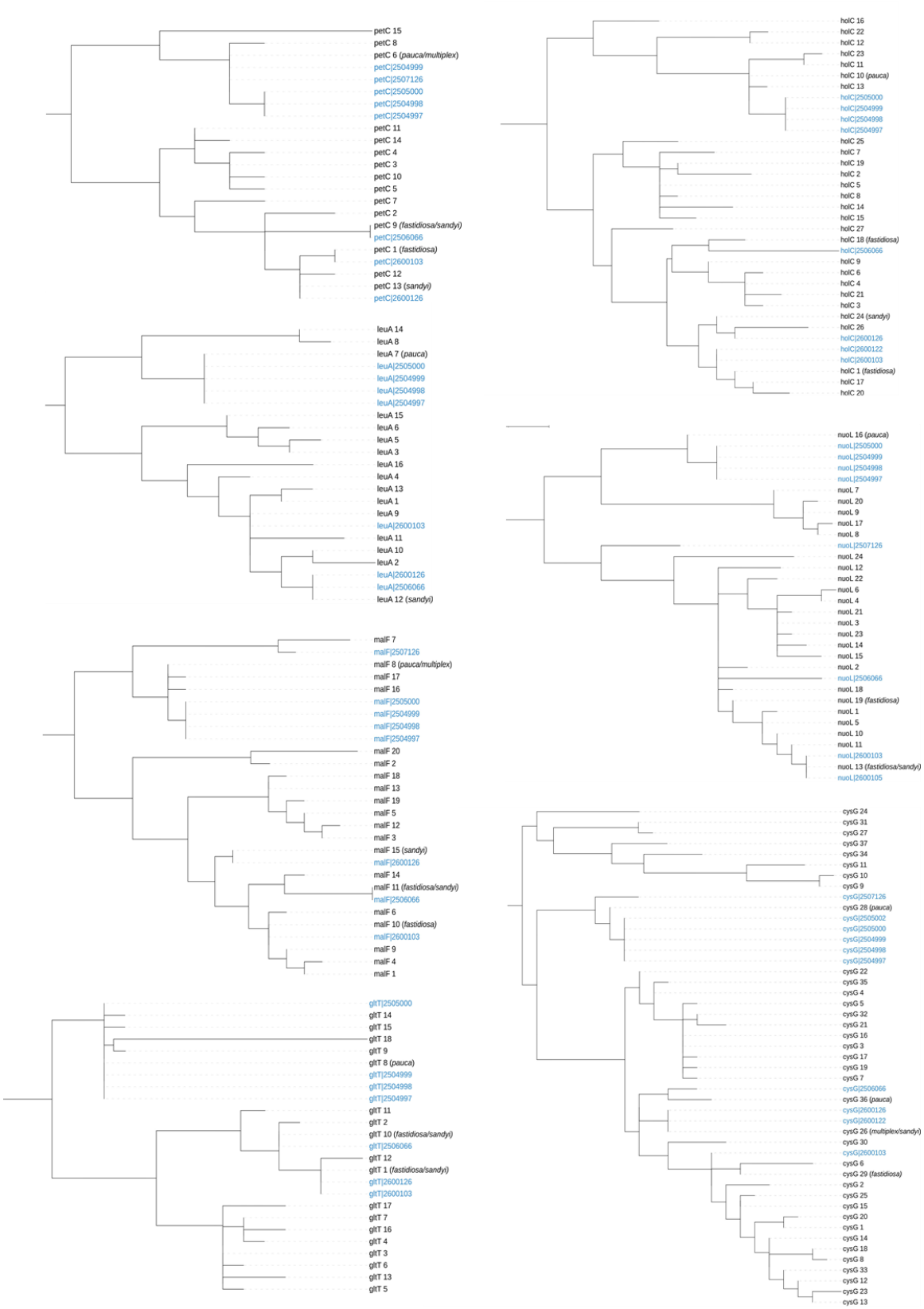


Figure 5.2. ML phylogenetic trees obtained for each gen of the MLST panel including the alleles sequenced from the samples positive for *X. fastidiosa* (blue color) and those alleles present in the pubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa).

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Among the samples for which ST assignment was possible, none matched previously described STs. Instead, all allelic combinations corresponded to novel ST variants not represented in the pubMLST reference dataset. This indicates that the characterized isolates harbour unique multilocus allelic profiles, expanding the known genetic diversity of *X. fastidiosa*.

Phylogenetically, the newly identified STs clustered within the major subspecies lineages rather than forming an external or basal group, confirming their affiliation with established *X. fastidiosa* diversity. The novel variants were distributed across different clades, within *fastidiosa* and *pauca*-related clusters. This pattern suggests that the detected diversity reflects diversification within recognised subspecies rather than the emergence of a deeply divergent lineage.

Branch lengths and topology indicate that the new STs are separated from their closest reference types by small but distinct genetic distances, consistent with SNP-level variation across multiple loci. The absence of identical matches with known STs, combined with their stable phylogenetic placement, supports the interpretation of previously undescribed multilocus genotypes circulating in the analysed populations.

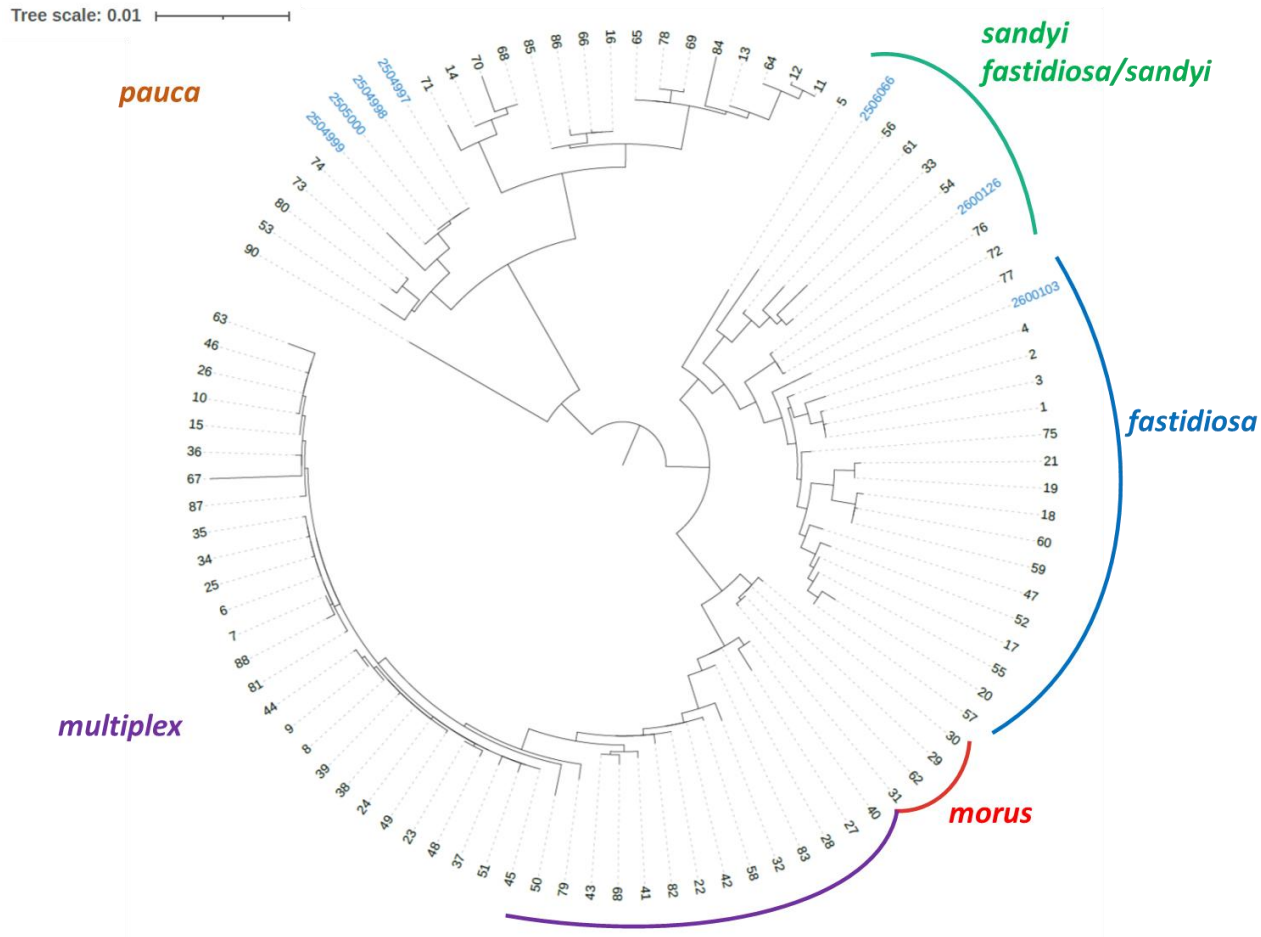


Figure 5.2. ML phylogenetic tree obtained with six of seven concatenated genes from the MLST panel from the samples positive for *X. fastidiosa* (blue color) in table 5.5 and the 90 Sequence types (ST) currently described in the pubMLST database (https://pubmlst.org/organisms/xylella-fastidiosa).

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5.3.2.2. Results obtained in the botanic garden of Corsica

Three of the 23 plant samples were tested positive for *X. fastidiosa* subsp. *multiplex*: A *Polygala* sp. (Cq 26.65) and a *Polygala myrtifolia* (Cq 23.02) had high titers, whereas a *Grevillea* sp. was just below our detection threshold (Cq 33.47) and will be resampled in spring (Table 5.2). Both *Polygala* plants were in an advanced state of decline, with many yellowing leaves in the process of drying out. Dupas' tetraplex qPCR confirmed that all three plants were infected with strains of the subsp. *multiplex*. MLST is under way to determine whether the strains present in the botanical garden belong to ST6 or ST7, which are common on the island. Official monitoring is ongoing in this site.

6. CONCLUSIONS

6.1 Pathogenicity experiments under controlled conditions

The pathogenicity experiments conducted under controlled conditions provided robust comparative evidence on host susceptibility, bacterial colonization dynamics, and strain-dependent variability in the infection process. The results confirmed that host responses to *X. fastidiosa* are highly heterogeneous, ranging from successful systemic colonization and symptom development to restricted bacterial persistence or progressive loss of detection over time.

The observation that bacterial detection declined markedly in some host species, while remaining stable in others, highlights the critical importance of host physiological compatibility and suggests that infection success is not solely determined by inoculation but by subsequent host-pathogen interactions. The limited correspondence between bacterial presence and visible symptom expression observed across several experiments further reinforces the complexity of disease development and cautions against relying exclusively on symptom-based assessments for identifying potentially susceptible host plants.

Strain-dependent differences in colonization efficiency and persistence indicate that genetic variability within *X. fastidiosa* contributes significantly to pathogenic behaviour. These findings emphasise the need to incorporate strain diversity into experimental designs, resistance screening programmes, and epidemiological interpretation.

Overall, the controlled experiments provided an essential mechanistic framework for interpreting field observations, supporting host-risk categorisation, and informing the refinement of predictive models of pathogen establishment and disease progression.

6.2 Surveys in participating countries

The surveys conducted across participating countries demonstrated the value of coordinated, multi-regional surveillance for capturing the spatial and biological variability of *X. fastidiosa* outbreaks. The results illustrate that pathogen occurrence, host associations, and

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symptom expression vary considerably across geographic regions, climatic contexts, and production systems.

Differences observed among regions in prevalence, host range, and genotype composition highlight the influence of local ecological and management factors. These patterns underscore the importance of sustained surveillance efforts and harmonised methodologies to ensure comparability of results and early recognition of epidemiological shifts.

Detection of *X. fastidiosa* in diverse hosts, including both symptomatic and asymptomatic plants, reinforces the necessity of systematic molecular diagnostics to complement visual inspections. The identification of novel host associations and previously undescribed sequence types (STs) confirms that the genetic and epidemiological landscape of *X. fastidiosa* is incompletely characterized and remains dynamic.

Collectively, the surveys conducted within the BeXyl project have contributed directly to improving outbreak characterisation, supporting risk assessment updates, and informed region-specific surveillance and containment strategies.

6.3 Surveys in sentinel plots

The sentinel plot network established within the BeXyl project has demonstrated its utility as both a proactive surveillance system and research tool. By monitoring selected plant species under standardized field conditions, these sentinel systems can enable early detection of pathogen introduction, documentation of host susceptibility, and evaluation of infection dynamics before large-scale outbreak development.

The results confirm that sentinel plots can reveal host–pathogen interactions not readily observable in conventional production systems, particularly in cases of asymptomatic infections or low pathogen prevalence occur. These systems additionally provided a structured platform for longitudinal observations, enabling systematic assessment of bacterial persistence, symptom progression, and environmental influences over extended time periods.

More importantly, sentinel plots effectively facilitated integration between experimental plant pathology studies and field surveillance, bridging controlled and real-world contexts. Their demonstrated role in validating host-risk hypotheses and supporting surveillance prioritisation frameworks can represent a significant methodological advancement for this field.

Recognising their proven value beyond the project timeframe, targeted efforts will be made to maintain selected sentinel plots to support long-term monitoring and ensure continued generation of epidemiologically relevant data for surveillance programs.

Overall, the sentinel network developed in this project has demonstrated its capacity to strengthen early-warning systems, enhance epidemiological understanding, and support adaptive surveillance strategies in response to evolving plant health risks.

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6.4 Surveys in botanical gardens and arboreta

The results obtained within this activity have reinforced the role of botanic gardens and arboreta as strategic infrastructure for phytopathological research and surveillance, particularly for challenging pathogens such as *X. fastidiosa*, characterised by broad host range, frequent asymptomatic infections, and complex epidemiology.

Botanic gardens provide a unique concentration of botanical and horticultural expertise, including accurate taxonomic identification, curated accession records, and long-term plant management. These capacities are essential for reliable host–pathogen association studies, where host misidentification can compromise epidemiological interpretation and risk assessment.

Beyond botanical knowledge, participating gardens demonstrated their ability to function as active surveillance nodes, contributing to symptom recognition, systematic sampling, traceability, and collaboration with diagnostic laboratories and plant health authorities. The preliminary detection of *X. fastidiosa* DNA further highlights the sensitivity of these systems and their significant value as early-warning observatories.

Importantly, surveillance activities conducted in botanic gardens also contributed to the detection of previously undescribed genetic variants of *X. fastidiosa*. The identification of novel allelic profiles and sequence types has contributed to expand the current understanding of pathogen diversity, improving resolution of population structure, and supporting more accurate tracing of introduction pathways and epidemiological links. Early recognition of new genetic variants is particularly critical for risk assessment and management, as genetic differences may influence host range, colonization behaviour, and adaptation to environmental conditions.

Integration of botanic gardens into sentinel surveillance networks can generate clear added value for EU and Mediterranean plant health preparedness. Scientifically, data from diverse plant collections can improve the understanding of *X. fastidiosa* host range, tolerance, and symptom expression. From a societal perspective, garden involvement effectively enhances awareness, transparency, and public engagement with plant health challenges.

Supporting materials developed within the project, including harmonised protocols, operational guidance, and communication tools, facilitated consistent implementation across all participating sites and demonstrated strong transferability to other stakeholder groups. This robust framework positions botanic gardens not only as valuable research partners but also essential long-term contributors to surveillance and science-based plant health strategies.

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7. PUBLICATIONS DERIVED FROM THIS DELIVERABLE

- Amoia, S. S., Saponari, M., Ligorio, A., Saldarelli, P., Boscia, D., and Giampetruzzi, A. 2025. Complete genome assembly of *Xylella fastidiosa* subsp. *fastidiosa* (ST1) strains "BA_GR31" and "BA_AL21" associated with a new outbreak in Apulia, Italy. J. Plant Pathol. 107:1549–1552.
- Briand, M., Jacques, M. A., and Dittmer, J. 2025. The hidden life of *Xylella*: mining the NCBI Sequence Read Archive reveals potential new species, host plants, and infected areas for this elusive bacterial plant pathogen. Appl. Environ. Microbiol. 91:e00913-25.
- Buisac, A., Gascon, B., Montesinos, E., Montesinos, L. 2026. Aggressiveness of subspecies and STs of *Xylella fastidiosa* and plant response in *Nicotiana benthamiana*. Phytopathology, doi: 10.1094/PHYTO-11-25-0366-R. Epub ahead of print.
- Fernández, F.D., Arias-Giraldo, L.F., Tolocka, P.A., Guzmán, F. A., Olivares, C., Roca, M.E., Otero, M.L., Conci, L.R., Landa, B.B., Haelterman, R.M. 2025. Phylogenomic analysis of *Xylella fastidiosa* subsp. *pauca* strains from olive and almond trees in Argentina. *Eur J Plant Pathol* 171:593–602.
- Montilon, V., Potere, O., Susca, L., Mannerucci, F., Nigro, F., Pollastro, S., Loconsole, G., Palmisano, F., Zaza, C., Cantatore, M., and Faretra, F. 2024. Isolation and molecular characterization of a *Xylella fastidiosa* subsp. *multiplex* strain from almond (*Prunus dulcis*) in Apulia, southern Italy. Phytopathol. Mediterr. 63:423–429.

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8. REFERENCES

Amoia, S. S., Saponari, M., Ligorio, A., Saldarelli, P., Boscia, D., and Giampetruzzi, A. 2025. Complete genome assembly of *Xylella fastidiosa* subsp. *fastidiosa* (ST1) strains "BA_GR31" and "BA_AL21" associated with a new outbreak in Apulia, Italy. J. Plant Pathol. 107:1549–1552.

Baró, A., Montesinos, L., Badosa, E., and Montesinos, E. 2021. Aggressiveness of Spanish isolates of *Xylella fastidiosa* to almond plants of different cultivars under greenhouse conditions. Phytopathology 111:1994–2001.

Baró, A., Saldarelli, P., Saponari, M., Montesinos, E., and Montesinos, L. 2022. *Nicotiana benthamiana* as a model plant host for *Xylella fastidiosa*: control of infections by transient expression and endotherapy with a bifunctional peptide. Front. Plant Sci. 13:1061463.

Bonants, P., Griekspoor, Y., Houwers, I., Krijger, M., van der Zouwen, P., van der Lee, T. A. J., and van del Wolf, J. 2019. Development and evaluation of a triplex TaqMan assay and next-generation sequence analysis for improved detection of *Xylella* in plant material. Plant Dis. 103:645–655.

Boscia, D., and Altamura, G. 2021. Searching for olives displaying resistance traits to *Xylella fastidiosa* subsp. *pauca* ST53: experimental evidence and challenges. In: Proceedings of the 3rd European Conference on *Xylella fastidiosa* and XF-ACTORS final meeting, 26–30 April 2021. Available at: https://dx.doi.org/10.5281/ZENODO.4903060

Bouras, G., Houtak, G., Wick, R. R., Mallawaarachchi, V., Roach, M. J., Papudeshi, B., Judd, L. M., Sheppard, A. E., Edwards, R. A., and Vreugde, S. 2024. Hybracter: enabling scalable, automated, complete and accurate bacterial genome assemblies. Microb. Genom. 10:001244.

Briand, M., Jacques, M. A., and Dittmer, J. 2025. The hidden life of *Xylella*: mining the NCBI Sequence Read Archive reveals potential new species, host plants, and infected areas for this elusive bacterial plant pathogen. Appl. Environ. Microbiol. 91:e00913-25.

Campbell, C. L., and Madden, L. V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, New York, NY, USA.

Cesbron, S., Dupas, E., Beaurepère, Q., Briand, M., Montes-Borrego, M., Velasco-Amo, M. P., Landa, B. B., and Jacques, M. A. 2020. Development of a nested-multilocus sequence typing approach for a highly sensitive and specific identification of *Xylella fastidiosa* subspecies directly from plant samples. Agronomy 10:1099.

Coletta-Filho, H. D., Francisco, C. S., Lopes, J. R. S., Muller, C., Almeida, R. P. P. 2017. Homologous Recombination and *Xylella fastidiosa* host-pathogen associations in south america. Phytopathology 107:305-312.

Cornara, D., Boscia, D., D'Attoma, G., Digiaro, M., Ligorio, A. M., Loconsole, G., Minutillo, S. A., Montilon, V., Palmisano, F., Ragone, G., Silletti, M. R., Verrastro, V., Zaza, C., and Saponari, M. 2024. An integrated strategy for pathogen surveillance unveiled *Xylella fastidiosa* ST1 outbreak in hidden agricultural compartments in the Apulia region (Southern Italy). Eur. J. Plant Pathol. 171:277–285.

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Davis, M. J. 1980. Isolation media for the Pierce's disease bacterium. Phytopathology 70:425.

Davis, M. J., French, W. J., and Schaad, N. W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6:309–314.

Dupas, E., Legendre, B., Olivier, V., Poliakoff, F., Manceau, C., and Cunty, A. 2019. Comparison of real-time PCR and droplet digital PCR for the detection of *Xylella fastidiosa* in plants. J. Microbiol. Methods 162:86–95.

EPPO. 2023. PM 7/24 (5) *Xylella fastidiosa*. EPPO Bull. 53:205–276.

Francis, M., Lin, H., Rosa, J. C.-L., Doddapaneni, H., and Civerolo, E. L. 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. Eur. J. Plant Pathol. 115:203–213.

Guilhabert, M. R., and Kirkpatrick, B. C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. Mol. Plant Microbe Interact. 18:856–868.

Harper, S. J., Ward, L. I., and Clover, G. R. G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology 100:1282–1288.

Hodgetts, J., Glover, R., Cole, J., Hall, J., and Boonham, N. 2021. Genomics informed design of a suite of real-time PCR assays for the specific detection of each *Xylella fastidiosa* subspecies. J. Appl. Microbiol. 131:855–872.

Li, W., Teixeira, D. C., Hartung, J. S., Huang, Q., Duan, Y., Zhou, L., Chen, J., Lin, H., Lopes, S., Ayres, A. J., and Levy, L. 2013. Development and systematic validation of qPCR assays for rapid and reliable differentiation of *Xylella fastidiosa* strains causing citrus variegated chlorosis. J. Microbiol. Methods 92:79–89.

Lopes, S. A., and Torres, S. C. Z. 2006. An effective and low-cost culture medium for isolation and growth of *Xylella fastidiosa* from citrus and coffee plants. Curr. Microbiol. 53:467–469.

Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, R. M. V. B. C., and Stall, R. E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456–461.

Montero-Astúa, M., Saborío-R., G., Chacón-Díaz, C., Garita, L., Villalobos, W., Moreira, L., Hartung, J. S., and Rivera, C. 2008. First report of *Xylella fastidiosa* in avocado in Costa Rica. Plant Dis. 92:175.

Montilon, V., Potere, O., Susca, L., Mannerucci, F., Nigro, F., Pollastro, S., Loconsole, G., Palmisano, F., Zaza, C., Cantatore, M., and Faretra, F. 2024. Isolation and molecular characterization of a *Xylella fastidiosa* subsp. *multiplex* strain from almond (*Prunus dulcis*) in Apulia, southern Italy. Phytopathol. Mediterr. 63:423–429.

Oliver, J. E., Cobine, P. A., and De La Fuente, L. 2015a. *Xylella fastidiosa* isolates from both subsp. *multiplex* and *fastidiosa* cause disease on southern highbush blueberry (*Vaccinium* sp.) under greenhouse conditions. Phytopathology 105:855–862.

Ouyang, P., Arif, M., Fletcher, J., Melcher, U., and Corona, F. M. O. 2013. Enhanced reliability and accuracy for field deployable bioforensic detection and discrimination of *Xylella*

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fastidiosa subsp. *pauca*, causal agent of citrus variegated chlorosis, using Razor Ex technology and TaqMan quantitative PCR. PLoS One 8:e81647.

Román-Écija, M., Navas-Cortés, J. A., Velasco-Amo, M. P., Arias-Giraldo, L. F., Gomez, L. M., Fuente, L. D. L., and Landa, B. B. 2023. Two Xylella fastidiosa subsp. multiplex strains isolated from almond in Spain differ in plasmid content and virulence traits. Phytopathology 113:960-974.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.

Shantharaj, D., Román-Écija, M., Velasco-Amo, M. P., Navas-Cortés, J. A., Landa, B. B., and De La Fuente, L. 2024. European *Xylella fastidiosa* strains can cause symptoms in blueberry. Plant Dis. 108:2658–2662.

Tolocka, P. A., Mattio, M. F., Paccioretti M. A., Otero, M. L., Roca, M. E., Guzmán, F.A., Haelterman, R. M. 2017a. *Xylella fastidiosa* subsp. *pauca* ST69 in olive in Argentina. Journal of Plant Pathology, 99:799-818.

Tolocka, P. A., Mattio, M.F., Otero, M. L., Paccioretti, M. D., Roca, M. E., Guzmán F.A., Haelterman, R.M. 2017b. Nueva secuencia tipo de *Xylella fastidiosa* subsp. *pauca* ST78, obtenida de un aislamiento de almendro de Argentina. IV Congreso Argentino de Fitopatología, Pp 177.

Wells, J. M., Raju, B. C., Nyland, G., and Lowe, S. K. 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. Appl. Environ. Microbiol. 42:357–363.

Yuan, X., Morano, L., Bromley, R., Spring-Pearson, S., Stouthamer, R., and Nunney, L. 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. Phytopathology 100:601–611.

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