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SEASONALITY, LIFE CYCLE AND VECTORIAL CAPACITY OF *XYLELLA FASTIDIOSA* INSECT VECTORS IN THE MAIN CROPS OF THE BALEARIC ISLANDS

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Doctoral Programme in Biomedical and Evolutionary Biotechnology

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“Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad”

-Albert Einstein-

To my grandparents, parents and brother

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"Llegar juntos es el principio; mantenerse juntos es el progreso; trabajar juntos es el éxito"

-Henry Ford-

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List of abbreviations

AAP: Acquisition Access Period

AIC: Akaike Information Criterion

BSL2: Biosecurity Laboratory Level

CoDIRO: *Complesso del Disseccamento Rapido dell'Olivo*

Ct: Cycle threshold

CVC: Citrus Variegated Chlorosis

DFS: Diffusible Signal Factor

EFSA: European Food Safety Authority

EPPO: European and Mediterranean Plant Protection Organization

EU: European Union

GFP: Green Fluorescent Protein

GLM: General Lineal Model

GLMM: Generalized Mixed Linear Models

GS: Growth Stage

HWT: Hot Water Treatment

IAP: Inoculation Access Period

ISPP-CTPPB: Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria

LAMP: Loop-mediated isothermal amplification

LS: Leaf Scorch

MENA: Middle East and North Africa

MLST: Multilocus sequence typing

N1: Nymphal stage 1

N2: Nymphal stage 2

N3: Nymphal stage 3

N4: Nymphal stage 4

N5: Nymphal stage 5

NAC: N-acetylcysteine

OQDS: Olive Quick Decline Syndrome

PACA: *Provence Alpes Cotes d'Azur región (France)*

PD: Pierce's disease

PCR: Polymerase Chain Reaction

PSU: Primary Sampling Unit

PPH: Preference-Performance Hypothesis

qPCR: quantitative Polymerase Chain Reaction

RH: Relative Humidity

SEM: Scanning Electron Microscopy

SSU: Secondary Sampling Unit

ST: Sequence Type

UIB: University of the Balearic Islands

US: United States

USA: United States of America

Abstract

Xylella fastidiosa (Wells *et al.* 1987) is a gram-negative Gammaproteobacterium pathogen of plants and limited to the xylem conduits. The bacterium is capable to infect more than 600 different plant species causing several types of diseases (i.e., Pierce's disease, citrus variegated chlorosis, leaf scorch). It is transmitted by xylem sap feeder insects from the order Hemiptera and suborder Cicadomorpha. In Europe, the major vectors belong to the family Aphrophoridae. *Xylella fastidiosa* was detected in 2013 in Italy and in 2016 in the Balearic Islands. The general objectives of the thesis are i) macrocosm and microcosm studies of the vectors in the major agrosystems of the Balearic Islands; ii) Prevalence of *X. fastidiosa* in the vectors and vectorial capacity of potential vectors; iii) Test a cultural method against vectors.

Nine organic farms (three olive, three vineyards and three almond) were selected in Majorca for conducting annual monitoring. The islands of Minorca, Ibiza and Formentera were sampled twice a year (summer and autumn). Insects were collected biweekly in each plot in Majorca by using a sweep net for adults and a wood frame of 0.25 m^2 for nymphs. In the microcosm study, 50 cages containing one male and one female of *Philaenus spumarius*, one plant species per cage (*Rosmarinus officinalis, Mentha x piperita, Ocimum basilicum, Pistacia lentiscus* or *Lavandula dentata*) and substrate for oviposition (straw) were placed at field conditions. Insects were placed inside the cages from September to November, then cages were checked to detect egg batches and monitor nymphs' development. From the adults collected from the field, the prevalence of *X. fastidiosa* was determined by qPCR. In a first approach, field collected insects were caged for the vector competence studies with *X. fastidiosa* free plants of *Medicago sativa* for 96 h. Then, insects were analysed by qPCR. Samples taken from plants were analysed 15, 30, 45 and 60d post inoculation. Moreover, in a second approach, nymphs were collected from the field, reared until adults and put in contact with vine and almond *X. fastidiosa* infected trees for 96 h acquisition access period. Then, adults were in contact with alfalfa plants for 96 h inoculation access period. Both adults and alfalfa plants were analysed by qPCR. Finally,

for the mechanical control method test, the ground cover vegetation was mowed or tilled from olive and vineyard farms to assess the control of nymphs of *X. fastidiosa* vectors.

Philaenus spumarius and *Neophilaenus campestris* (Aphrophoridae) were recorded in the Balearic Islands. Results indicated that nymphs were present from early-May to early-June. *Philaenus spumarius* nymphs were more abundant in the cover vegetation of olive crops, followed by vineyard and almond ones, while *N. campestris* was more abundant in olive and almond.

The highest abundance of *P. spumarius* adults was recorded in May and October in the cover vegetation. Presence of adults increased in trees in June, while presence in the border vegetation of the crop increased in August and decreased around October. In the case of *N. campestris*, the highest abundance of adults was detected in the cover plants in May and November, however its presence in trees and border vegetation can be considered negligible.

The prevalence of *X. fastidiosa* from the insects collected (1059 insects analysed) was 22.8 %; *P. spumarius* showed a prevalence of 23.6 % and *N. campestris* of 20.8 %. The island with the highest prevalence was Majorca reaching the 24 %, followed by Menorca (21.5 %) and Ibiza (21 %), Formentera remained free of *X. fastidiosa*. *Xylella fastidiosa* subsp. *fastidiosa* and *multiplex* were detected in insects form Majorca, *X. fastidiosa* subsp. *multiplex* was determined in Minorca and *X. fastidiosa* subsp. *pauca* was identified in Ibiza.

The first approach of transmission test showed a 16.3 % of positive insects (N = 264). Inoculation to *M. sativa* was confirmed since the plants were positive by qPCR 15, 30, 45 and 60 days after inoculation. In the second approach, *P. spumarius* acquired the bacteria from almond (34.4 % of insects) and from vineyard (87.7 %). Only insects that acquired the bacterium from almond, transmitted *X. fastidiosa* to alfalfa plants.

Results on the mechanical control test showed that mowing and tilling affect nymphs of X. *fastidiosa* vectors by reducing its density significantly below that of control zones.

Resum

Xylella fastidiosa (Wells *et al*. 1987) és un fitopatogen gramnegatiu que pertany als Gammaproteobacteris i només envaeix els conductes del xilema. El bacteri és capaç d'infectar més de 600 espècies vegetals diferents causant diversos tipus de malalties (com per exemple, la malaltia de Pierce, la clorosi variegada dels cítrics o el Síndrome del decaïment sobtat de l'olivera). Es transmet per insectes de l'ordre dels hemípters i del subordre dels cicadomorfs que s'alimenten del fluid xilemàtic. A Europa, els principals vectors de propagació pertanyen a la família Aphrophoridae. *Xylella fastidiosa* es va detectar a Europa l'any 2013 i al 2016 a les Illes Balears. Els objectius generals de la tesi són: i) Estudi del macrocosmos i microcosmos dels vectors en els agrosistemes més rellevants de les Illes Balears; ii) Prevalença de *X. fastidiosa* als vectors i capacitat vectorial dels potencials vectors; iii) Assaig d'un mètode de control cultural enfront els vectors.

S'han seleccionat a Mallorca nou explotacions ecològiques (tres d'olivera, tres de vinya i tres d'ametllers) per fer un seguiment anual. Les illes de Menorca, Eivissa i Formentera es van mostrejar dos cops l'any (estiu i tardor). Els insectes es van recollir quinzenalment a cada parcel·la de Mallorca, els adults mitjançant màniga entomològica i les nimfes amb un marc de fusta de 0,25 m^2. A l'estudi del microcosmos, es van col·locar al camp 50 gàbies que contenien un mascle i una femella de *P. spumarius*, una planta per gàbia (*Rosmarinus officinalis*, *Mentha x piperita*, *Ocimum basilicum*, *Pistacia lentiscus* o *Lavandula dentata*) i substrat per a l'oviposició (palla). Els insectes es van col·locar dins de les gàbies de setembre a novembre, després es van comprovar per detectar postes d'ous i controlar el desenvolupament de les nimfes. A partir dels vectors adults recollits al camp, es va determinar la prevalença de *X. fastidiosa* mitjançant qPCR. Per als estudis de competència vectorial, es van realitzar dos tipus d'assajos. Per al primer tipus, els insectes adults recollits al camp es van posar en contacte amb *Medicago sativa* lliures de *X. fastidiosa* durant 96 h. A continuació, es van analitzar els insectes mitjançant qPCR. Les mostres de plantes es van analitzar els 15, 30, 45 i 60 dies després de la inoculació. Per al segon tipus, es van recollir nimfes del camp, es van criar fins a adults i es van posar en

contacte amb arbres de vinya i ametller infectats de *X. fastidiosa* durant 96 h. Aleshores, els adults es van posar en contacte amb plantes d'alfals durant un període d'inoculació de 96 h. Tant les plantes d'alfals com els vectors es van analitzar mitjançant qPCR. Finalment, per a l'assaig del mètode de control mecànic, es va segar o llaurar la vegetació de la coberta del sòl de granges d'olivera i vinya per avaluar el control de les nimfes dels vectors *X. fastidiosa*.

A les Illes Balears s'han detectat els insectes vectors *P. spumarius* i *N. campestris* (Aphrophoridae). Les nimfes estaven presents des de principis de maig fins a principis de juny. Les nimfes de *P. spumarius* eren més abundants a la vegetació de coberta dels cultius d'olivera, seguides de vinya i ametller, mentre que *N.campestris* era més abundant en olivera i ametller.

La major abundància d'adults de *P. spumarius* es va registrar a maig i octubre a la vegetació de coberta. La presència d'adults va augmentar als arbres al juny, mentre que la presència a la vegetació de vora del cultiu va augmentar a l'agost i va disminuir cap a l'octubre. En el cas de *N. campestris*, la major abundància d'adults es va detectar a les plantes de coberta durant els mesos de maig i novembre, però la seva presència en arbres i vegetació de vorera es pot considerar insignificant.

La prevalença de *X. fastidiosa* dels insectes recollits a camp va ser del 22.8 %, *P. spumarius* va mostrar una prevalença del 23.6 % i *N. campestris* del 20.8 %. L'illa amb major prevalença va ser Mallorca arribant al 24 %, seguida de Menorca (21.5 %) i Eivissa (21 %), Formentera es va mantenir lliure de *X. fastidiosa*. *Xylella fastidiosa* subsp. *fastidiosa* i *multiplex* es va detectar a insectes capturats a Mallorca, *X. fastidiosa* subsp. *multiplex* a Menorca i *X. fastidiosa* subsp. *pauca* a Eivissa.

El primer tipus de prova de transmissió va mostrar un 16.3 % d'insectes positius (N = 264). Es va confirmar la inoculació a *M. sativa* ja que les plantes eren positives per qPCR 15, 30, 45 i 60 dies després de la inoculació. En el segon tipus de prova, *P. spumarius* va adquirir el bacteri de l'ametller (34.4 % dels insectes) i de la vinya (87.7 %), i, només d'insectes d'ametller, es va confirmar la inoculació a plantes d'alfals.

Els resultats de la prova de control mecànic van mostrar que la sega i el conreu afecten a les nimfes de vectors de *X. fastidiosa* reduint la seva densitat significativament a les zones de control.

Resumen

Xylella fastidiosa (Wells et al. 1987) es una Gammaproteobacteria gramnegativa patógena que invade los conductos del xilema de las plantas. La bacteria es capaz de infectar a más de 600 especies de plantas diferentes y causar varios tipos de enfermedades (p.ej. la enfermedad de Pierce, la clorosis variegada de los cítricos y el síndrome del declive rápido del olivo). Se transmite mediante insectos que se alimentan de la savia del xilema perteneciente al orden Hemiptera y el suborden Cicadomorpha. En Europa, los principales vectores de propagación pertenecen a la familia Aphrophoridae. *Xylella fastidiosa* se detectó en 2013 en Europa y en 2016 en las Islas Baleares. Los objetivos generales de la tesis son: i) estudio del macrocosmos y microcosmos en los agrosistemas más relevantes de las Islas Baleares; ii) Prevalencia de *X. fastidiosa* en los vectores y capacidad vectorial de los potenciales vectores; iii) Ensayo de un método de control cultural frente a los vectores.

Se seleccionaron nueve fincas ecológicas (tres de olivo, tres de viña y tres de almendro) en Mallorca para realizar un seguimiento anual. Las islas de Menorca, Ibiza y Formentera se muestrearon dos veces al año (verano y otoño). Los insectos se recolectaron quincenalmente en cada parcela de Mallorca utilizando una manga entomológica para capturar los adultos y un marco de madera de 0,25 m^2 para ninfas. En el estudio de microcosmos, se colocaron en campo 50 jaulas que contenían un macho y una hembra de *P. spumarius*, una planta por jaula (*Rosmarinus officinalis*, *Mentha x piperita*, *Ocimum basilicum*, *Pistacia lentiscus* o *Lavandula dentata*) y sustrato para la oviposición (paja). Los insectos se colocaron dentro de las jaulas de septiembre a noviembre, luego se revisaron para detectar puestas de huevos y monitorear el desarrollo de las ninfas. De los adultos recolectados en el campo, se determinó la prevalencia de *X. fastidiosa* por qPCR. Para los estudios de competencia vectorial, se llevaron a cabo dos tipos de ensayos. Para el primer tipo, los insectos recolectados en el campo se pusieron en contacto con plantas de *Medicago sativa* libres de *X. fastidiosa* de durante 96 h. Luego, los insectos fueron analizados por qPCR. Las muestras tomadas de las plantas se analizaron 15, 30, 45 y 60 días después de la inoculación. Para el segundo tipo, las ninfas se recolectaron del campo, se

criaron hasta adultos y se pusieron en contacto con árboles de almendro y vid infectados con *X. fastidiosa* durante un periodo de adquisición de 96 h. Pasado este tiempo, los adultos estuvieron en contacto con las plantas de alfalfa durante un período de inoculación de 96 h. Tanto los adultos como las plantas de alfalfa se analizaron mediante qPCR. Finalmente, para la prueba del método de control mecánico, se segó o labró la vegetación de la cubierta vegetal de fincas de olivos y viñedos para evaluar el control de las ninfas de los vectores *X. fastidiosa*.

En las Islas Baleares se han encontrado dos especies de vectores, *P. spumarius* y *N. campestris* (Aphrophoridae). Las ninfas estuvieron presentes desde principios de mayo hasta principios de junio. Las ninfas de *P. spumarius* fueron más abundantes en la cubierta vegetal de los cultivos de olivo, seguidas de las de vid y almendro, mientras que *N. campestris* fue más abundante en el olivo y el almendro.

La mayor abundancia de adultos de *P. spumarius* se registró en mayo y octubre en la cubierta vegetal. La presencia de adultos aumentó en los árboles en junio, mientras que la presencia en la vegetación de borde del cultivo fue mayor en agosto y disminuyó en octubre. En el caso de *N. campestris*, la mayor abundancia de adultos se detectó en la cubierta en mayo y noviembre, sin embargo, su presencia en árboles y vegetación de borde puede considerarse negligible.

La prevalencia de *X. fastidiosa* en los insectos recolectados fue de 23 %, *P. spumarius* mostró una prevalencia de 23.8 % y *N. campestris* de 20.8 %. La isla con mayor prevalencia fue Mallorca con un 24 %, seguida de Menorca (21.5 %) e Ibiza (21 %), Formentera se mantuvo libre de *X. fastidiosa*. *Xylella fastidiosa* subsp. *fastidiosa* y *multiplex* se detectó en insectos capturados en Mallorca, *X. fastidiosa* subsp. *multiplex* en Menorca y *X. fastidiosa* subsp. *pauca* en Ibiza.

El primer tipo de prueba de transmisión mostró un 16.3 % de insectos positivos (N = 264). Se confirmó la inoculación a *M. sativa* ya que las plantas fueron positivas por qPCR a los 15, 30, 45 y 60 días después de la inoculación. En el segundo tipo, *P. spumarius* adquirió la bacteria del almendro (34.4 % de los insectos) y del viñedo (87.7 %), y, solo de los insectos del almendro, se confirmó la inoculación a las plantas de alfalfa.

Los resultados de la prueba de control mecánico mostraron que la siega y la labranza afectan a las ninfas de los vectores *X. fastidiosa* al reducir su densidad de forma significativa a las zonas de control, disminuyéndola.

1. General introduction

1.1. The bacteria *Xyella fastidiosa*

The genus *Xylella* (Gammaproteobacteria: Xanthomonadaceae) has two known species: *X. fastidiosa* (Wells *et al.* 1987) and *X. taiwanensis* (Su *et al.*, 2016). *Xylella fastidiosa* is a pathogen of plants limited to the xylem and capable of infecting more than 600 plant species (EFSA 2015, 2018, 2021, 2022). This species has great number of genotypic and phenotypic diversity, that allows the bacterium to have a wide host range (Schuenzel *et al.*, 2005; Nunney *et al.*, 2013; EFSA PLH 2015a; EFSA 2018). There are six *X. fastidiosa* subspecies described: *fastidiosa*, *multiplex*, *pauca*, *morus*, *tashke* and *sandyi* (Schaad *et al.* 2004; Schuenzel *et al.* 2005; EFSA PLH 2018). But spp. *fastidiosa* and *multiplex* are the only recognized by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) (Bull *et al.*, 2012; EFSA 2021). Moreover, *X. fastidiosa* exhibits a high degree of genetic diversity among strains that can be identified as sequence types (ST) (Schuenzel *et al*., 2005). The ST can be recognised by the multilocus sequence typing (MLST) (Maiden *et al.*, 1998) that characterize *X. fastidiosa* and estimate the recombination rate (Scally *et al*., 2005). The MLST has been crucial to understand the processes involved in the evolution of pathogenicity of *X. fastidiosa* and in identifying new disease-causing strains (Scally *et al.*, 2005; Yuan *et al.*, 2010; Elbeaino *et al.*, 2014; Nunney *et al.*, 2014; Denancé *et al.*, 2017, 2019; EFSA, 2021). The bacteria are associated with important diseases in a wide range of plants, being an emerging important agricultural issue (Redak *et al.*, 2004; EFSA, 2013). Each subspecies and ST have different host range causing diseases such as the Pierce's disease in grapevine (*Vitis vinifera*), citrus variegated chlorosis, leaf scorch (almond, elm, oak, oleander, American sycamore, mulberry and maple), alfalfa dwarf, olive quick decline, plum leaf scald and peach phony disease (Fig. 1, Table 1) (Hopkins and Purcell, 2002; Chatterjee *et al.*, 2008; Janse and Obradovic, 2010; Krugner *et al.*, 2019; EFSA 2021). Nevertheless, many plant species may remain symptomless (EFSA PLH 2018; EFSA, 2013).

Table 1. Subspecies of *X. fastidiosa*, susceptible host plants and geographic distribution. Modified from EFSA 2013.

Subspecies	Susceptible host plant	Geographic distribution
X. fastidiosa subsp. *fastidiosa*	Grapevine, alfalfa, almond, citrus, coffee	Costa Rica, Mexico, States, Spain, Taiwan, United
X. fastidiosa subsp. *multiplex*	Almond, peach, plum, apricot, elm, ginko, sunflower, oak, blueberry, pecan, olive	Brazil, France, Italy, Iran, Portugal, Spain, United States
X. fastidiosa subsp. *pauca*	Citrus, olive, coffee	Argentina, Brazil, Costa Rica, Ecuador, France, Italy, Netherlands, Paraguay, Spain
X. fastidiosa subsp. *sandyi*	Oleander and ornamentals	Brazil, Costa Rica, France, Honduras, United States,
X. fastidiosa subsp. *morus*	Red mulberry	United States
X. fastidiosa subsp. *tashke*	Pink dawn	United States

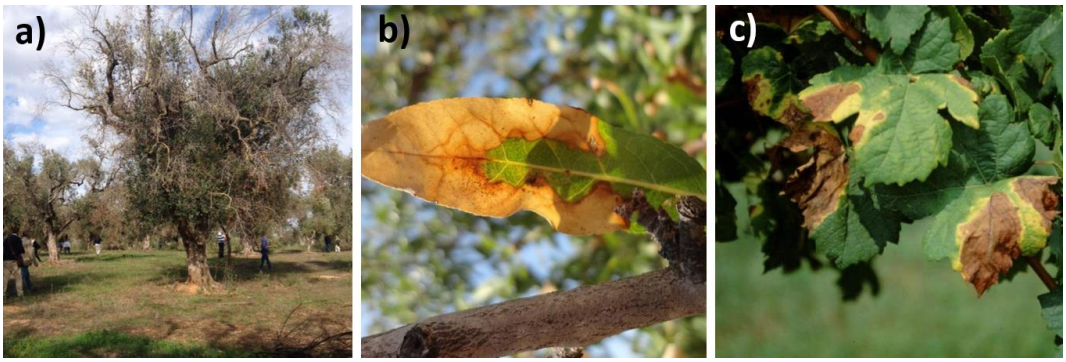

Figure 1. Visual symptoms of Olive quick decline in olive (a), Almond leaf scorch in almond (b) and Pierce's disease in grapevine (c) caused by *X. fastidiosa* infection. © https://gd.eppo.int/taxon/XYLEFA/photos.

The symptomatology is related to the occlusion of the xylem vessels when bacteria multiplicate inside and develops into a biofilm blocking the fluid transport (EFSA, 2018). In the course of time, symptomatology develops to leaf scorch or drying of leaf margins, dieback of branches and even death of the plant when several infections occur (EFSA, 2013; EFSA, 2018). However, the symptoms are not specific and can be confused as drought or mineral deficiencies (EFSA, 2018; EPPO, 2017). The symptom development depends on the host plant species, the *X. fastidiosa* genotype that is infecting and the bacterial population load (Almeida and Purcell, 2003; EFSA PLH 2015a).

It is an obligatory declaration pathogen within the European Union regulated as a harmful organism and its introduction and spread within the EU is banned (Directive 2000/29/EC and Decision 2015/789/EU) (EFSA 2013; EFSA 2018). Also, it has a specific contingency plan at national level in Spain (MAGRAMA 2015). Out of the EU, it is known to occur over a wide range of climatic zones in tropical countries and subtropical aeras (e.g., Brazil, Costa Rica and southern California) and in more temperate or even continental climate regions (e.g., Canada, north-eastern regions of the USA and Argentina) (EFSA PLH, 2019).

1.2. Biology of *Xylella fastidiosa*

Xylella fastidiosa is and strictly aerobic, non-flagellated and gram-negative bacterium that lives exclusively in xylem cells or tracheary elements (Fig. 2) (Chatterjee *et al.*, 2008; Janse and Obradovic, 2010; ESFA, 2013; EFSA PLH 2015a). The optimum growth temperature is between 26 °C and 28 °C (Janse and Obradovic, 2010; ESFA, 2013).

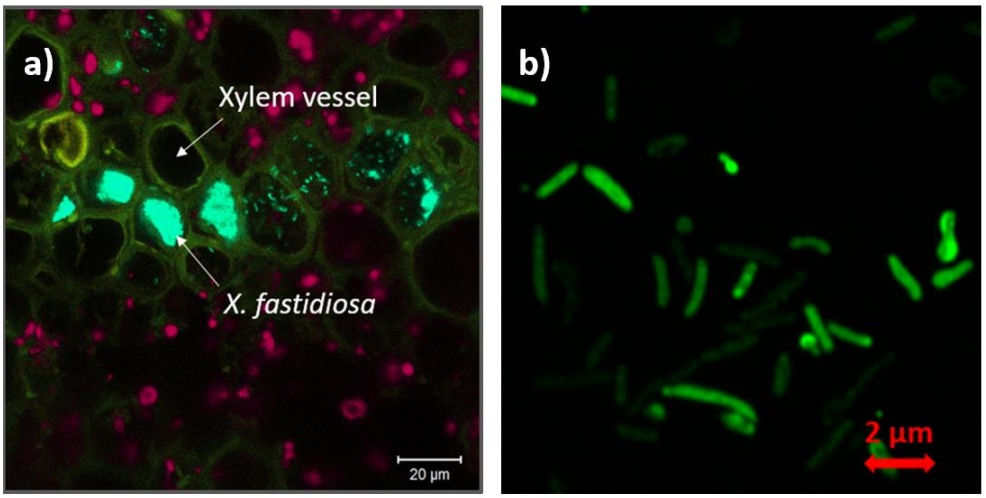

Figure 2. *Xylella fastidiosa* marked with green fluorescent protein (GFP) under the confocal microscope. a) Biofilm of *X. fastidiosa* in the xylem vessels. b) individuals of GFP-*X. fastidiosa*. © N. Casarin.

In susceptible host plants, it multiplies and spreads downstream or upstream widely from the site of infection by attaching cells to vessel walls forming a biofilm-like colony by a Type I short pili (Almeida *et al.*, 2001; Meng *et al.*, 2005; Chatterjee *et al.*, 2008; Janse and Obradovic, 2010; EFSA, 2013). The invasion of the bacterium to the xylem vessels and among them it is supposed to occur throughout the pit membrane (Chatterjee *et al.*, 2008; Janse and Obradovic, 2010; Sicard *et al.*, 2018). After that, the twitching motility and migration is due to a functional Type IV long pili located at one pole of the cell (Meng *et al.*, 2005; Chatterjee *et al.*, 2008). The biofilms produced by the pathogen are different in composition in the plant and in the vector (Li *et al.*, 2007; Meng *et al.*, 2005; Janse and Obradovic, 2010).

1.3. Distribution *Xylella fastidiosa*

1.3.1. Worldwide distribution

First records of Pierce disease originated from California (USA) in the 1880s in grapevine, however, the aetiology of the disease remained unclear until the pathogen was isolated in 1987 by Wells *et al.* (Janse and Obradovic, 2010; ESFA, 2013; EFSA PLH 2015a; EFSA, 2021). Then, it was isolated in Brazil in 1995 from *Citrus*, and found in many crop

plants and wild plants (Janse and Obradovic, 2010). After that, it spread worldwide and it is currently present in North, Central and South America, Caribbean, Asia and Europe (Fig. 3) (EFSA PLH, 2018; EFSA, 2021).

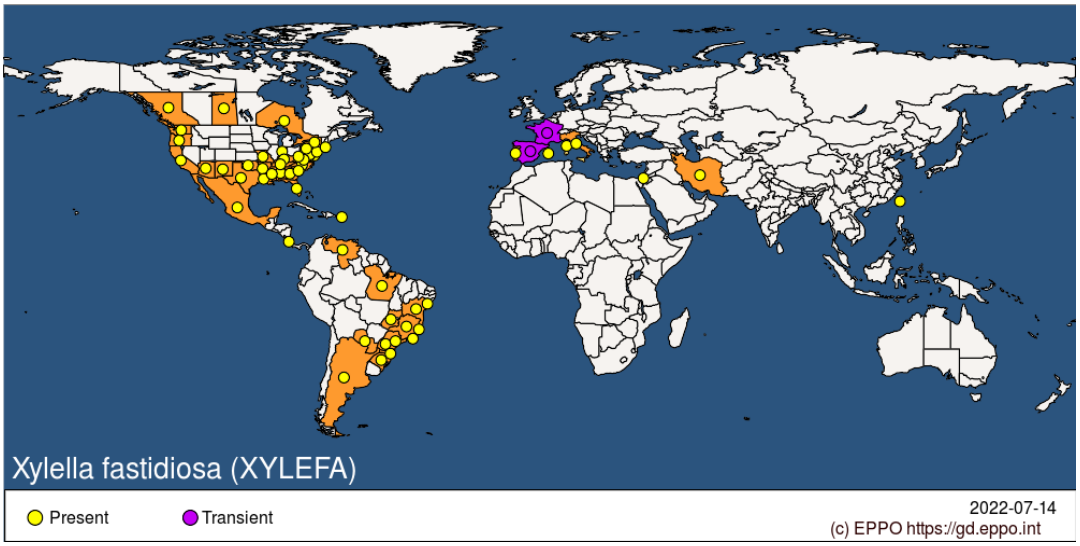

Figure 3. Worldwide distribution of *X. fastidiosa* by EPPO.

About the different subspecies of *X. fastidiosa*, spp. *fastidiosa* is the causal agent of Pierce Disease in the USA (EFSA PLH, 2015; Nunney *et al.*, 2010). This subspecies is distributed also in central America and in Taiwan (Nunney *et al.*, 2010; Su *et al.*, 2013; EFSA PLH, 2015). In the case of ssp. *pauca*, it was the causing agent of the citrus variegated chlorosis in Brazil (Nunney *et al.*, 2012; EFSA PLH, 2015). Furthermore, it caused the first outbreak of *X. fastidiosa* in Europe in the Italian region of Apulia in 2013 (Saponari *et al.*, 2013; Cariddi *et al.*, 2014; EFSA PLH, 2015). The ssp. *multiplex* is the one showing the widest host range of plants expressing disease symptoms (Nunney *et al.*, 2013; EFSA PLH, 2015). It was detected in USA, Brazil and Europe.

1.3.2. European Union distribution of *Xylella fastidiosa*

The potential distribution of *X. fastidiosa* according to climate change in Europe was predicted by Godefroid *et al.* (2019), confirming the spread of the plant pathogen across Europe (Fig. 4).

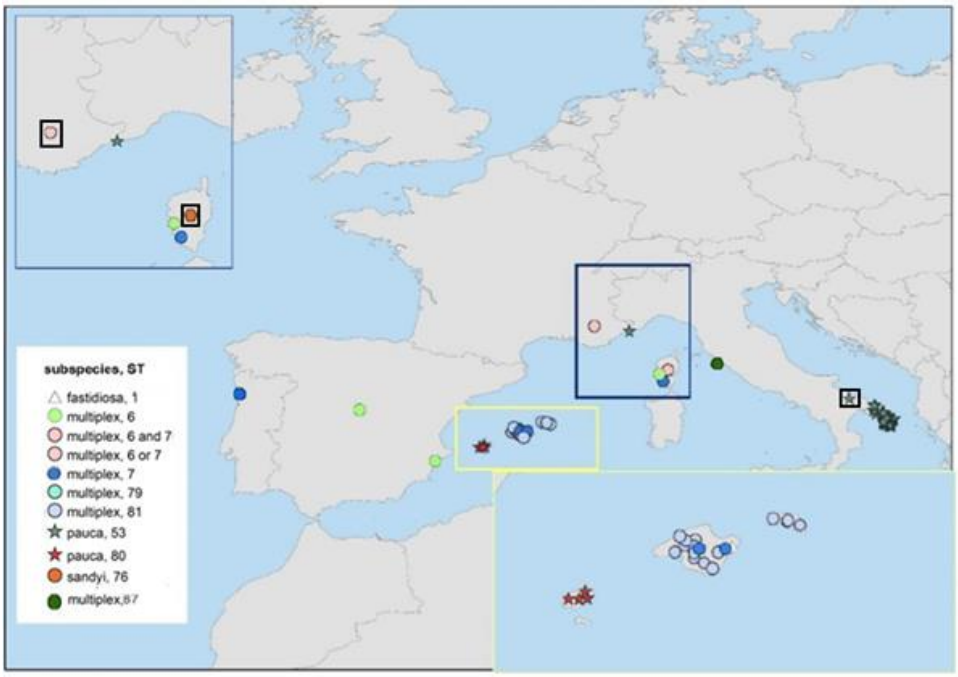

Figure 4. European distribution of *X. fastidiosa* (EFSA, 2021).

The first unconfirmed report of Pierce disease in Europe was in Kosovo in 1998 (Berisha *et al.*, 1998). So, it is considered that Italy was the first country in Europe where *X. fastidiosa* was extensively detected in olive trees in October 2013 (Cariddi *et al.* 2014; Saponari *et al.* 2014; Loconsole *et al.* 2016) associated with the CoDIRO (*Complesso del Disseccamento Rapido dell'Olivo*) syndrome, now more correctly named Olive Quick Decline Syndrome (OQDS) (Saponari *et al.* 2014; Martelli *et al.* 2016). This outbreak was also related to oleander and almond (EFSA, 2015; Saponari 2013; Cariddi *et al.*, 2014).

Subsequently, since summer 2015, other outbreaks of *X. fastidiosa* belonging to other subspecies (e.g.: *multiplex*) were detected in Corsica in 2019 and continental France in 2020 (*Provence Alpes Cotes d'Azur region*) (EFSA 2021), while recent outbreaks were located in the region of Tuscany in 2018 and Lazio in 2021 (Italy), Porto in 2019 and Lisbon and Algarve in 2021 (Portugal) (EFSA PLH Panel 2019; EFSA 2021).

In October 2016, during an official survey, *X. fastidiosa* was detected in *Prunus avium* (cherry) and *Polygala myrtifolia* plants located in a garden center in the locality of

Manacor (Majorca, Balearic Islands, Spain). From four symptomatic cherry trees, three were positive for *X. fastidiosa* and, from seven *P. myrtifolia*, four resulted also positive (Olmo *et al.* 2017). Surveys were extended to all the Balearics, detecting the bacterium in 1223 samples within ornamental, cultivated and wild plants from all the archipelago, in exception of Formentera. Since then, various outbreaks have been detected, leading to the detection of different subspecies: spp. *fastidiosa* (Majorca), spp. *multiplex* (Majorca and Minorca) and spp. *pauca* (Ibiza).

In June 2017, *X. fastidiosa* subsp. *multiplex* (ST6) was detected in the Alicante region (Valencia, Spain) infecting almond trees (Generalitat Valenciana, 2017) and the same subspecies and ST were also detected in April 2018 in an olive tree in the Autonomous Region of Madrid (EFSA, 2021) that was eradicated. Detections were not only in crops, in 2011 *X. fastidiosa* was found in coffee plants from a French garden centre and, since 2016 there were some positives in greenhouses located in Holland, Germany and Portugal (EFSA 2018 and 2021).

1.4. Economic importance of *Xylella fastidiosa*

In Europe, there are hosts with a high economic value such as *Olea europaea*, *Prunus dulcis*, *Vitis vinifera*, *Prunus avium*, *Prunus domestica*, *Prunus salicina* or *Citrus* spp. (EFSA PLH, 2019). If *X. fastidiosa* was fully spread, would cause an annual production loss of 5.5 billion euros that affects the 70 % of older olive trees (over 30 years old) and the 35 % of younger olive production; 13 % of almond, 11 % of citrus and 1-2 % of grapevine (European Commission, 2021). In Italy, it is estimated that olive producers have already lost between 0.2 and 0.6 billion euros in investments, and it could increase until 1.9 to 5.5 billion of euros over the next 50 years (Schneider *et al.*, 2020; Albre *et al.*, 2021), as well as socio-ecological damages with a loss of 1,017 € and 1,059 € per ha (Frem *et al*., 2021).

According to EFSA PLH (2015), infected and symptomatic plants often die within the years, so the yield of most infected symptomatic plants is negligible or not commercially acceptable. Pierce's disease has been described as a major constrain for the grapevine commercial sector in USA and tropical America (EFSA, 2013). Tumber *et al.*, (2014)

estimated that *X. fastidiosa* costs 104 million of dollars per year in California in reduced yield, regulatory costs and management costs (Sicard *et al.*, 2018). Also, there was a high mortality of oleander in California in the 1990s associated with the leaf scorch caused by *X. fastidiosa*, resulting in a loss of more than 125 million of dollars (EFSA PLH, 2015). Furthermore, it is estimated that up to 50 % of oaks in the southern New York to Georgia are showing oak leaf scorch due to *X. fastidiosa* infections (EFSA PLH, 2015).

In the case of Sao Paulo (Brazil), the 40 % of 200 million citrus plants show *X. fastidiosa* symptoms and in 2005 there was an annual loss of 120 million of dollars (EFSA PLH, 2015; Lindow, 2019). Numerous orchards and small growers have been eliminated from the industry and replanted because of *X. fastidiosa* infections (EFSA PLH, 2015). Furthermore, in Argentina, the bacterium killed around 500,000 plum trees between 1935 and 1954 (EFSA PLH, 2015).

In Australia, it was estimated that the economic impact of *X. fastidiosa* would vary from 2.3 to 7.9 billion of dollars over 50 years in wine grapes and wineries (ABARES, 2018; Frem *et al*., 2021).

Finally, in Middle East and North Africa (MENA) region, production losses have been estimated as 10 million dollars in grapes, 218.35 million dollars in citrus and 1 billion in olives (Cardone *et al*., 2021; Frem *et al*., 2021).

1.5. Vectors of *Xylella fastidiosa*

To determine if an insect is a vector of pathogens, it is necessary to do transmission bioassays and confirm both acquisition and inoculation of the pathogen by the vector (Nault *et al.*, 1997; Chatterjee *et al.*, 2008). *Xylella fastidiosa* multiplies within the vector mouth parts and it can be transmitted without a latent period (Purcell and Finlay, 1979; Hill and Purcell, 1995; Redak *et al.*, 2004; Almeida *et al.*, 2005; Chatterjee *et al.*, 2008). The natural transmission of the bacterium is via insects that feed in the xylem of plants, but insect transmission seems to be poorly specific and therefore all xylem-fluid feeding insects

can be considered as potential vectors, until proven (Frazier, 1944; Purcell, 1990; Almeida *et al.*, 2005; EFSA 2013). In fact, transmission efficiency varies substantially depending on insect species, host plant, and *X. fastidiosa* genotype (Redak *et al.*, 2004; Lopes *et al.*, 2009; EFSA, 2013).

The bacterium is restricted to the anterior part of the alimentary canal of the insects, where the bacterium adheres to, multiplies and persist in the precibarium and cibarium (i.e., sucking pump) foregut parts (Fig. 5) (Almeida *et al.*, 2005; Overall and Rebek, 2017; EFSA 2018). The sucking mouthparts of the insect (mandibular and maxillary stylets) allow them to reach the xylem of plants, from which they ingest sap (Fig. 5). Due to very poor nutritional value of xylem fluid, they ingest large amounts of crude sap and produce big amounts of liquid excretions. They are usually not direct pest unless present at very high population levels (EFSA, 2013). Either nymphs and adults are able to acquire and inoculate the bacteria to healthy plants (EFSA, 2013), however, nymphs lose their infectivity due to the foregut is ectodermal and renewed with moulting (EFSA, 2018). Then, newly emerged adults are the potential vectors, and they will transmit the bacteria during its whole lifetime because *X. fastidiosa* multiplies within the vectors (Almeida *et al.*, 2005; Chatterjee *et al.*, 2008; EFSA, 2018). Conversely, the bacterium is not transovarially transmitted (Freitag, 1951; Redak *et al.*, 2004; EFSA, 2018).

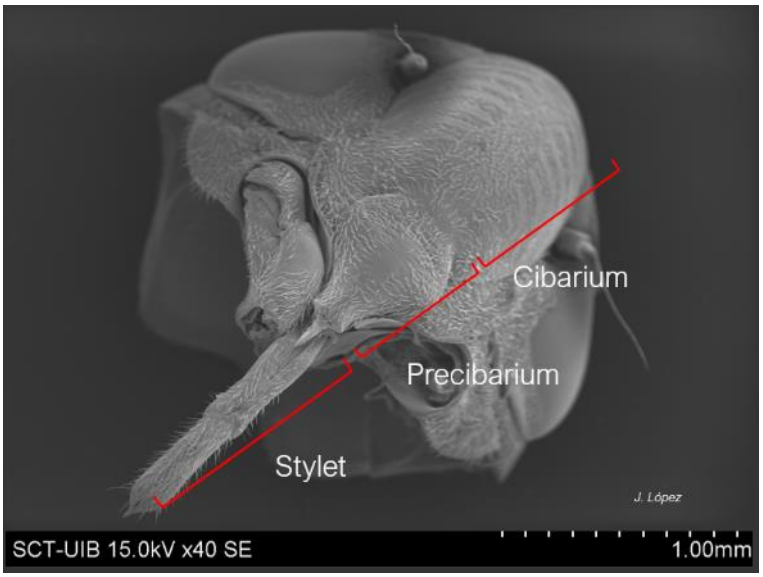

Figure 5. Dissected head of *P. spumarius* under a Scanning Electron Microscopy (SEM). Mouthparts: stylet, precibarium and cibarium. © J. López-Mercadal.

All strict xylem fluid-feeding insects belong to Auchenorrhyncha (Hemiptera) and infraorder Cicadomorpha (= Clypeorrhyncha) (Redak *et al.*, 2004; Almeida *et al.*, 2005; EFSA, 2021). The table 2 summarizes the main families of hemipteran xylem-feeders. In general, those insects cause little damage to plants, in some cases related to the xylem sucking activity (Almeida *et al.*, 2005). Some damages of spittlebugs are described in Mozaffarian and Wilson (2015) such as chlorosis, affecting the photosynthetic rate, reduction of the plant growth, number of flowers and lateral branches, phytotoxemia, modifying plant community structure and host population genetic structure destroying the pastures and soil degeneration. But nevertheless, in some cases they can cause huge damage, for example sharpshooters in California are considered pests. Those insects consume huge quantities of sap producing excreta that causes physiological damage to infested flora (Percy *et al*., 2008).

Table 2. Taxonomy of *X. fastidiosa* insect vectors.

Suborder	Infraorder	Superfamily	Family	Subfamily	Common name
Auchenorrhyncha	Cicadomorpha	Cicadoidea	Cicadidae		Cicadas
		Cercopoidea	Aphrophoridae		Spittlebugs
			Machaerotidae		Tube-building spittlebugs
		Membracoidea	Cicadellidae	Cicadellinae	Sharpshooter Leafhoppers

According to Janse and Obradovic (2010), worldwide record of vectors of *X. fastidiosa* includes 5 species of Cercopoidea and 39 species of 19 genera of Cicadellinae. This last subfamily includes the most important vectors of *X. fastidiosa* in some regions (e.g., Americas) and it is a highly diverse group with 1950 species approximately, representing the 9 % of all Cicadellidae (Knight and Webb, 1993; Mejdalani, 1998; Redak *et al.*, 2004). The Aphrophoridae family has about 900 species described worldwide and most of them inhabit the tropical regions, while only about 29 species are present in Europe (Richards and Davies, 1977; Shih and Yang, 2002; Albre *et al.*, 2021).

In Europe, *Philaenus spumarius* (Linnaeus, 1758), *Philaenus italosignus* Drosopoulos and Remane 2000 and *Neophilaenus campestris* (Fallén, 1805) (Aphrophoridae) are the only vector species identified to date in Italy based on vector acquisition trials (Saponari *et al.* 2014; Cornara *et al.* 2017; EFSA, 2021). *Philaenus spumarius* was related in 2013 with the Apulian outbreak of *X. fastidiosa* (Saponari *et al*., 2014; Cornara *et al.*, 2017a; Cornara *et al.*, 2017b). *Philaenus spumarius* is widely distributed and common throughout Europe and present in North Africa, Soviet Union, Afghanistan, Japan, United States, Canada, Azores, Hawaii and New Zealand (Cornara *et al.*, 2018; EPPO 2020). *Philaenus italosignus* is endemic to Southern mainland Italy and Sicily (EPPO 2020). Regarding *N. campestris*, it has a western Paleartic distribution (EPPO 2020).

Other species such as *Euscelis lineolatus* Brullé, 1832 (Cicadellidae) have tested positive for *X. fastidiosa* by PCR in Italy, however, their vector role has not been

demonstrated yet (Elbeaino *et al.* 2014). Also, the highly widespread *Cicadella viridis* is considered a potential vector (Janse and Obradovic, 2010; EFSA, 2013; EFSA PLH, 2018).

In Corsica (Cruaud *et al.* 2018), an extensive surveillance of potential vectors also found *P. spumarius* tested positive to *X. fastidiosa* by PCR and nested PCR. In Spain, Aphrophoridae species (*P. spumarius*, *N. campestris* and *N. lineatus*) have been demonstrated to be present in olive groves and other host plants, but their potential vectorial role was not demonstrated (Lopes *et al.* 2014; Miranda *et al.* 2017; Morente *et al.* 2017).

In the Americas the main family of vectors is the Cicadellidae (Redak *et al.*, 2004). Numerous leafhopper species have been reported in the south-eastern part of the United States (Mizell and French, 1987; Ball, 1979; Alderz, 1980; Hopkins and Purcell 2002). The species related to Pierce's disease and phony peach disease are: *Homalodisca coagulata* (Say) (glassy-winged sharpshooter), *H. insolita*, *H. vitripennis*, *Graphocephala versuta*, G. *atropunctata* (Signoret) (blue-green sharpshooter)*, Oncometopia orbona*, *Cuerna costalis*, *Xyphon fulgida* (Nottingham) and *Drachocephala minerva* (Ball) (green sharpshooter) (Turner and Pollard, 1959; Ball *et al.*, 1979; Mizell and French, 1987; Hopkins and Purcell 2002). All the american vector species are absent in Europe according to the Fauna Europea database (de Jong, 2013).

1.6. Biological cycle of *P. spumarius* and *N. campestris*

Philaenus spumarius and *Neophilaenus campestris* have hemimetabolous development and univoltine cycle (Fig. 6) (Yurtsever, 2000; Miranda *et al.*, 2017 Morente and Fereres, 2017; Cornara *et al.*, 2018; EFSA, 2018), but Drosopoulos and Asche (1991) recorded bivoltine cycle in certain parts of Greece. In the Mediterranean area, Aphrophoridae species overwinter in egg form until end of February/March when hatching occur. Nymphs develop until May throughout five nymphal stages and adults start to emerge since end April and early May. During May and June cover vegetation desiccates, and adult spittlebugs disperse to trees and shrubs. Adults start copulating during the

summer. As the summer season advances, abundance of males declines in proportion to females (Halkka, 1962). They remain in canopies until September/October, when ground vegetation appears again. Oviposition starts in early September as females are induced to lay eggs in dried grass because of short daylight and decrease of temperatures (Witsack, 1973; Morente *et al.*, 2018a). Then, eggs undergo wintering diapause again. This diapause stops when they are exposed to chill period (less than 5 °C about 100 days) (West and Lees, 1988).

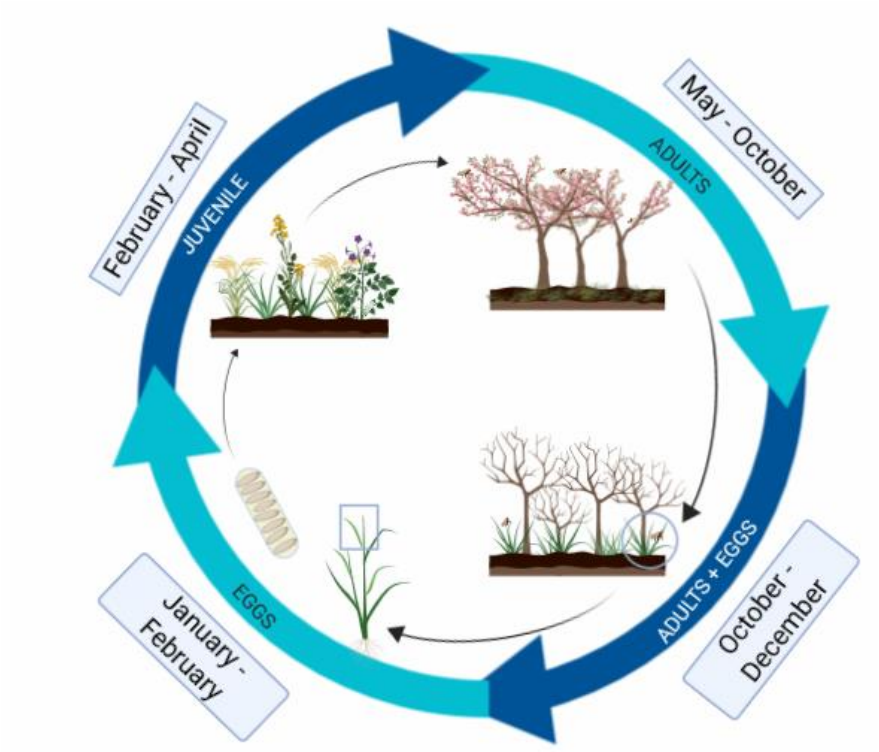

Figure 6. General life cycle of *P. spumarius*. © J. López-Mercadal.

1.6.1. Eggs

Eggs are ovoid and elongated with about 1 mm long and 0.35 mm wide, approximately a third as wide as long (Weaver and King, 1954; Yutsever, 2000; EPPO 2020) (Fig. 7). After oviposition, eggs are yellowish white and has a dark orange pigmented spot in the shell at one end. When fertilized, the spot gets bigger and black coloured, developing in a lid in about 90 days (Fig. 7) (Yurtsever, 1997; Yurtsever, 2000).

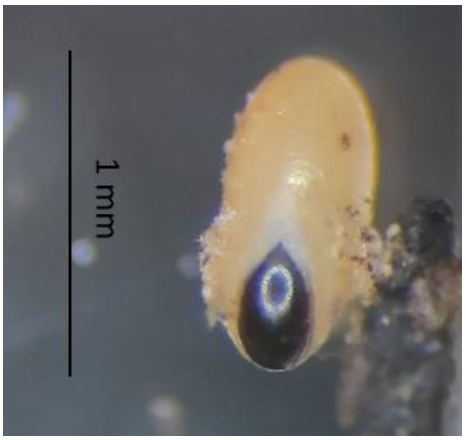

Figure 7. Egg of *P. spumarius* under binocular microscope. © J. López-Mercadal.

According to Yurtsever (1997), the number of eggs laid is variable, but in general a female is able to produce up to 350-400 eggs packed in batches of 1 to 30 eggs by frothy cement (EPPO, 2020). The egg batches are placed at the base of herbaceous plants in between two apposed surfaces (i.e., the stem and the leaf sheath, on stubble, dead parts of plants, plant residues, cracks and tree trunk barks, or in the litter) (Cornara *et al.*, 2018; EPPO, 2020) (Fig. 8).

Figure 8. *Philaenus spumarius* egg batch from where nymphs have already emerged. © J. López-Mercadal.

1.6.2. Nymphs

There are five nymphal stages. As nymphs are developing, the length of legs gets longer in proportion with the body length, the abdomen flattens dorso-ventrally and wing pads appear (Yurtsever, 2000). While developing, nymphs produce a white spit around them to protect its body against environmental conditions, desiccation, predators and parasitoids (Weaver and King, 1954; Yurtsever, 2000; Cryan and Svenson, 2010). The development to adulthood requires an amount of 700-800 day-degrees above 5 °C (Yurtsever, 2000). Several nymphs can aggregate and share the same spittle mass and different spits can be found in the same plant (EPPO, 2020). Nymphs of *P. spumarius* can be found in a wide range of dicotyledonous plant species (Drosopoulos, 2003), while *N. campestris* nymphs are found mainly in Poaceae plants (Bodino *et al.*, 2020) (Fig. 9). The spit is made of the semi-digested fluid of the xylem discharged from the alimentary channel, combined with mucopolysaccharides and polypeptides produced by malpighian tubules (Mello *et al.*, 1987; Rakitov, 2002; Mozaffarian and Wilson, 2015).

Figure 9. Spits of *P. spumarius* on *Cardus* spp. (a) and *N. campestris* (b). © J. López-Mercadal.

The first instar is approximately 1.35 mm long (EPPO, 2020). The newly first instar is light orange, and it gradually turns green from the first to the fifth instar (Yurtsever, 2000)

(Fig. 10 a). On the contrary, *N. campestris* nymphs show a beige/light brown/orange colour with a black pattern on the dorsum (Fig. 10 b). Spittle on this stage is usually settled on the basal part of the plants (EPPO, 2020). In the third instar wing pads begin to appear (Yurtsever, 2000). From the fourth instar, spittle masses are larger, and nymphs can be sexed according to their external genitalia (Yurtsever, 2000). Finally, in the fifth instar, nymphs ceases to form the spittle and forms the chamber where the adult stage will appear (Weaver and King, 1954; EPPO, 2020).

Figure 10. Differences between *N. campestris* (b) and *P. spumarius* (a) nymphal stages. © J. López-Mercadal.

1.6.3. Adults

Body length of *P. spumarius* is approximately of 6 mm but females are slightly larger than males. Adult emergence usually occurs after 50 days of nymphal emergence (Fig. 11). Firstly, adults remain in the spittle mass until their cuticle is hard and fully pigmented (Fig. 12). Adults will be fully mature ten days after leaving the spittle and females will start to mate (Yurtsever, 2000).

Philaenus spumarius is highly polyphagous and can be detected in meadows, waste grounds, abandoned fields, stream sides, roadsides, hayfields, parks, marshlands, gardens, vineyards, orchards and other cultivated fields (Yurtsever 2000; EPPO, 2020).

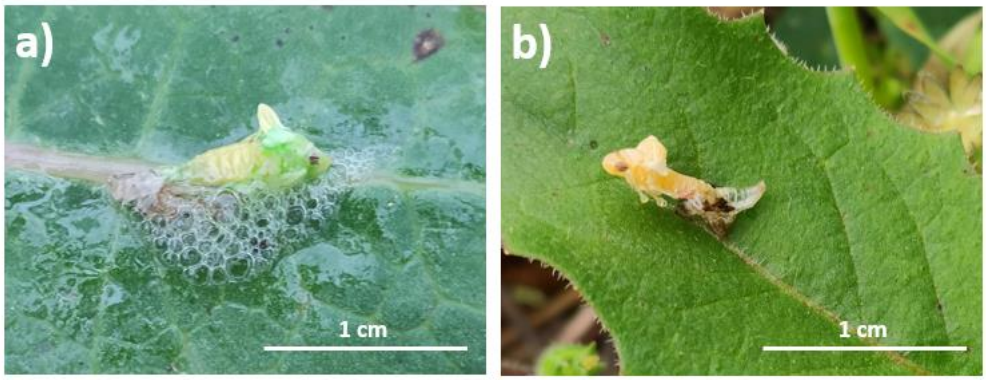

Figure 11. Adult emergence of *P. spumarius* (a) and *N. campestris* (b). © J. López-Mercadal.

Figure 12. Newly emerged adult of *P. spumarius* remaining in the spittle. © J. López-Mercadal.

1.6.3.1. Morphological features of *P. spumarius* adults

Philaenus spumarius is a polyphagous insect with a high diversity of color polymorphism (Yutsever, 2000; Tishechkin, 2013). Identification of this species is based on external characteristics and genitalia (Fig. 13, 14, 15) (Elbeaino *et al.*, 2014; Tishechkin,

2013). Males are smaller than females: 5.3 to 6.0 mm and 5.4 to 6.9 mm, respectively (EPPO, 2020). Body shape is rounder than *N. campestris*, with the outer margin of fore wings along whole length convex providing them with this oval shape (Biederman and Niedringhaus, 2009; EPPO, 2020). The colour of individuals is very variable, from yellowish to black, having numerous morphotypes described (Stewart and Lees, 1996; Yurtsever, 2000; Kunz *et al.*, 2011; EPPO, 2020). The male genitalia are characterized with, apically, six antler-like appendages and circle-shaped for the upper one (EPPO, 2020) (Fig. 14).

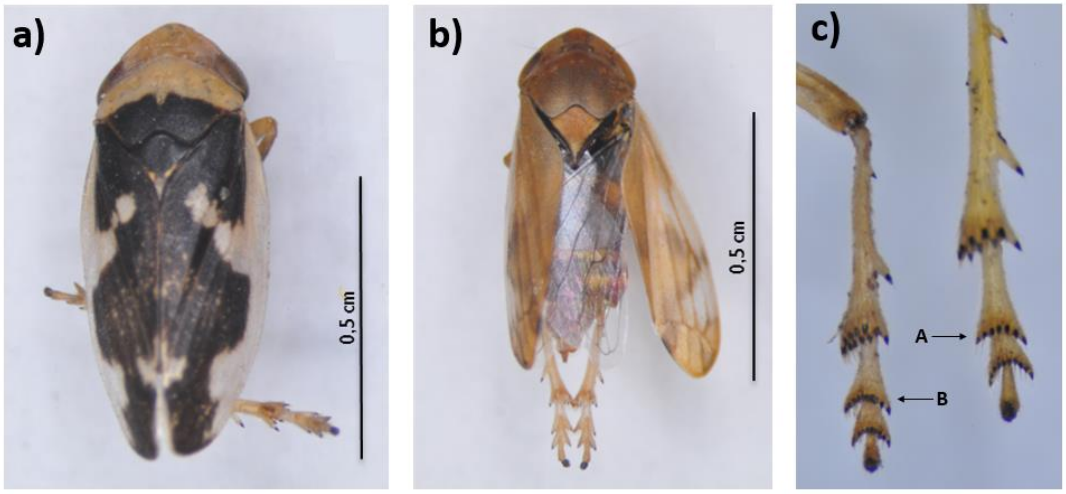

Figure 13. Difference between adults of Aphrophoridae that can be distinguished by size, colour and other morphological features. a) *P. spumarius*; b) *N. campestris* (right); c) apical spines of the tarsus I based on Elbiano *et al*., (2014): *P. spumarius* (A) and *N. campestris* (B). © J. López-Mercadal and M. A. Miranda.

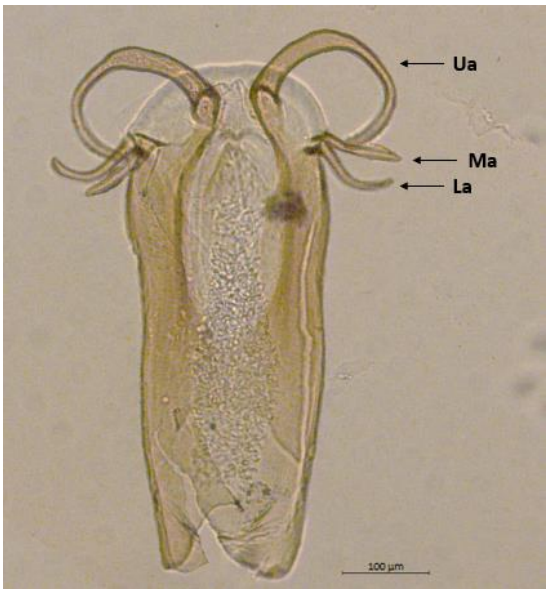

Figure 14. Front view of aedagous from the genitalia of a *P. spumarius* male. Ua: Upper appendages; Ma: Middle appendages; La: Lower appendages. © J. López-Mercadal.

1.6.3.2. Morphological features of *N. campestris* adults

Males are smaller than females: 5.0 to 5.3 mm and 5.4 to 5.7 mm, respectively. Body shape is slenderer than in *P. spumarius*, with the first third of the fore wings straight (Fig. 13 b). Base colour is greyish yellow to greyish brown, sometimes with reddish undertone. Generally, *N. campestris* shows a dark longitudinal strip extending from the vertex towards the scutellum (median keel). The forewing outer margin has two light spots (Biedermann and Niedringhaus, 2009; EPPO, 2020). The male genitalia are characterized as a hooklike and ear-shaped in the lateral view, lateral lobe in elongate triangle and shortened side serrated (EPPO, 2020) (Fig. 15).

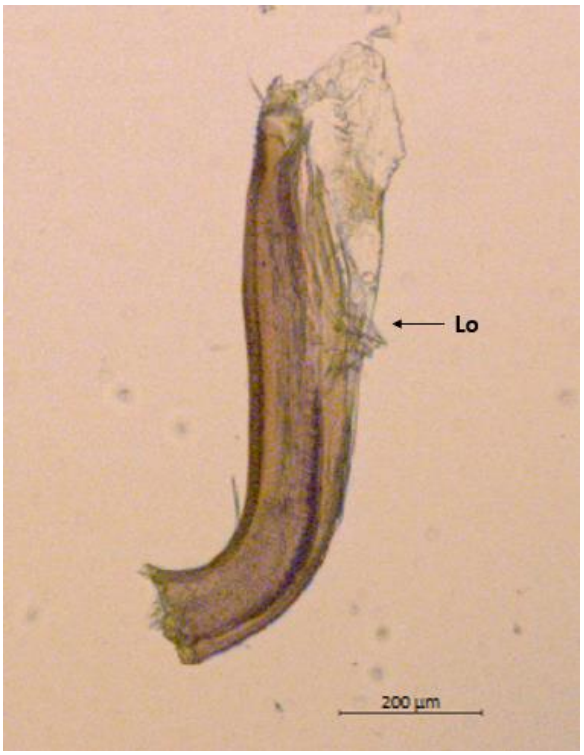

Figure 15. Lateral view of aedagous from the genitalia of a *N. campestris* male. Lo: lateral lobe. © J. López-Mercadal.

1.7. Control of *Xylella fastidiosa*

Plant infection by *X. fastidiosa* has no cure. Once detected in a territory, the first step of prevention for the bacteria spread include administrative measures such as the implementation of quarantine and phytosanitary procedures (Janse and Obradovic, 2010). Then, the control of *X. fastidiosa* requires the disruption of the interactions among the main characters involved in the pathosystem, such as the vectors (Almeida *et al.*, 2005). These measures are based on prevention using resistant varieties, cultural and hygienic measures, and chemical and biological vector control (Janse and Obradovic, 2010). Nevertheless, control of *X. fastidiosa* remains difficult because there are many symptomless plants (weeds, ornamentals and crops), possible unknown vectors and neighbouring reservoirs areas in orchard (Janse and Obradovic, 2010). Taking that into account, it is recommended to implement and combine multiple techniques to achieve the control of the disease (Almeida *et al.*, 2005).

1.7.1. Resistant hosts

The susceptibility of host plants to be infected vary on the different plant species. For example, coffee and plum cultivars seem to be relatively more susceptible to infection than citrus because of the anatomy of the xylem vessels (Alves *et al.*, 2004; Rashed *et al*., 2013).

Genotypes of *V. vinifera* grapes showed more susceptibility to *X. fastidiosa* than other *Vitis* spp. For example, most of cultivars of Europe-type (*V. vinifera*), American-type (*V. labrusca*) and French American hybrid grapes are susceptible to Pierce's Disease, but some resistance species were found in south-eastern US (Mortensen *et al.*, 1977; Hopkins and Purcell, 2002; Janse and Obradovic, 2010). For example, the muscadine grape (*V. rotundifolia*) is characterized by its hight resistance and tolerance to *X. fastidiosa*, showing vigor and longevity (Loomis, 1958; Hopkins and Purcell, 2002). The variation of susceptibility of grape cultivars has been attributed to genotype differences in bacterial quantities and the occlusion rate of the xylem tissue (Rashed *et al*., 2013).

In the case of *Citrus* species, *X. fastidiosa* subsp. *pauca* systematically infects *C. sinensis* more than other species. Orange cultivars are very susceptible but for example Navelina ISA 315 shows very low bacterial titre. *Citrus aurantifolia*, *C. paradisi*, *C. limon, C. grandis*, *C. resituclata*, *Poncirus trifoliata,* kumquats and tangors seem hight tolerant or resistant to *X. fastidiosa* (EFSA, 2016b).

For *Quercus* spp., it is known that *Q. suber*, *Q. robur* and *Q. ilex* are systematically infected by *X. fastidiosa*. The widespread in Balearic Islands *Q. ilex* has been shown to be infected with the CoDiRO strain in Apulia (Italy).

Regarding olive plants, most studies in Europe has been focus on the varieties Leccino, Ogliarola salentina, Cellina di Nardò, Corantina, Frantoio, Cima di Melfi, Nocellara and Picholine, that were the most representative in the Apulia outbreak (EFSA, 2017).

Leccino was identified as tolerant to *X. fastidiosa* ST53 infections (Saponari *et al*., 2016; EFSA, 2017). Also, ST53 bacterial populations were two orders lower in Leccino than in

Cellina di Nardò and Ogliarola salentina (Giampetruzzi et al., 2016; Boscia et al., 2017). Also, olive variety FS-17® has been showed to have a genotype with possible resistance traits against the bacterium because, in a heavily infected olive orchard, this variety showed a half less bacterial load than in Leccino (Boscia *et al.,* 2017; EFSA, 2017).

1.7.2. Biological control of vectors

Among the invertebrates, several Arachnida, Hymenoptera, Diptera, and Coleoptera have been reported as predators of the meadow spittlebug. Identified egg parasitoids (Hymenoptera) in America are *Ooctonus* spp. (Mymaridae), *Tumidiscapus* sp. (Aphelinidae), *Centrodora* sp. (Aphelinidae) and some dipterans (Weaver and King, 1954). The parasitoid *Gonatocerus* sp. (Mymaridae) has been tested against *H. coagulata*, but its population decreases in winter when vector egg is produced (Morgan *et al.*, 2001). It was observed a decrease of 90 % and 95 % of *H. vitripennis* one year after the release of *Cosmocomoidea ashmedi* (Mymaridae) in the French Polynesia (Grandginard *et al.*, 2008 and 2009; Krugner *et al.*, 2019) and 56 % of *H. vitripennis* eggs were found parasitised in California after the release of the same parasitoid (CDFA, 2017; Krugner *et al.*, 2019). It is calculated that the mortality of sharpshooters in California due to egg parasitism, predation by spiders, green lacewings, praying mantis, ants and several predacious bugs reach from 2.3 % to 15.5 % (Fournier *et al.*, 2008; Hagler *et al.*, 2013; Krugner *et al.*, 2019).

In the case of Europe, Hasbroucq *et al*., (2020) found 3.48-11.77 % of parasitism in *Cicadella viris* eggs collected from La Hulpe (Belgium) by *Anagrus incarnatus* (Mymaridae). Also, Arzone (1972) identified four Mymaridae egg parasitoids of *C. viridis*: *Polynema woodi*, *Gonatocerus longicornis* and *Anagrus incarnatus*.

Adults of *P. spumarius* are observed to be attacked by the dipteran parasitoid *Verralia aucta* (Pipunculidae) in the United States (Whittaker, 1973) and also in Italy (Europe) (Molinatto *et al*., 2020), the nematode *Agamermis decaudata* (Mermithidae) (Weaver and King, 1954), and spiders like *Mitopus morio* (Phalangiidae) (Phillipson, 1960; Harper and Whittaker, 1976). In fact, Benhadi-Marín et al., (2020) developed a guild-based protocol to target spiders as potential natural enemies of *P. spumarius*. In the case of *P.*

spumarius nymphs, the prairie ant *Formica montana* (Formicidae) has been reported to prey on them (Henderson *et al.*, 1990).

Vertebrates can also play a role as natural enemies, such as birds, reported as predators of the meadow spittlebug (Yurtsever, 2000). Evans (1964) and Halkka and Kohila (1976) found *P. spumarius* in the diet of *Pooecetes gramineus* (Passerellidae), *Spizella pusilla* (Passerellidae), *S. passerine* (Passerellidae), *Tetrao urogallus* (Phasianidae), *Phasianus colchicus* (Phasianidae), *Perdix perdix* (Phasianidae), *Delichon urbica* (Hirundinidae), *Corvus frugilegus* (Corvidae), *Turdus viscivorus* (Turdidae), *T. philomelos* (Turdidae), *Phylloscopus trochilus acredula* (Phylloscopidae) and *Sturnus vulgaris* (Sturnidae). Furthermore, the common frog *Rana temporaria* was found to predate on *P. spumarius* in Ireland (Blackith *et al.*, 1974).

Entomopathogenic fungi was reported by Kaya *et al.*, (2003) affecting particularly to overwintering of *H. coagulata* adults in citrus. In the case of *P. spumarius*, the genus *Entomophthora* was recorded to infect adults (Whittaker, 1973).

Other lines of work are under study these last years (EFSA PLH, 2019b) such as the Diffusible Signal Factor (DSF), palmitoleic acid (C16-cis), macadamia oil and related DSF homologues in grapevine (Lindow *et al.*, 2014, 2017, 2018) that inhibit X. fastidiosa growth. Endophytic microorganisms that have been capable to control *X. fastidiosa* populations within the plant like *Paraburkholderia phytofirmans* (Burkholderiaceae) in grapevine (Lindow *et al.*, 2017; Lindow *et al.*, 2018; Baccari *et al.*, 2019); *Curtobacterium flaccumfaciens* in *Catharanthus roseus* (Lacava *et al.*, 2007); *Pseudomonas fluorescens* (Pseudomonadaceae), *Achromobacter xyloxosidans* (Alcaligenaceae) and *Cochliobolus* sp. (Pleosporaceae) in grapevine (Rolshausen *et al.*, 2018). Also, bacteriophages in grapevine

(Das *et al.*, 2015) and in cowpea (Hao *et al.*, 2017; EFSA PLH, 2019). Biological control using an avirulent strain of *X. fastidiosa* in grapevine (Hao *et al.*, 2017); and biological control using weakly virulent strains of *X. fastidiosa* in grapevine (Hopkins *et al.*, 2005, 2012a and 2012b).

1.7.3. Chemical control of *X. fastidiosa* and vectors

Insecticides are considered the most expensive and efficient method to suppress vector populations and reduce *X. fastidiosa* spreading (Kruegner *et al.*, 2019). The most important information to know for the applications is what, when, where and how apply them to maximise the mortality of the target pest while minimising non-target effects (Kruegner *et al.*, 2019).

Systemic insecticides such as neonicotinoids and pyrethroids are the most efficient against vectors, but most of the products tested are not registered by the authorities yet (Prabhaker *et al.*, 2006 and 2007; Tubajika *et al.*, 2007; Janse and Obradovic, 2010; Cornara *et al.*, 2018; Dongiovanni *et al.*, 2017a and 2017b; Sabaté and Izquierdo, 2018; Dáder *et al.*, 2019; Kruegner *et al.*, 2019). In this context, Dongiovanni *et al.*, (2017c) tested in Italy the efficacy of the neonicotinoids imidacloprid and acetamiprid, and the pyrethroids deltamethrin and lambda-cyalothrin showing a high mortality rate of *P. spumarius* adults. Not only with adults, Sabaté and Izquierdo (2018) conducted in Spain trials with *P. spumarius* nymphs, deltamethrin caused a huge shock down on the population with a 100 % of mortality after one day of application.

Spittlebugs and sharpshooters are susceptible to insecticides, and it is reported that their control reduced the incidence of *X. fastidiosa* diseases (Krewer *et al.*, 1998; Bethke *et al.*, 2001; EFSA; 2013). Furthermore, they do not develop resistance to insecticides because they only have one or two generations per year (EFSA; 2013). Also, repellents such as kaolin (aluminium silicate) that is accepted for organic management have been also tested for *X. fastidiosa* vectors (Tubajika *et al.*, 2007; Janse and Obradovic, 2010).

Apart from chemical substances to supress vectors, chemical compounds that decrease the biofilm formation of *X. fastidiosa* in different crops are under development (EFSA PLH, 2019) such as the N-acetylcysteine (NAC) in sweet orange (Muranaka *et al.*, 2013), zinc in tobacco (Navarrete de la Fuente, 2015) and Dentamet (zinc, copper, citric acid biocomplex) in olive trees (Scortichini *et al.*, 2018; Tatulli *et al.*, 2021). Also, it is

reported the efficacy of formulations such as bioactive compounds as activators to enhance plant resistance response (EFSA PLH, 2016a) such as the harpin (*Erwinia amylovora*) that works as a natural defence system (SAR) (Tubajika *et al.*, 2007; Janse and Obradovic, 2010). Finally, formulations of growth promoting micronutrients or biological compounds are tested such as soil-applied systemics and insect growth regulators that are compatible with biocontrol due to do not affect to non-target species (Akey *et al.*, 2002; Redak and Bethke, 2003; Almeida *et al.*, 2005; EFSA, 2016a).

1.7.4. Thermotherapy

New phytosanitary methods were developed for controlling *X. fastidiosa* such as heat treatment of dormant plant material (EFSA PLH, 2018; Hilton *et al.*, 2021). The mechanism comprises the submersion in water of dormant, woody planting material such as grafts and cutting (with or without roots) for a given time and temperature (EFSA PLH, 2015b). First steps on this technology to fight against *X. fastidiosa* were developed by Goheen *et al*. (1973) by using hot water treatment (HWT) in vivo to inhibit the causal agent of Pierce's disease in symptomatic vineyards of Napa Valley (California, United States). They conducted a HWT in *X. fastidiosa* infected grapevines (*V. vinifera*) for 3 hours at 45 °C and observed that the disease was eliminated. In Pecan (*Carya illinoinensis*), hot water was useful for eliminating the bacteria in scions before grafting with an immersion of 30 min and 46 °C. Thus, it was reduced the possibility of *X. fastidiosa* graft-transmission (Sanderlin and Melanson, 2008; EFSA, 2013). In Europe, Mannini *et al.*, (2007) tested HWT to combat phytoplasmas in vineyard for three years in the region of Piedmont (Italy) showed a maximum of 20 % of reduction of vine in the non-treated plants, confirming the reliability of the method. EFSA (2015b) recommends a HWT of 50 °C during 45 min for a general

sanitation of the material treated. Recently, Hilton *et al.*, (2021) improved the technique using microwave irradiation in pecan graft wood with combination of dH_2O (sterile deionized water) and CNT_s (carbon nanotubes) that remediate *X. fastidiosa* of grafts.

1.7.5. Cultural practices

Cultural practices have to be adopted to reduce population density and to keep plants in optimum condition (Janse and Obradovic, 2010). These measures will depend on the country legislation and the farmer management. For example, in the Balearic Islands (Spain) it is recommended to tillage, mowing and use herbicides to reduce the appearance of weeds and stop vectors life cycle. Also, it is recommended to manage properly the fertilization and irrigation to avoid plant stress and strengthen the trees against diseases. Moreover, it is suggested to grind and/or burn the remains of pruning. All the recommendations are collected in the guide "Manual de bones pràctiques agronòmiques per a la prevenció de *Xylella fastidiosa*" (GOIB, 2017).

Here is a list of examples of other kind of measures recommended that would be adapted in order to disease epidemiology:

- Iron deprivation.
- Cultivar selection (mostly used in grape).
- Removal of diseased trees in 2 to 5 years old.
- Survey for the disease in June and July and pruning after tree removal with avoidance of heavy summer pruning. Very severe pruning can cure infected trees.
- Rouging wild plums and cherries or other hosts within a ca. 400 m of an orchard.
- Establishing new plantings with no peach or plums closer than 400 m to others.
- Weed control in and around orchards.
- Elimination woods, especially oaks around orchards.
- No routine spraying with insecticides.
- Screen barrier to avoid the movement of insects. It was tested in *H. coagulata* in vineyards and nurseries, but its incompatible with other techniques such as insecticides and biocontrol.

1.8. Sampling methods of *Xylella fastidiosa* vectors

Sampling methods for vectors and their harmonisation are the key to understand the epidemiology of vector-borne diseases in a particular area regarding to the abundance, distribution and phenology. Also, sampling method and repetition frequency should be implemented according each pathosystem (European Commission, 2021; EFSA, 2021). The techniques implemented are those mostly used in general entomology without specific attractants neither special traps for targeted sampling.

The quadrat sampling (i.e., 0.25 m^2 woody frame) is the most frequent method for sampling nymphs of Aphrophoridae and Cercopidae, concretely it has been used for the species *P. spumarius*, *N. campestris*, *N. albipennis* and *Callitettix versicolor* (Bieman *et al*., 2011; Chen and Liang, 2015; Bodino *et al*., 2019; Dongiovanni *et al*., 2019). For other family of vectors, the quadrat is not often, and it is mainly used the direct observation and occasionally other methods (e.g., sweep net, sticky traps, aspirators and beating tray) (Halkka *et al*., 1971; Purcell and Frazier, 1985; Castle and Naranjo, 2008; Albre and Gibernau, 2019).

Regarding the adult sampling, they can be collected using aspirators, sticky traps, sweep net and beating tray (European commission, 2015; EPPO, 2020). For surveillance, the most effective and preferred method for collecting Aphrophoridae is the sweep net (EFSA, 2021) and it has been widely used by several authors (Yurtsever, 2004; Cornara *et al*., 2017; Miranda *et al*., 2017; Cruaud *et al*., 2018; Bodino *et al*., 2019; Cavalieri *et al*., 2019 Bodino *et al*., 2020). If insects cannot be processed immediately, they should be stored under 90-99 % ethanol or at -20 °C. Sticky traps can also be stored at -20 °C (EPPO, 2020).

2. Objectives

The objectives of the present work were the following:

1. To study the bioecology of the *Xylella fastidiosa* insect vectors present in the Balearic Islands:
   1.1. Adult abundance and seasonal dynamics in olive, almond and vineyard crops in Majorca, Minorca, Ibiza and Formentera.
   1.2. Nymph abundance and seasonality in olive, almond and vineyard crops in Majorca.
   1.3. Microcosm study of vectors to increase knowledge on their life cycle.
   1.4. Control *X. fastidiosa* vectors by cultural methods.
2. To assess the prevalence of *X. fastidiosa* in the vectors.
3. To check the vectorial capacity of the major vectors presents in the Balearic Islands.

CHAPTER 1

Seasonal and population dynamics of the potential vectors of *Xylella fastidiosa* in the Balearic Islands.

Abstract

Xylella fastidiosa is an endophytic pathogenic bacterium transmitted by spittlebugs (Hemiptera: Aphrophoridae) that was detected in Europe in 2013. Then, in October 2016, it was detected for the first time in the Balearic Islands (Spain). In this work, a surveillance of vectors (both nymphs and adults) in representative crops of the Balearics, such as olives, almonds, and vineyards from late 2017 to 2021 has been conducted. The nymphal abundance in the cover vegetation was estimated using a woody rectangle of 0.25 m^2 and adults were collected by sweep net from cover vegetation, tree canopies and bordering woody shrubs. We confirmed the presence of two species of Aphrophoridae: *Philaenus spumarius* and *Neophilaenus campestris* in Majorca, Ibiza, Minorca and Formentera. Nymphs were more abundant in the cover vegetation from early March to late May in olive, followed by vineyard and almond. Vectors of *X. fastidiosa* were present in all crops, being more abundant in olive, followed by almond and vineyard crops. Two peaks of adult abundance were observed in all crops, one in April-May coinciding with the end of nymphal stage, and one in October-November. In general, the highest number of adults were collected from the cover vegetation, while presence of adults in crop trees was scarce, particularly during summer months. The present study aims to describe the vectors bioecology in Mediterranean agrosystems and provide useful information to design effective control programs against these insects in infected areas to avoid *X. fastidiosa* spread.

Introduction

Xylella fastidiosa (Proteobacteria: Xanthomonadaceae) (Wells *et al*., 1987) is a Gram-negative bacterium pathogen of plants limited to the xylem and capable of infecting more than 600 plant species. This bacterium causes important diseases in crops such as Pierce's disease (PD) in vineyards, variegated chlorosis in citrus (CVC) and leaf scorch (LS) in different species of *Prunus* sp. *Xylella fastidiosa* species is a notifiable pathogen within the European Union (Decision 2015/789 / EU) and it has a specific contingency plan at national level in Spain (Orden APM/21/2017). The bacterium invades the plant xylematic vessels where replicates and develops into a biofilm that blocks the vessels and can provoke the death of the plant. The time lapse between *X. fastidiosa* inoculation and symptom appearance is highly variable, it depends on the type of plant and ranges from months to years. The host symptoms are not specific and can be confused with mineral deficiencies or drought effects, however, many host plants remain symptomless. The main vectors of *X. fastidiosa* are xylem-sap feeding insects from the Order Hemiptera, suborder Cicadomorpha and three superfamilies: Cercopoidea, Cicadoidea and Membracoidea (Redak *et al*., 2004). In Europe, vector species are included in the family Aphrophoridae, known as spittlebugs (EFSA, 2021). Whereas, in regions such as the USA and Brazil major vectors include species of the family Cicadellidae known as sharpshooters (Redak *et al.*, 2004). However, all xylem-sap feeders should be considered as potential vectors of *X. fastidiosa*. The insect acquires the bacterium when feeding from the xylem of infected trees and replicates in the foregut of the insects (precibarium and cibarium).

Italy was the first country in Europe where *X. fastidiosa* was extensively detected in olive trees in 2013 (Saponari *et al.*, 2013). Subsequently, other outbreaks have been detected in France (Corsica and PACA region) and in greenhouses of Holland, Germany and Portugal. In 2016, it was also detected in the Balearic Islands in Majorca (Olmo *et al.*, 2017) and after that, numerous positives cases were found in Minorca and Ibiza.

Identification and surveillance of *X. fastidiosa* vectors are highly important to understand the epidemiology of the disease in each affected area. Until now, *Phlilaenus*

spumarius L., *Philaenus italosignus* (Drosopoulos and Remane, 2000) and *Neophilaenus campestris* (Fallen, 1805) are considered the *X. fastidiosa* vectors in Europe (EFSA, 2021). In Spain, Aphrophoridae species have been recorded in olive and other host plants (EFSA, 2018). First field surveys of Aphrophoridae in Spain were conducted in Andalusia, Madrid and Murcia in grapevine, citrus and olive groves during autumn 2004 using yellow sticky traps, but no insect resulted positive for the bacteria (Lopes *et al.*, 2014). Preliminary surveillance conducted in the Balearic Islands in 2017 after *X. fastidiosa* detection, showed the presence of *P. spumarius*, *N. campestris* and *N. lineatus* (Miranda *et al.*, 2017).

Morente *et al.* (2018) carried out a survey of the vectors in 2016 and 2017 in olive groves from Portugal, Madrid, Valencia and Andalusia with sweep nets and yellow sticky traps. In that study, *P. spumarius* and *N. campestris* were found in canopy and in cover vegetation, showing peaks of abundance in late-spring and autumn. Finally, Bodino *et al.,* (2019, 2020, 2021) conducted an extensive surveillance in olive groves and vineyards from Italy (Apulia, Liguria and Asti) between 2016 and 2018, showing the presence and seasonality of *P. spumarius*, *N. campestris* and *Aphrophora alni*. Here, the main objective was to study the phenology, abundance and dynamics of the different species of potential vectors in the main crops of the Balearics (olive, almond and vineyard), for both adults and nymphs.

Material and methods

Study sites

The Balearic Islands archipelago is located in the Western Mediterranean (South-Eastern Spain) and includes four inhabited islands: Majorca (3635 km^2), Minorca (695 km^2), Ibiza (571 km^2) and Formentera (82 km^2) (IBESTAT, 2021). The climate in the Balearics is the Mediterranean type, characterized by dry and hot summers and wet mild winters. The annual mean temperature is 21.8 °C and the annual mean precipitation 456 mm (Homar *et al.*, 2010). The natural vegetation in the Balearic Islands is mainly composed by pinewood, oaks and garrigue with mastic and wild olive, being 1322 km^2 the total land used for agriculture where almond, olive and grapevines are considered the main crops (Table 3). Crops in the Balearic Islands are generally set in mosaic mixed crop type landscape,

meanwhile extensive monoculture farms are rare. The agrosystems in where systematic sampling of vectors were conducted included: olive (*Olea europaea* L.), almond (*Prunus dulcis* (Mill.)), and vineyard (*Vitis vinifera* L.) orchards.

Table 3. Area (km^2) devoted to the main crops (almond, olive and vineyard) in the Balearic Islands (IBESTAT, 2021).

	Almond	**Olive**	**Vineyard**
Majorca	155.53	47.59	16.17
Ibiza	9.75	0.96	0.57
Minorca	-	0.06	0.38
Formentera	0.005	0.003	0.13

A total of nine organic farms (±1 ha) were selected in Majorca Island to conduct a long-term sampling consisting in a biweekly surveillance of the insect vectors. We selected three olive farms, three vineyards and three almond orchards located in the municipalities of Algaida, Manacor, Inca and Felanitx (Fig. 16) (Annex I).

Samplings in Ibiza, Formentera and Minorca Islands, were conducted twice a year (spring and autumn) during two intensive sampling days. In Ibiza we selected three almond orchards, three olive farms and three vineyards located in the municipalities of Santa Eulària des Riu and Sant Joan de Labritja (Fig. 16). In Formentera three vineyards and two olive farms located in the sole municipality of Formentera, in the residential areas of Sant Francisco, Sant Ferran de Ses Roques, Es Pujols, Es Caló de Sant Agustí and El Pilar de la Mola (Fig. 16). Samplings in Minorca were conducted in one almond orchard, seven olive orchards and seven vineyard orchards, distributed in the municipalities of Ciutadella, Ferreries, Es Mercadal, Alaior, Maó and Sant Lluís (Fig. 16) (Annex I).

All the plots selected are characterized to carry out an organic management of the crop.

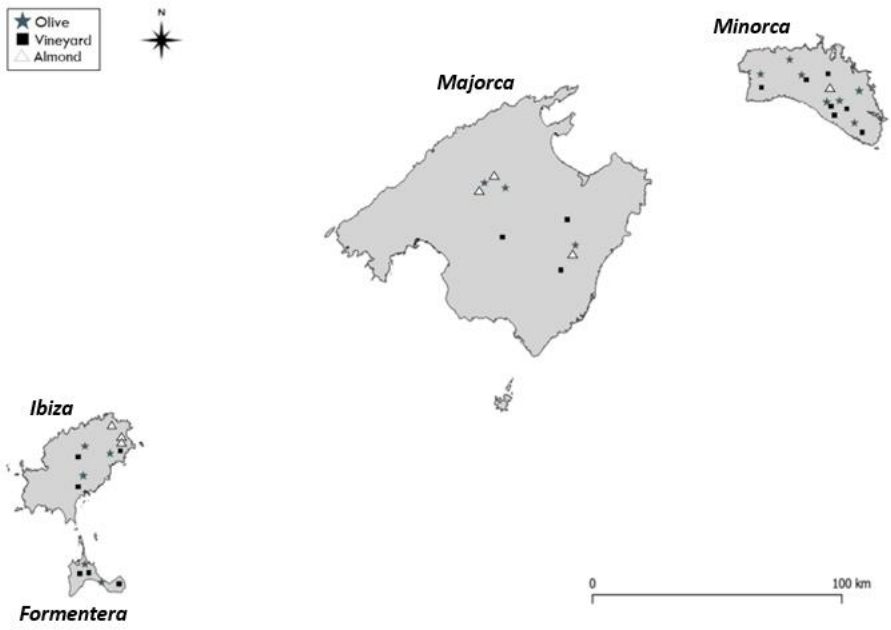

Figure 16. Location of the orchards in the Balearic Islands (Spain) where sampling for adults and nymphs was conducted. Majorca: three olive farms, three vineyards and three almond orchards. Ibiza: three almond orchards, three olive farms and three vineyards. Formentera: three vineyards and two olive farms. Minorca: one almond orchard, seven orchards and seven vineyard orchards.

Insect surveillance

In Majorca, sampling started from February 2018 until February 2021. The sampling in the other islands started in November 2017 in Ibiza, in June 2018 in Minorca and June 2019 in Formentera. Sampling in these islands were conducted until November 2020.

The insect collection was performed inside a homogenous 1 ha area (primary sampling unit (PSU) in the selected field plots. The secondary sampling unit (SSU) included the herbaceous cover vegetation (SSU-cover), the tree canopy (SSU-tree) and the bordering woody shrubs (SSU-border) following the methodology of Bodino *et al*., (2019).

Nymphs were sampled in Majorca in the herbaceous cover vegetation from February until May-June by direct observation of spits produced by the nymphs. In order to determine the moment of zero nymphs and the early detection in the following

month, the sampling started in February. Nymphal population was monitored using a quadrat sampling method. At each sampling, 30 quadrats of 0.25 m^2 (woody rectangle of 100 x 25 cm each) were randomly positioned within a transect of 100m in the cover vegetation of each SSU (Bodino *et al.*, 2019). In each quadrat, we recorded the number, stage and species of the nymph. Nymphs were identified in the field following Vilboste (1982) and Zenner *et al.* (2005).

Adults were sampled using a triangle-shaped sweep net (38 cm each side) on the three types of vegetation described by the SSU (Fig. 17). Methodology followed Di Serio *et al.*, (2019) with some modifications.

Figure 17. Sampling of adult *X. fastidiosa* vectors from tree canopy with sweep net. © J. López-Mercadal.

In the case of the SSU-tree, eighteen randomly selected trees were sampled in each plot, sweeping around the full tree canopy. For vineyards, five rows of 100 m of vineyard canopy were sampled. Due to the sampling effort (number of sweeps) was higher in vineyards (100 sweeps/row) compared to almond and olive trees (average of 20 sweeps/tree), the results are represented by number of insects/sweeps. Sampling on SSU-border was carried out on five randomly selected shrubs from each PSU, sweeping 20 times

for each individual. Finally, sampling in SSU-cover was carried out by sweeping 5 transects line of 20 m inside the crop.

The adult spittlebug samplings were carried out in a conservative sampling strategy considering that previous data (Miranda *et al.*, 2017), indicated that population of Aphrophoridae in the Balearics are scarce compared to elsewhere. For this, only one in three collected insects were kept for further analysis. All captured insects were sexed and identified on field before being released. Aphrophoridae species were morphologically identified according to the morphological keys (Bieman *et al.*, 2011; Kunz *et al.*, 2011; Mozzaffarian *et al.*, 2015; Wilson *et al.*, 2015).

Statistical analysis

Firstly, we used a negative binomial general lineal model (GLM) to assess the effect of the kind of crop, species (*P. spumarius* or *N*. campestris), temperature, month, year, week, day+day^2, locality and plot on the density of spittlebug nymphs. Models included the density of spittlebug nymphs as the dependent variable, expressed as the number of nymphs per square meter. In each case, type of crop, vector species, month, year, locality and plot were included as fixed factors. The other independent variables were included as covariates.

To analyse differences in the densities of *P. spumarius* and *N. campestris*, in the different years and crops, we performed a lrtest comparing the null model with the model that included the year or the crop.

Secondly, we used a zero-inflated model was performed to assess the effect of the kind of crop, species (*P. spumarius* or *N. campestris*), temperature, month, year, week, day+day^2, locality, plot, season and vegetation on the density of adult spittlebug. Models included the density of adult spittlebug as the dependent variable, expressed as the number of adults per sweeps. In each case, kind of crop, species, month, year, locality, plot, season and vegetation were included as fixed factors. The other independent variables were included as covariates. To analyse differences in the densities of *P. spumarius* and *N.*

campestris, in the different years and crops, we performed a lrest comparing the null model with the model that included the year or the crop.

Analyses were performed in R software V.3.2.5 (R Core Team 2017). All GLMs were fitted using the packages "glm.predict" (Schlegel, 2021) and "pscl" (Jackman *et al.*, 2015). Model selection was performed using the Akaike Information Criterion corrected for small sample sizes, AIC (Burnham and Anderson, 2002).

Results

Environmental parameters

Among the years of study, the mean temperature was 17 °C and the precipitation had a mean value of 40.1 mm, reaching the maximum in 2018 with 47 mm. In the municipality of Inca the mean temperature among the years was about 17.1 °C with a precipitation of 41.4 mm. In Manacor, the mean value of temperature was 16.4 °C and the precipitation 46.4 mm. In Felanitx, the mean value of temperature from 2018 to 2020 was 17 °C and 42.6 mm of precipitation. Finally, in the municipality of Algaida the mean temperature was 17.3 °C and the mean precipitation 30mm.

The description of the phenology of olive, almond and vineyard crops was conducted during 2020 according to BBCH standards about growth stages (GS). In the case of olives, during January until 19th of February trees showed the buds in development (01-03-07-09 GSs) and apical leaves starting its development (11 GS). The 19th of February started inflorescence development (53 GS) and flowering (60-65 GS) until early May, when 12th of May fruit development (71 GS) was detected. Fruit ripening (81-89 GS) lasted until October, when olives were collected.

In the case of almond trees, in January bud (00-01 GS) and leaf development (11-19 GS) were observed in the field until 5th of February when the inflorescence appeared (51-53 GS). Fully flowering (60-61-65-67 GS) and leaf development were observed until mid-March as the 19th we detected the fruit development (72 GS). Fruit ripening (81 GS) occurred from May until the 9th of July when the separation of the fruit exocarp started (87

GS), being completely matured (90 GS) by the end of the month until October. Then, trees lost leaves and the rest of exocarps.

For vineyard, in January 2020 we observed different GGs from dormant bud (00 GS), bud sweeling (01 GS), bud opening (09 GS) and first leave opening (11 GS). Then, the 5th of February vine plants were pruned and the 20th of March leaves started to open (13 GS). The 15th of April leaves were entirely open (19 GS) and inflorescence were clearly visible (53 GS). The 29th of April, flowers were closely pressed together (55 GS). The 13th of May flowers started separating (61-68 GS) and the 27th development of fruit started (71 GS). Then, fruit started ripening (81) until grape collection in August-September and leave decoloration and falling started (91-95) until the end of the year. Total leave falling (97 GS) was observed by 26th December.

Xylella fastidiosa *vectors nymphal abundance, seasonality and host preference*

In general, considering all data collection from 2018 to 2020, nymphs of the vector species of *X. fastidiosa*, *P. spumarius* and *N. campestris*, were observed in field conditions (macrocosm) in Majorca from March to early June (Fig. 18). The nymphs of *P. spumarius* were more abundant (0.03 nymphs/m^2), while the density of nymphs of *N. campestris* (0.005 nymphs/m^2) was twelve times lower than *P. spumarius*. The peak of nymph abundance was between end-March and early-April (Fig. 18).

The density of *P. spumarius* nymphs was significantly higher than *N. campestris* (Estimate: 2.462; Std. Error: 0.873; P-value = 0.005). Both species, *P. spumarius* and *N. campestris* adults were detected each spring from 2018 to 2020 in olive, almond and vineyard crops, with no effect on the nymphal density because of the year sampled (d.f. =2; X^2= 0.882) and the kind of crop (d.f. =2; X^2= 0.691).

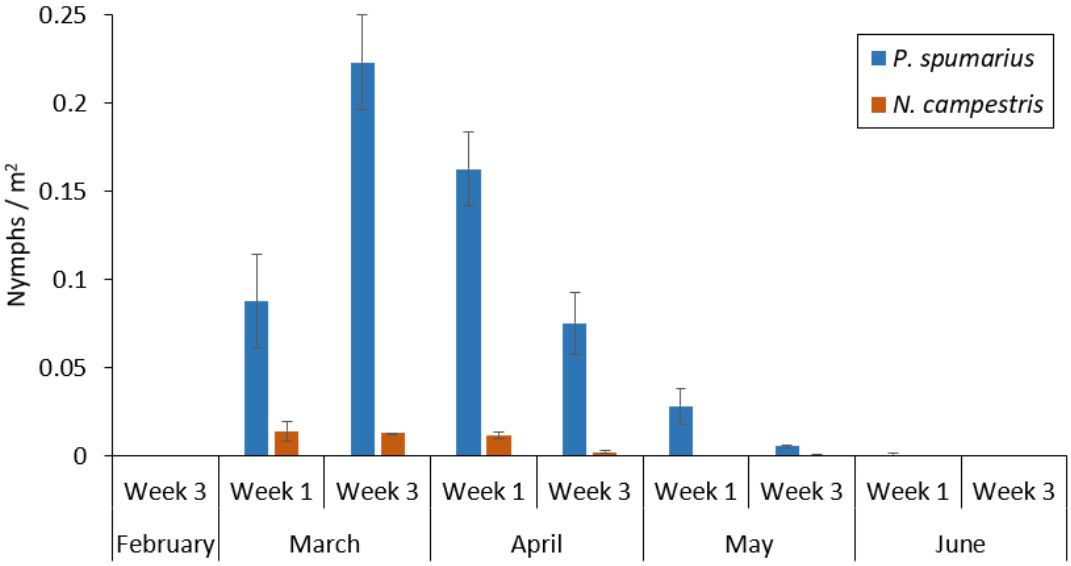

Figure 18. Seasonal pattern and abundance of *P. spumarius* and *N. campestris* nymphs in Majorca from 2018 to 2020.

In regard to the density of nymphs per crop (almond, olive and vineyard), for *P. spumarius*, the highest density (nymph/m^2) was detected in the olive plots (0.08-5.55 nymph/m^2) followed by vineyard and almond crops (Fig. 19) both in 2018 and 2019. Contrary, in 2020 the highest nymph density was detected in vineyard plots (0.05-0.45 nymph/m^2), followed by olive and almond plots (Fig. 19). In general, all crops showed the highest density of nymphs between the 3rd week of March and the 1rst of April. In 2018 no nymphs were detected during the 1rst week of March, but they were detected in all crops during the 3rd week of March. In 2019 the sampling started in February and the first nymphs were detected during the 1rst week of March. In 2020 the sampling started in January and first nymphs were detected the first week of March in vineyard and olive crops and the third week of March in olive crop. In our area, the first populations of nymphs were detected between the 4th week of February and the 1st-2nd week of March, depending on the climatic conditions of the year that may drive nymphal survival. In 2018 and 2020 nymphs were detected in all crops until the 1st week of May, while in 2019 nymph detection was extended until the 1rst week of June. From the obtained results, nymphs of *P. spumarius* are detected in the cover vegetation of the different crops (olive, vineyard

and almond) from early March to the end of May, depending on the climatic conditions of the year.

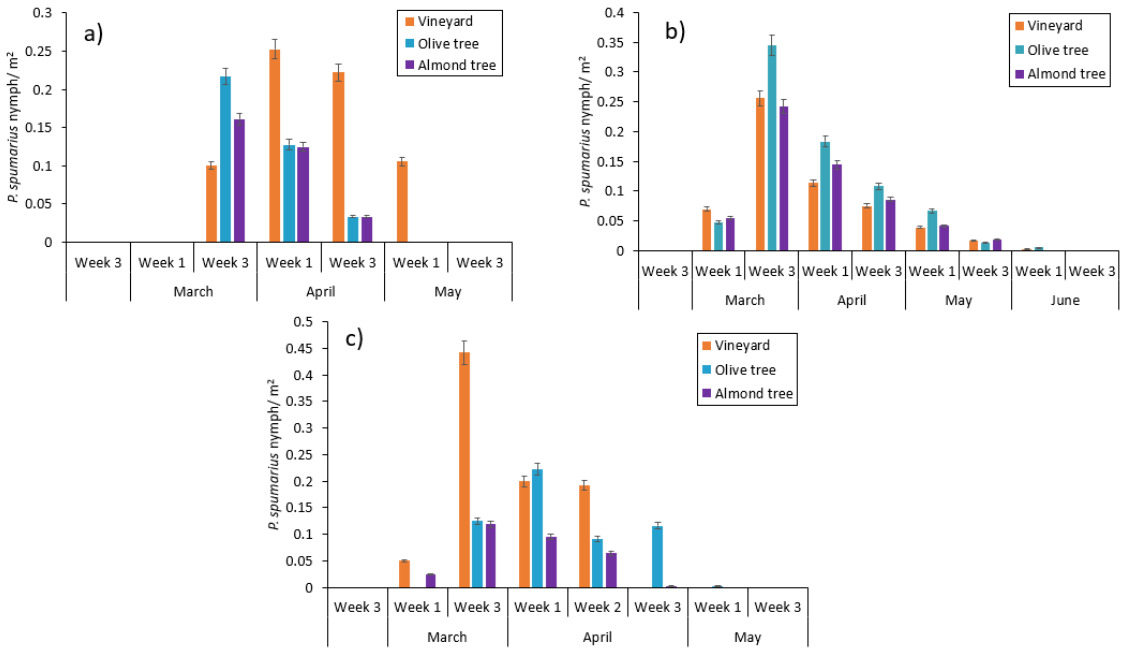

Figure 19. Seasonality of *P. spumarius* nymphs per crop in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

In regard to the instar distribution of nymphs (Fig. 20), instars distribution was different over the time from N1 to N5 for 2018, 2019 and 2020. Nymphs N1 were not recorded in 2018 and only in low population during the 1rst week of March in 2019 and 2020. Instars N2 and N3 were more frequent in March and early April, while N4 and N5 instars were more frequent in late April-May. We detected nymphs (low population) in early June 2019.

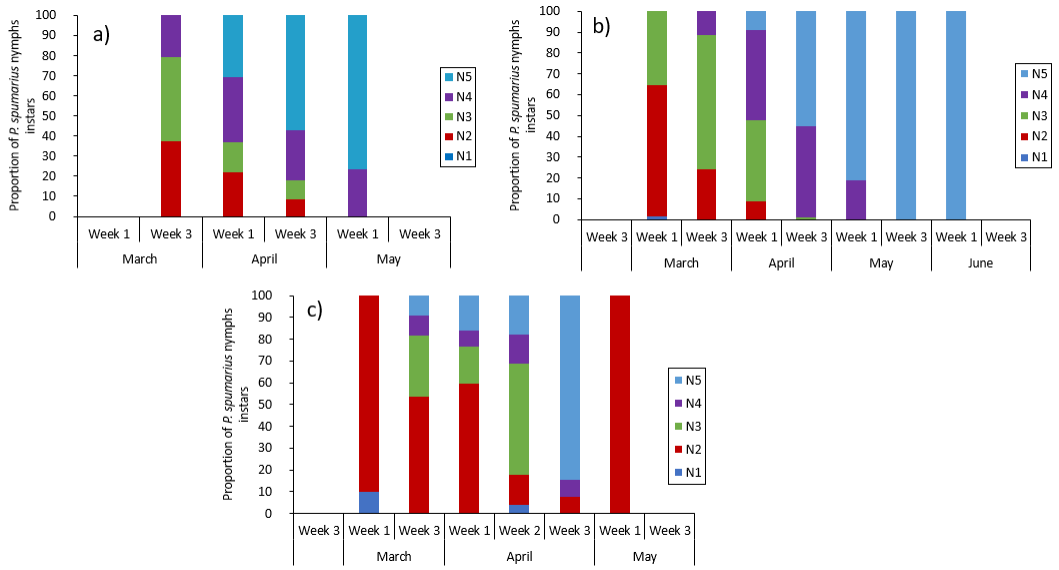

Figure 20. *P. spumarius* nymph instars proportion in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

The highest density of nymphs of *N. campestris* was detected in olive and almond crops during the 3rd week of March in 2018, the 1st week of March in 2019 and the 1st week of April in 2020 (Fig. 21). Density of nymphs decreased substantially from the first week of April being similar in all crops (0.2-0.7 nymphs /m^2) except in 2020. Nymphs were absent in the almond crop during the 3rd week of April in 2018, during the 1rst and 3rds weeks of May in 2019 and from the 3rd week of April in 2020. Last detection of nymphs was in vineyard and olive crops during the 3rd week of May in 2019. Considering the timeframe of sampling we applied (biweekly) and the results from three years, *N. campestris* nymphs were detected from the 1st - 2nd week of March to the 4th week of April in vineyard and olive crops, while nymphs seemed to be absent earlier (3rd week of April) in the almond crop. In 2020, *N. campestris* nymphs were hard to detect, as few nymphs were observed from the 3rd week of March until the 2nd week of April. We did not find significant differences between the density of *N. campestris* nymphs among crops (d.f. = 2; X^2= 0.925)

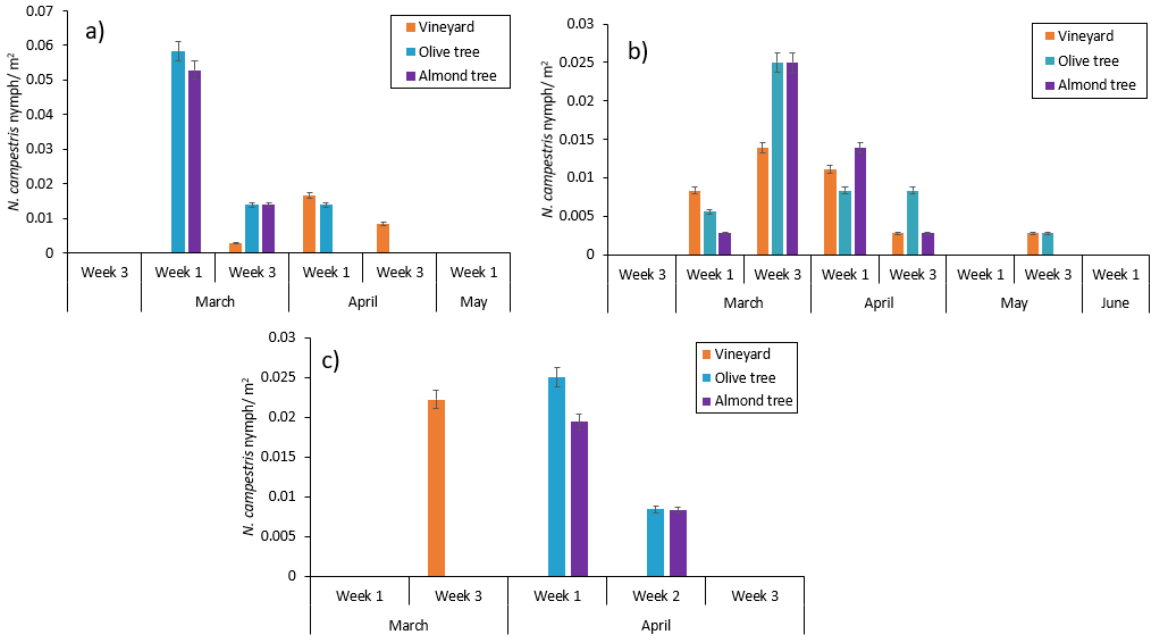

Figure 21. Seasonality of *N. campestris* nymphs in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

The seasonal distribution of nymph instars of *N. campestris* showed similar pattern as in the case of *P. spumarius*. Youngest nymphs (N1-N2) were found from early March to early April, while N4 and N5 were found mainly in late April (Fig. 22).

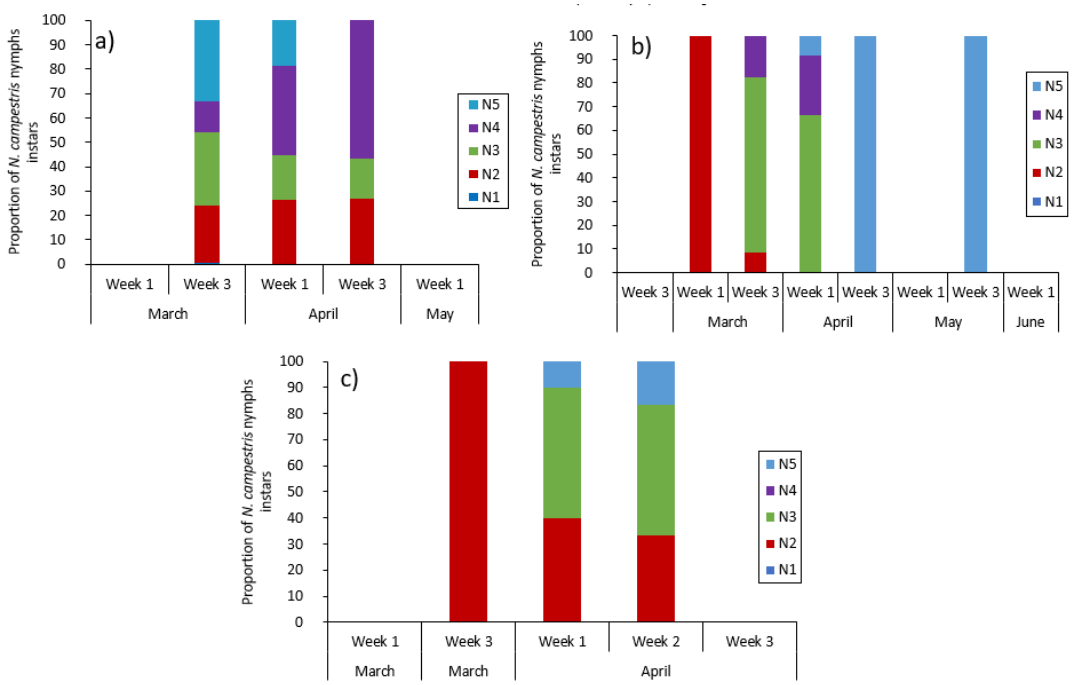

Figure 22. *N. campestris* nymph instars distribution in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

Regarding the position of nymphs in the plant cover vegetation (upper, medium, bottom), we observed a trend for the bottom position in *N. campestris* in all years of sampling (Fig. 23). For *P. spumarius*, in 2018 we did not observe any clear pattern of position, however in 2019 the highest number of nymphs was found in the medium part of the plant.

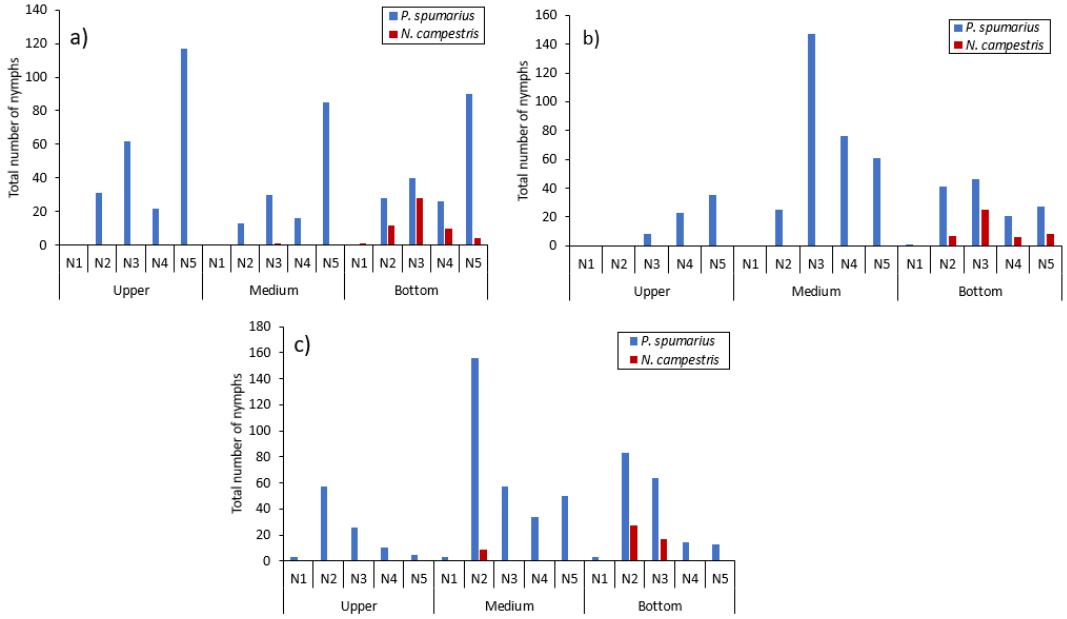

Figure 23. Position (upper, medium, bottom) on the plant of the nymph instars of *P. spumarius* and *N. campestris* in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

When analysing the position of the nymphs in the cover vegetation by crop, in 2019 and 2020 nymphs of *P. spumarius* were detected most frequently at the medium part of the plants for all the crops (Fig. 24), while in 2018, nymphs were detected similarly distributed among all positions in the plant. In the case of *N. campestris*, in 2018 and 2019 nymphs were detected at the bottom of the plants for all crops, but in 2020 some were detected at the upper position of in the olive crop cover plants and at the medium position in vineyard cover plants (Fig. 24).

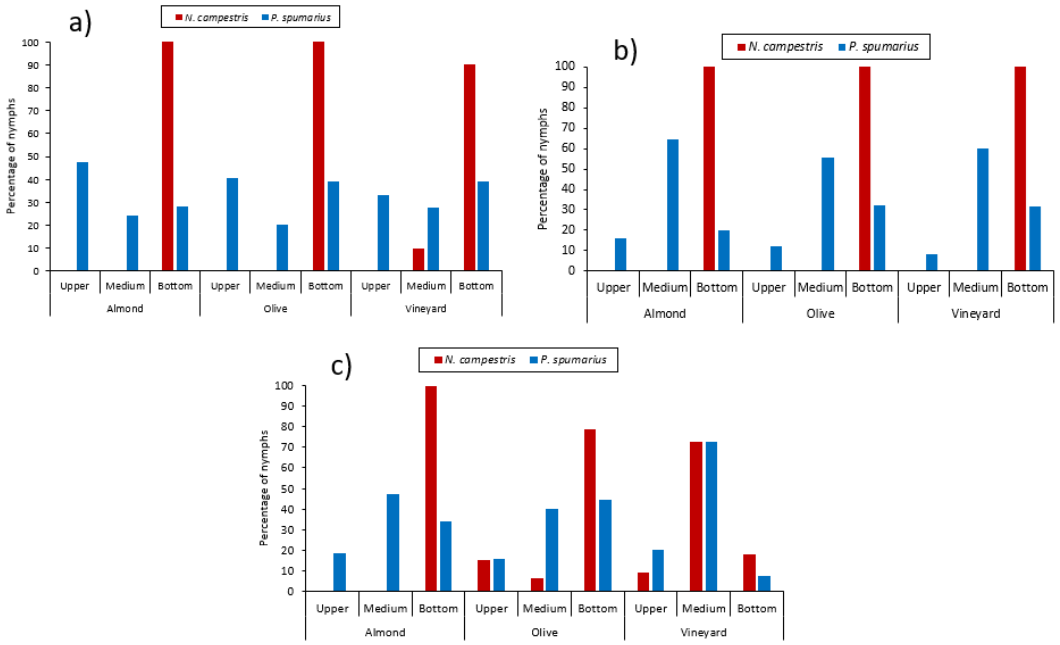

Figure 24. Position on the plant of the cover vegetation by crop of the nymph instars of *P. spumarius* and *N. campestris* in 2018 (a), 2019 (b) and 2020 (c) in Majorca.

Nymphs were observed in 31 plant species, grouped into 17 genera and 12 plant families: Compositae, Cruciferae, Geraniaceae, Guttiferae, Labiatae, Leguminosae, Malvaceae, Plantaginaceae, Poaceae, Primulaceae, Rubiaceae, and Umbelliferae (Fig. 25, 26 and 27). *Philaenus spumarius* nymphs were observed in all the reported family plants, showing preference for Compositae and Leguminosae, in particular for the genera *Glebionis*, *Sonchus*, *Carduus*, *Plantago* and *Erodium*. In 2018 and 2019, *P. spumarius* nymphs were more abundant in *Glebionis* and *Sonchus* species, while in 2020 in *Medicago* and *Calendula* species.

Meanwhile, *N. campestris* nymphs were only observed in Poaceae plants in 2018 and 2019, but in 2020 were sporadically observed in Compositae (*Carduus* spp.) and species of Geraniaceae, but still showing high preference for Poaceae plants.

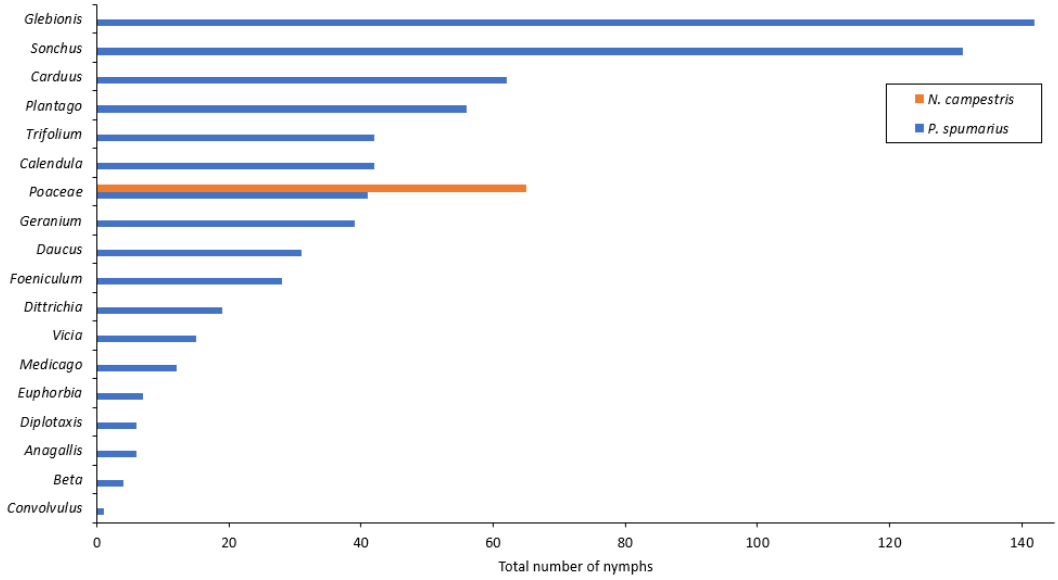

Figure 25. Host-plant preference of *P. spumarius* and *N. campestris* in Majorca in 2018. Preference is showed at genera level except for the Poaceae (family).

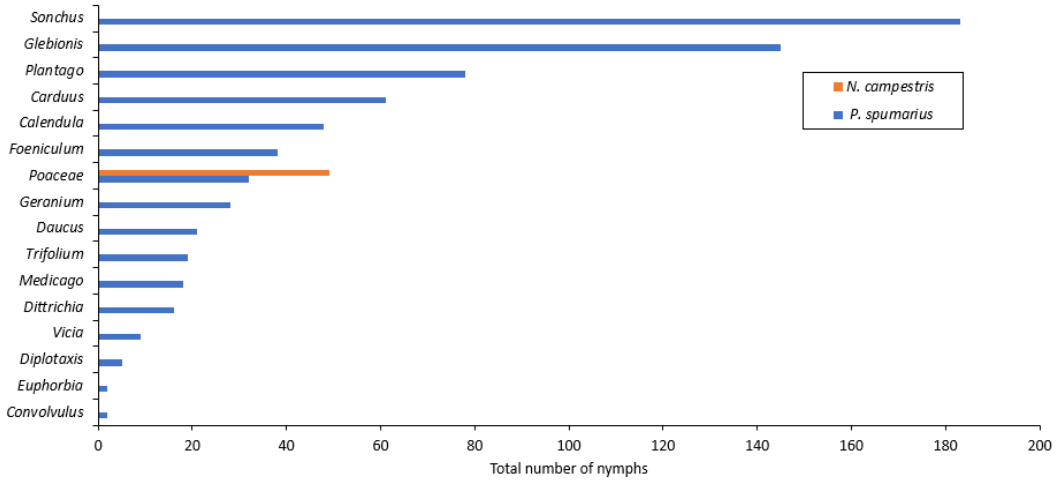

Figure 26. Host-plant preference of *P. spumarius* and *N. campestris* in Majorca in 2019. Preference is showed at genera level except for the Poaceae (family).

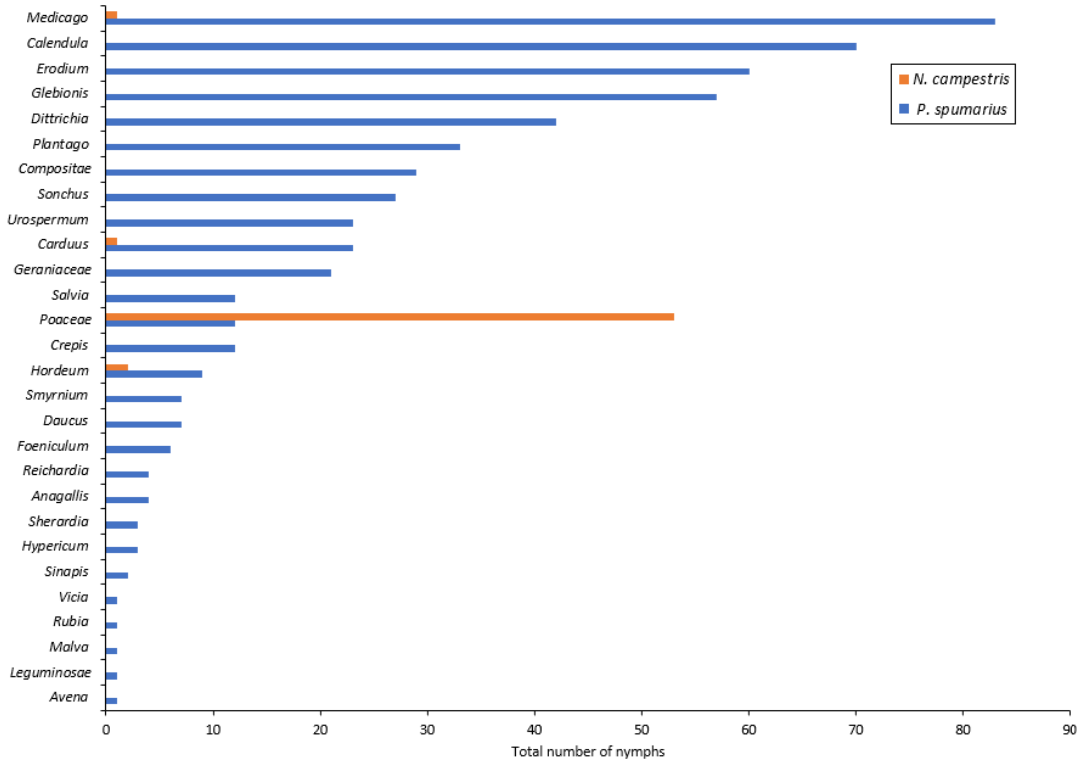

Figure 27. Host-plant preference of *P. spumarius* and *N. campestris* in Majorca in 2020. Preference is showed at genera level except for the Poaceae and Leguminosae (families).

The distribution of plants was equal among crops (Fig. 28, 29, 30), but *P. spumarius* was more abundant in cover of olive crops. In 2018 and 2019, *P. spumarius* was recorded in a high number of plant taxon in vineyard, followed by almond and olive (Fig. 28, 29). In the case of 2020, olive crops seem to have higher variety of cover plant composition than vineyard and almond (Fig. 30).

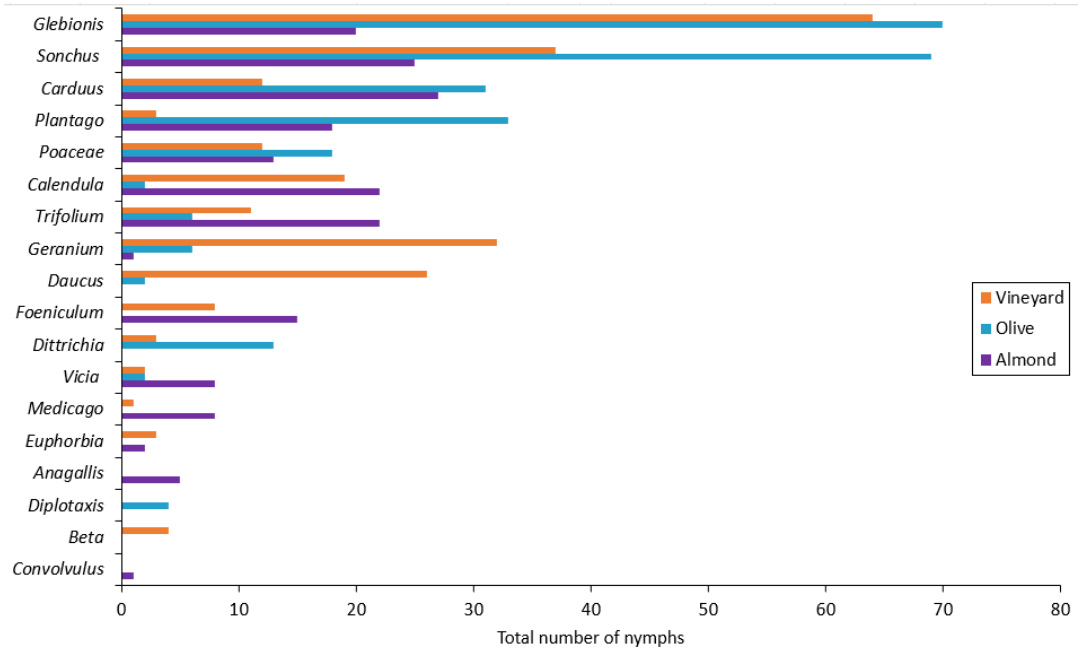

Figure 28. Host-plant preference of *P. spumarius* in Majorca in 2018. Preference is showed at genera level except for the Poaceae (family).

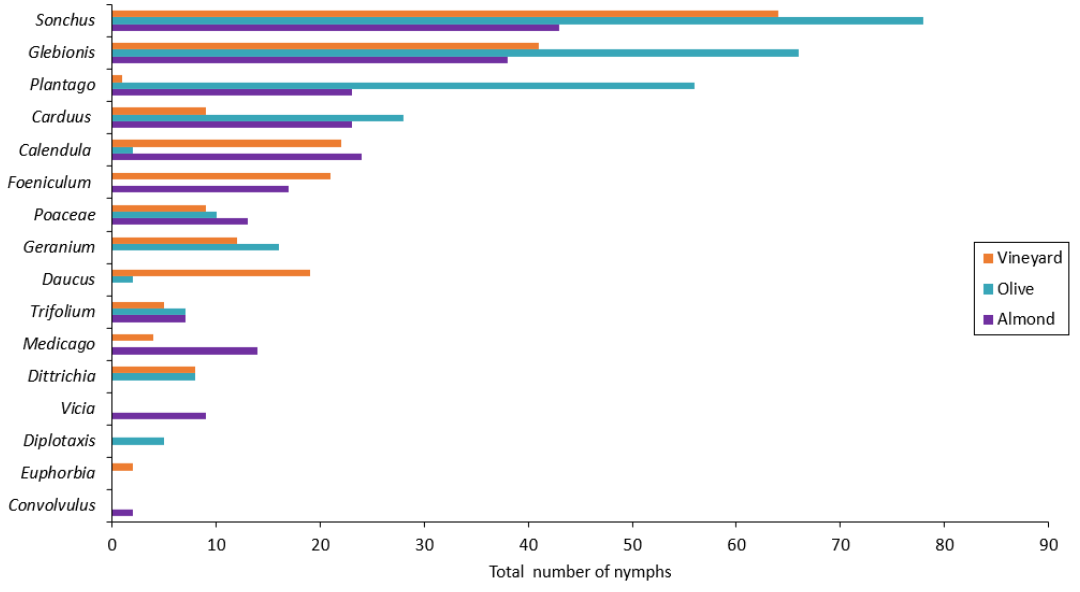

Figure 29. Host-plant preference of *P. spumarius* in Majorca in 2019. Preference is showed at genera level except for the Poaceae (family).

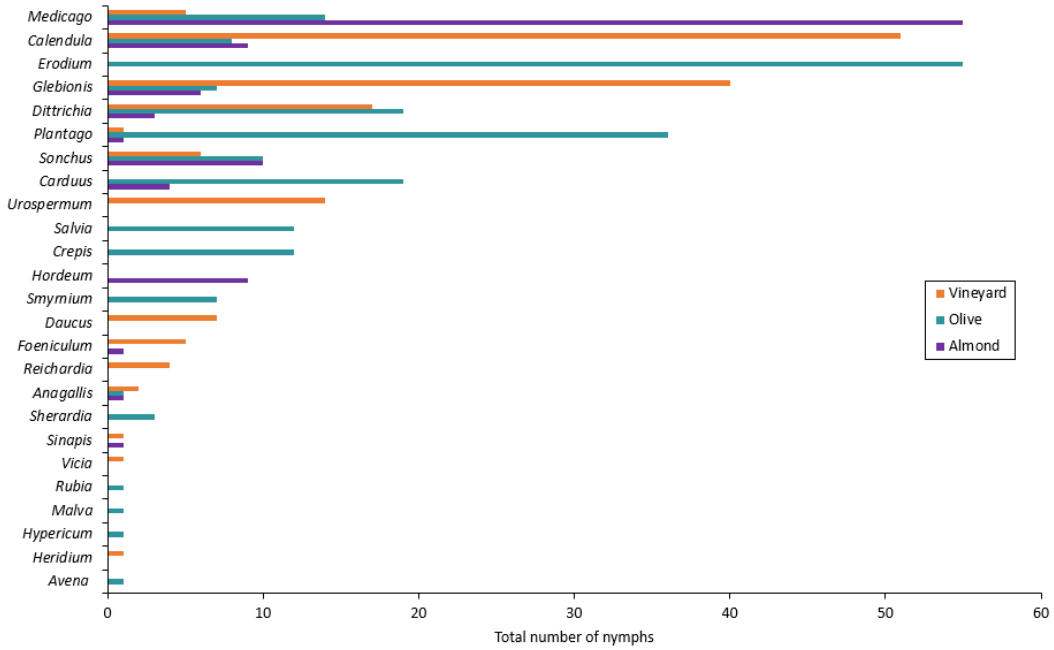

Figure 30. Host-plant preference of *P. spumarius* in Majorca in 2020. Preference is showed at genera level except for the Poaceae (family).

For *N. campestris*, it was only found in Poaceae plants in 2018 and 2019 in almond, olive and vineyard. But, in 2020 nymphs were also recorded in *Medicago* spp. (Fabaceae) in almond and in *Carduus* spp. (Compositae) plants in olive (Fig. 31).

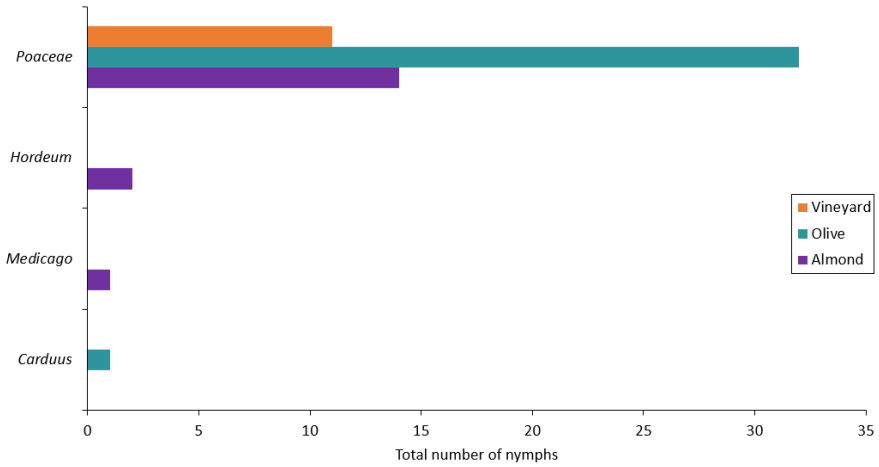

Figure 31. Host-plant preference of *N. campestris* in Majorca in 2020. Preference is showed at genera level except for the Poaceae (family).

Xylella fastidiosa *vectors adult abundance and seasonality*

The general seasonal pattern of Aphrophoridae from 2018 to 2020 in the Balearic Islands for each of the SSU is represented in Fig. 32. Adults of Aphrophoridae were collected from all SSU, but seasonal pattern differed from one each other. Adults were found in the SSUa predominantly in spring and autumn, in the SSUt in spring- summer and in the SSUs in summer.

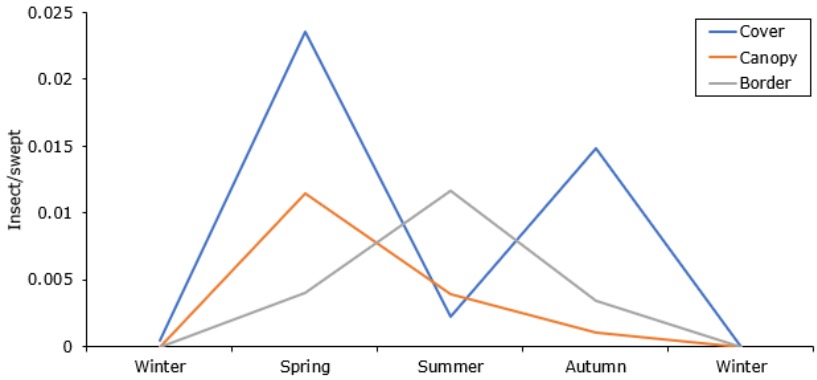

Figure 32. General adult seasonal pattern of Aphrophoridae in the Balearic Islands from 2017 to 2020. Each of the SSU are represented: SSUa: cover; SSUt: canopy; SSUs: border.

The species *P. spumarius* was more abundant than *N. campestris* in each of the SSU (Fig. 33). Both species were more abundant in the SSUa (cover) compared to SSUt (canopy) and SSUs (border).

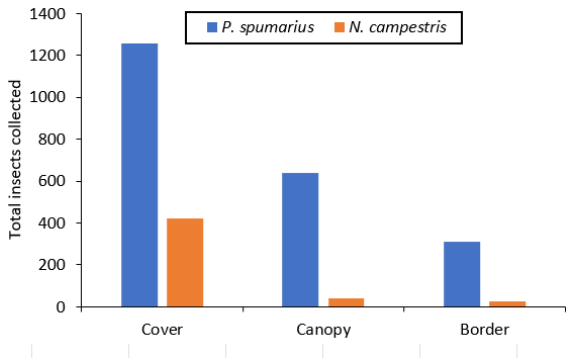

Figure 33. Abundance of *P. spumarius* and *N. campestris* per SSU in the Balearic Islands from 2017 to 2020. Each of the SSU are represented: SSUa: cover; SSUt: canopy; SSUs: border.

Philaenus spumarius was also the most abundant species in all sampled crops (Fig. 34). This species was more abundant in olive crop, followed by vineyard and almond orchards. The abundance of *N. campestris* was similar in all crops.

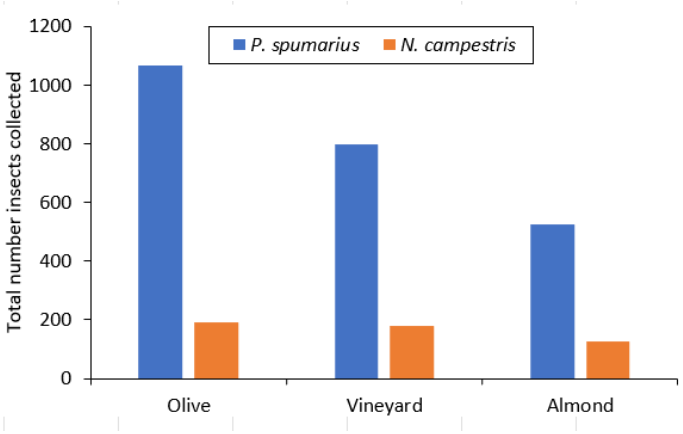

Figure 34. Abundance of *P. spumarius* and *N. campestris* per crop (olive, vineyards and almond) in the Balearic Islands from 2017 to 2020.

The number of Aphrophoridae collected varied from one year to another despite the similar effort of sampling (except for 2017) (Table 4). The highest number of adults for both species was collected in 2018, followed by 2020 and 2019. For all years, *P. spumarius* resulted more abundant than *N. campestris*. In Majorca, the sampling was annual and resulted in 1990 Aphrophoridae collected from 2018 to 2020. From those, 1723 were *P. spumarius* (86.6 %) and 267 *N. campestris* (13.4 %). In Minorca, Ibiza and Formentera samplings were conducted only twice per year (two days in spring and autumn) all years, except for Formentera where the sampling in 2018 was not conducted. In Minorca, out of 269 Aphrophoridae collected, 177 were *P. spumarius* (65.8 %) and 92 *N. campestris* (34.2 %). In the case of Ibiza, from 440 Aphrophoridae collected, 221 were *P. spumarius* (50.2 %) and 219 *N. campestris* (49.8 %). Finally, in Formentera, 51 Aphrophoridae were collected from those 28 were identified as *P. spumarius* (54.9 %) and 23 as *N. campestris* (45.1 %).

Table 4. Number of adults of Aphrophoridae (males and females) collected in the Balearic Islands 2017-2020. NS: No Sampling; PS: *Philaenus spumarius*; NC: *Neophilaenus campestris*.

	2017				2018				2019				2020					
	PS		NC		PS		NC		PS		NC		PS		NC		PS/NC	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀		TOTAL
Majorca	NS	NS	NS	NS	496	531	74	66	105	175	21	35	163	253	24	47	1723/267	1990
Minorca	NS	NS	NS	NS	23	55	12	11	11	25	8	8	26	37	25	28	177/92	269
Ibiza	19	74	16	70	17	60	21	63	2	9	9	10	14	26	7	23	221/219	440
Formentera	NS	NS	NS	NS	NS	NS	NS	NS	7	15	2	11	1	5	5	5	28/23	51
TOTAL	19	74	16	70	536	646	107	140	125	224	40	64	204	321	61	103		2751

From the 1723 *P. spumarius* collected in Majorca, 11 morphotypes were recognized, from which Populella, Fasciata and Quadrimaculata (Fig. 36) were the most common found (Fig. 35). Some morphotypes were not previously described in the literature and were categorized as "Without name" following Biedermann and Niedringhaus (2009). Finally, the morphotype of 82 specimens of *P. spumarius* was not recognized because of preservation reasons or specimen deterioration (labelled as unidentified).

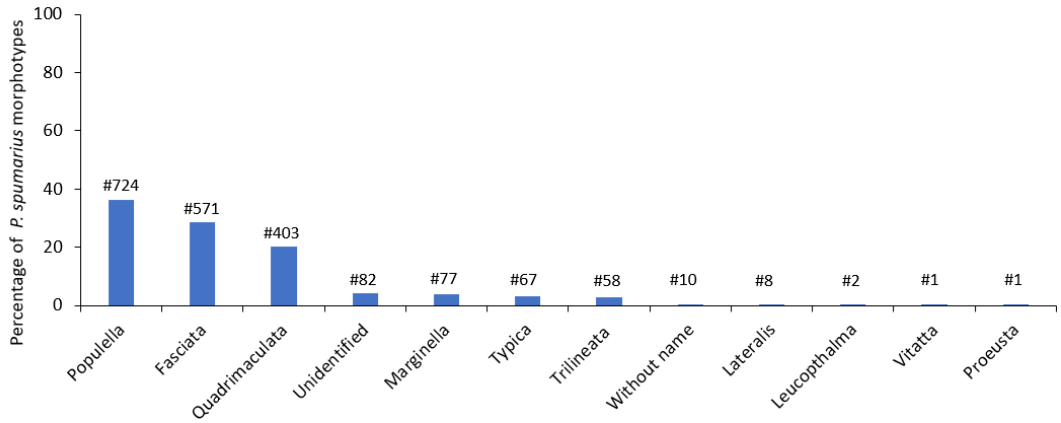

Figure 35. *Philaenus spumarius* morphotypes identified in Majorca between 2018 and 2020.

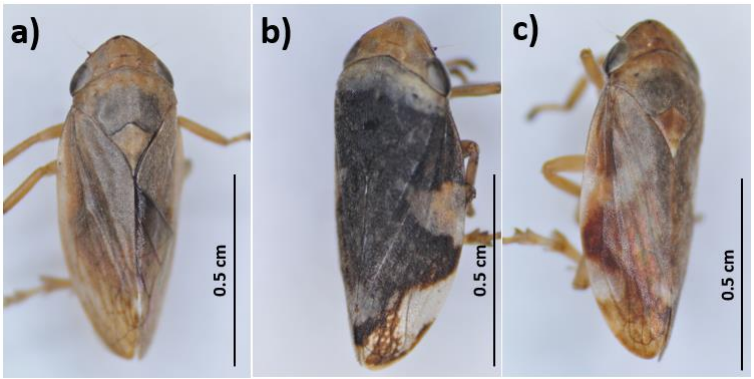

Figure 36. The most common *Philaenus spumarius* morphotypes identified in Majorca between 2018 and 2020. a) Populella; b) Fasciata; c) Quadrimaculata.

For Minorca, Ibiza and Formentera, 7 morphotypes of *P. spumarius* were recognized (Fig. 37), from which Quadrimaculata, Populella and Fasciata were the most abundant as in Majorca.

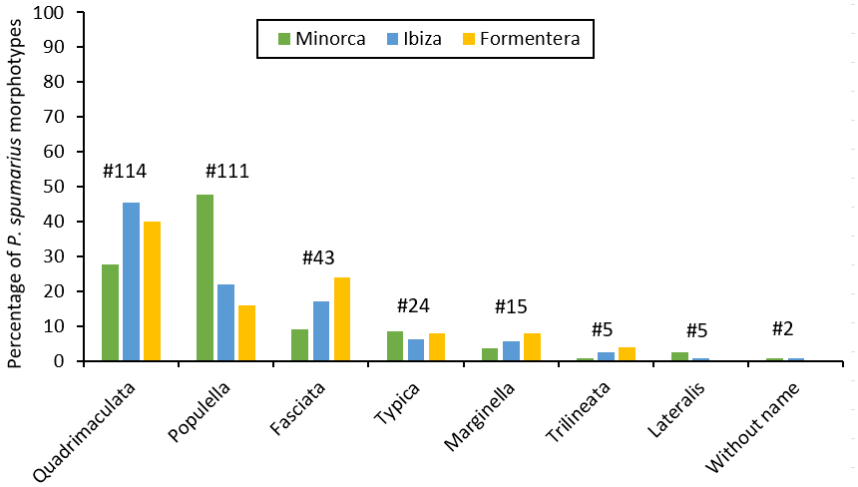

Figure 37. *Philaenus spumarius* morphotypes identified in Minorca, Ibiza and Formentera between 2017 and 2020.

Seasonal pattern of *P. spumarius*:

Adults of *P. spumarius* showed different seasonal pattern depending on the SSU (Fig. 38) for the three years of sampling (2018 to 2020). For all years, the highest abundance

of adults was recorded in May in the cover vegetation (SSUa- herbaceous plants) followed by a second peak located in October. The first peak in May was related to the completion of the development of nymphs on the herbaceous plants. The second peak in October corresponded to the presence of adults in the cover vegetation for mating and oviposition. The presence of adults decreased in the SSUa and increased in the SSUt (adults on trees) in June in all years. In 2018 adults in SSUt decreased in August and adults in the SSUs (adults on shrubs) increased, however, the pattern was different in 2019 and 2020, when we found adults from May to October in higher proportion in the SSUs compared to SSUt. Both types of vegetation, trees and particularly border vegetation species (e.g., *P. lentiscus*), seemed to play a relevant role in the dynamics of adults during the summer months.

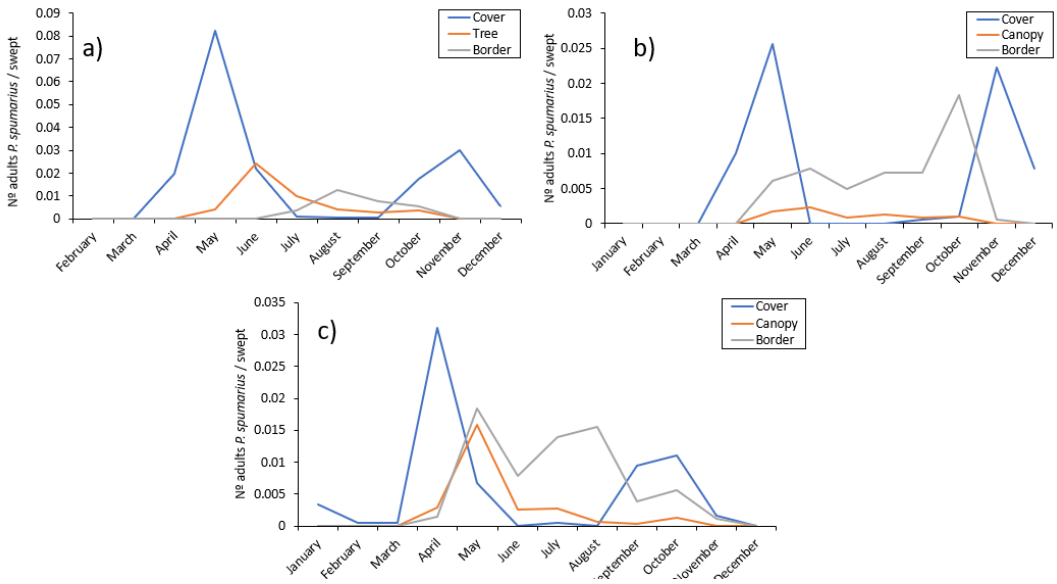

Figure 38. Seasonal pattern and abundance of *P. spumarius* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the different SSU.

Seasonal pattern of *N. campestris*:

In the case of *N. campestris* (Fig. 39) the difference among adults captured in the SSU was greater than *P. spumarius*. The highest abundance of adults was detected in the SSUa in May and November, similarly to the peaks of abundance described for *P.spumarius*.

The peak in May was always the highest one, except in 2019. The presence of adults of *N. campestris* can be considered as negligible in the SSUt and SSUs since the number of adults collected on those SSU was extremely low (less than 0.025 adults/sweeps), in particular during 2018 and 2019. In general, *N. campestris* was found in lower abundance in all SSU when compared to *P. spumarius*.

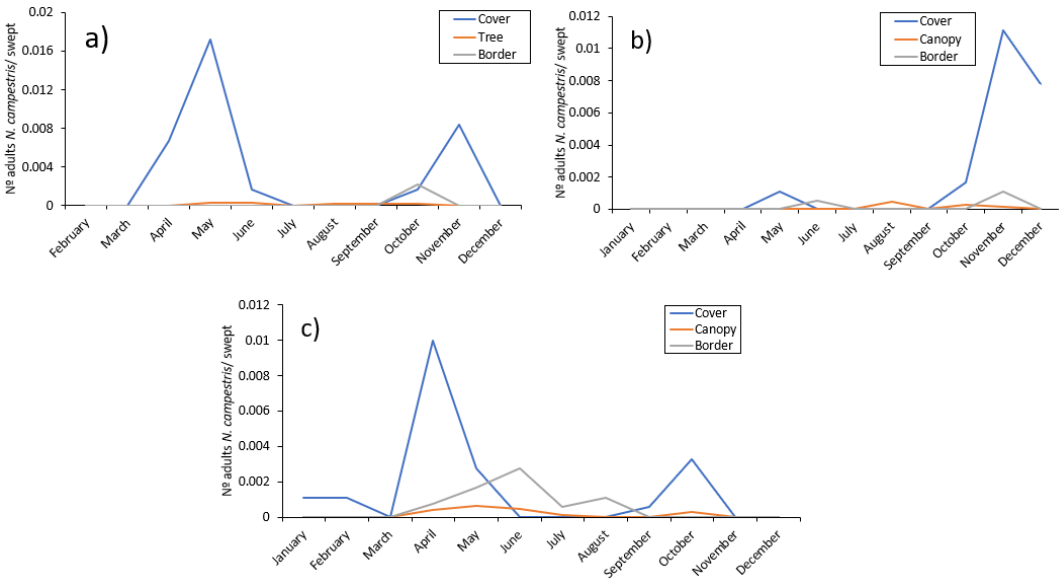

Figure 39. Seasonal pattern and abundance of adults of *N. campestris* in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the different SSU.

Seasonal pattern of *P. spumarius* per crop and SSU:

The seasonal pattern for *P. spumarius* was similar in all crops for the SSUa (cover vegetation) (Fig. 40). Adults showed similar pattern in olive, vineyard and almond crops in all years of sampling, with a peak of adults in May and a second one between October-November. Almond crop showed lower abundance of adults compared to olive and vineyard crops. In particular, the peak of October- November showed that adult population in the cover vegetation associated to the almond crop was three to four times lower than in vineyard and olive crops.

In the case of SSUt the pattern was different compared to SSUa (Fig. 41). For all years, the highest number of adults was recorded in June for olive and almond trees, while

presence of adults in the vineyard plants could be considered anecdotal in terms of abundance. Finally, seasonal pattern of adults in SSUs (Fig. 42) showed a more variable seasonal pattern compared to SSUa and SSUt. In general, adults were more abundant in the SSUs from April up to November, showing a peak in August for 2018, between September and October for 2019 and between May and August for 2020. Abundance found in the SSUs was similar to that found in the SSUa and SSUt.

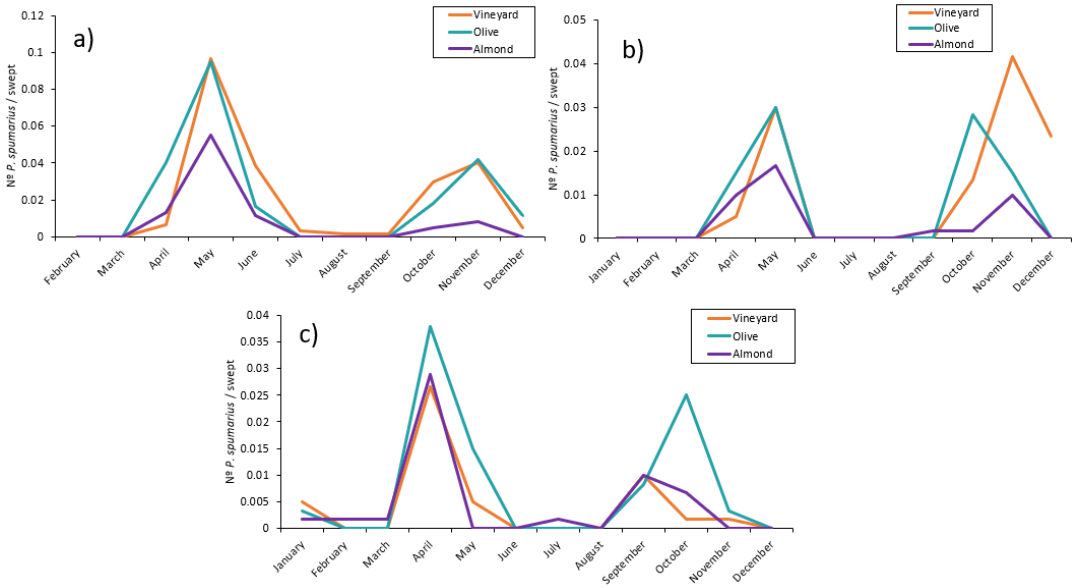

Figure 40. Seasonal pattern and abundance of *P. spumarius* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUa (herbaceous cover vegetation) in almond, olive and vineyard crops.

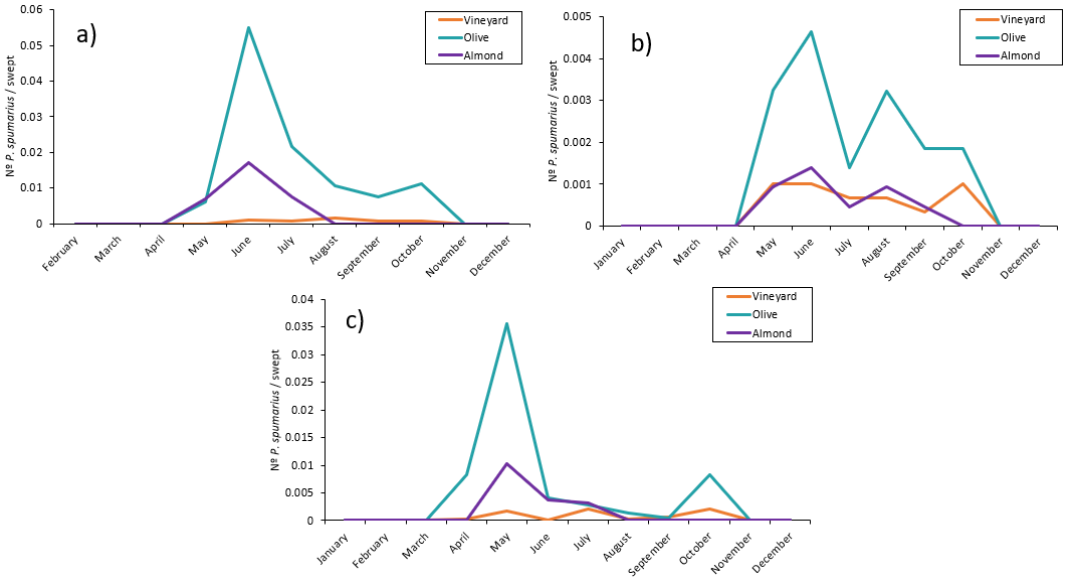

Figure 41. Seasonal pattern and abundance of *P. spumarius* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUt (tree canopy) in almond, olive and vineyard crops.

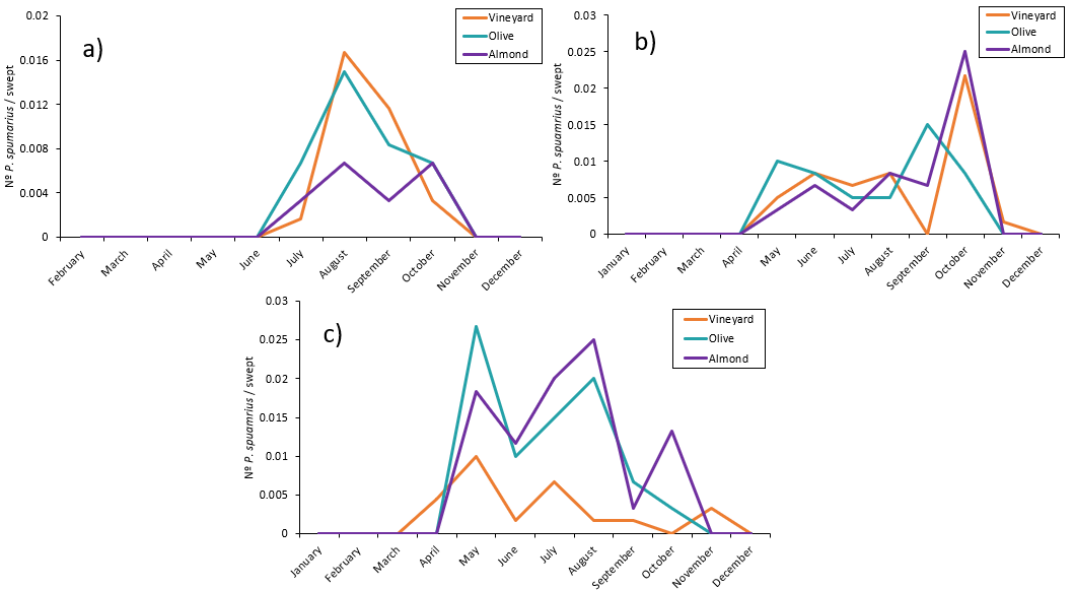

Figure 42. Seasonal pattern and abundance of *P. spumarius* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUs (border vegetation) in almond, olive and vineyard crops.

Seasonal pattern of *N. campestris* per crop and SSU:

The seasonal pattern for *N. campestris* was similar to *P. spumarius*, however *N. campestris* showed lower density in all plots (Fig. 43, 43, 45). Adults showed two peaks of abundance in the SSUa (Fig. 43) similarly to *P. spumarius*. In 2018 adults were more abundant in SSUa of vineyard compared to that of olive and almond crops. For the following years, adults were more abundant in SSUa of olive crops, while abundance in SSUa of almond and vineyard were similar. Abundance of *N. campestris* in SSUt and SSUs was very low (less than 0.01 adult /sweep) (Fig. 44, 45) and therefore no clear seasonal pattern was observed in any of the years.

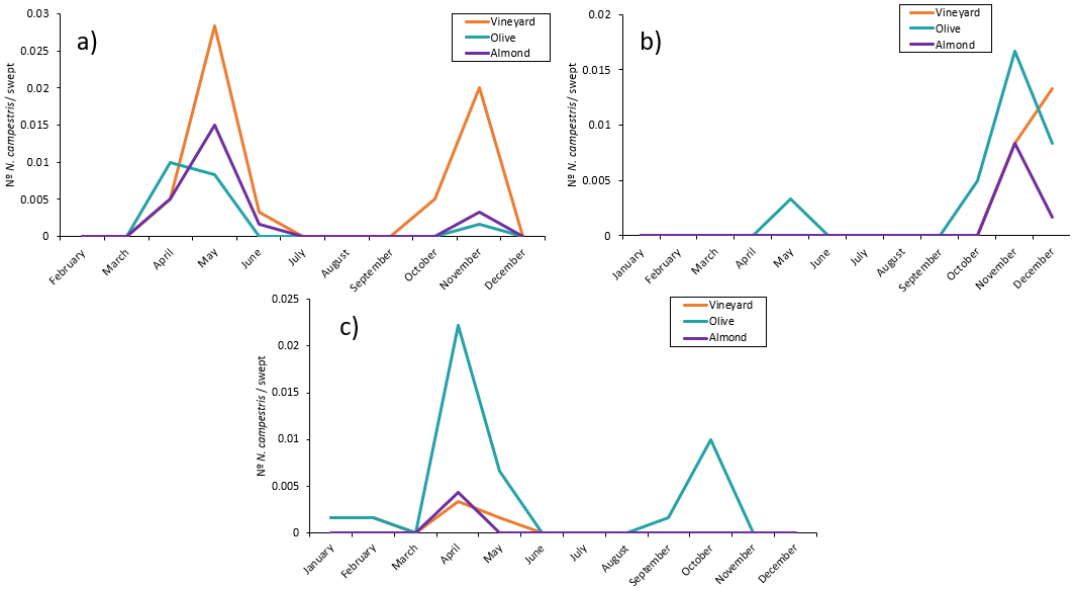

Figure 43. Seasonal pattern and abundance of *N. campestris* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUa (herbaceous cover vegetation) in almond, olive and vineyard crops.

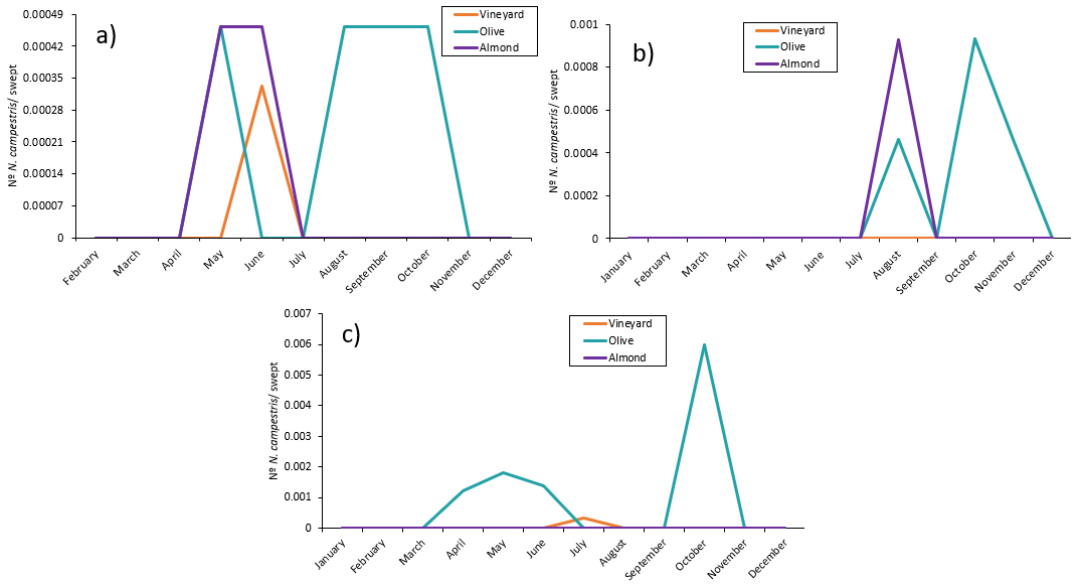

Figure 44. Seasonal pattern and abundance of *N. campestris* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUt (tree canopy) in almond, olive and vineyard crops.

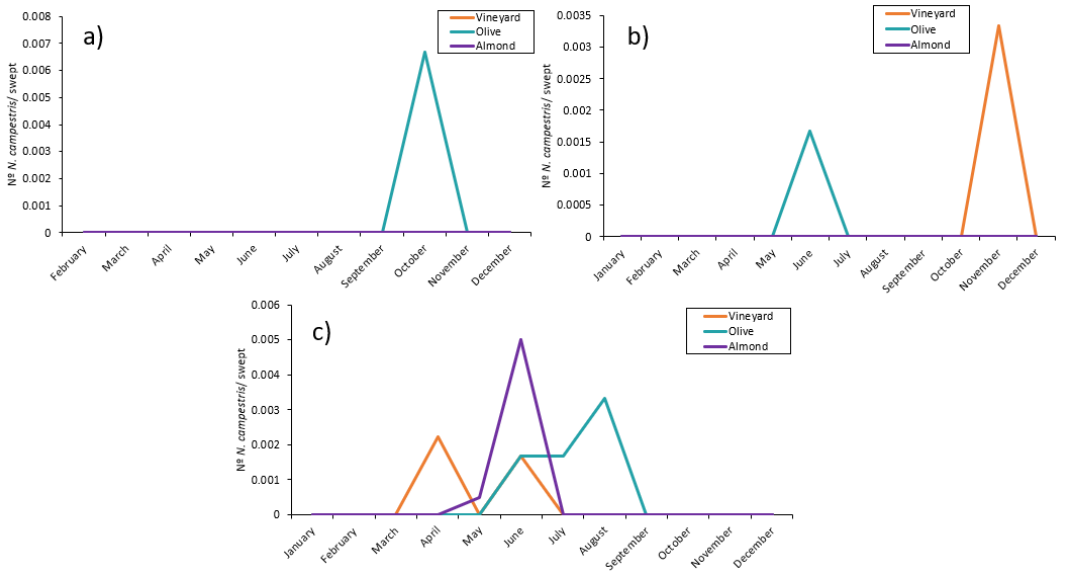

Figure 45. Seasonal pattern and abundance of *N. campestris* adults in Majorca in 2018 (a), 2019 (b) and 2020 (c) in the SSUs (border vegetation) in almond, olive and vineyard crops.

Statistical results

Model selection indicated that the most influential variables explaining the presence and abundance of *P. spumarius* were canopy cover from the vegetation structure (Table 5) (Annex II). Regarding the types of crops, the presence of the vector was higher in vineyards than in olive or almond crops. On the other hand, when present, the vector was more abundant in almond crops (Table 5). We also found a spatial and temporal variation in vector presence abundance, being 2018 the year with the highest density (Table 5). Regarding inter annual variation, the presence of *P. spumarius* was highest in spring and lowest in summer (Table 5, Fig 36). Accordingly, with high temperatures and evapotranspiration vector abundance decreased. Finally, precipitation favoured the presence of the vector but not its abundance (Table 5).

Table 5. Summary of the efect (positive or negative) of the different variables on P. spumarius density by running zero-inflated models. P-value <0.001: **+ / -** (most influent factor on the dependent variable); P-value = 0.05 – 0.001: + / -; Ø: no effect.

Variables	Presence	Abundance
Canopy	+	Ø
Cover	+	Ø
Border	Ø	Ø
Olive	Ø	-
Vineyard	+	-
Almond	Ø	+
2018	Ø	+
2019	-	Ø
2020	-	+
Felanitx	Ø	Ø
Inca	+	-
Manacor	+	-
Algaida	Ø	+
Spring	+	Ø
Summer	-	Ø
Autumn	Ø	Ø
Winter	-	Ø
Precipitation	+	-
Et0	Ø	-
Temperature	Ø	-

For *N. campestris*, the best model (Annex II) supported that it was more present on cover and border vegetation compartments, being less abundant in this last one (Table 6). As in the case of *P. spumarius*, when present it was more abundant in almond than in olive

and vineyard crops (Table 6). We also found a spatial and temporal variation for both presence and abundance, again, with higher density in 2018 (Table 6). Moreover, *N. campestris* was more present (i.e., distributed spatially) and particularly abundant in autumn, being negatively influenced by temperature (Table 6).

Table 6. Summary of the effect (positive or negative) of the different variables on *N. campestris* density by running zero-inflated models. P-value <0.001: **+ / -** (most influent factor on the dependent variable); P-value = 0.05 – 0.001: + / -; Ø: no effect.

Variables	Presence	Abundance
Canopy	Ø	Ø
Cover	+	-
Border	+	+
Olive	Ø	-
Vineyard	Ø	-
Almond	Ø	+
2018	+	+
2019	Ø	Ø
2020	+	+
Felanitx	-	Ø
Inca	-	-
Manacor	-	Ø
Algaida	+	+
Spring	-	Ø
Summer	Ø	+
Autumn	+	+
Winter	-	Ø
Temperature	-	-

Potential vectors in Ibiza

We conducted one sampling in November 2017, and from 2018 to 2020 samplings were conducted in June or July and November.

In November 2017 only samples from SSUa were collected since this sampling was conducted previously to the start of the EFSA grant. In this case, only adults from vegetation were collected in mixed crop plots, which are characteristic of Ibiza (e.g., almond and carob trees *Ceratonia siliqua*). We defined the crop (i.e., olive, vineyard and almond) according to the dominant species in the plot. In 2017 we collected adults of *P. spumarius* and *N. campestris* in the SSUa of all crops (Fig. 46). The highest number of *P. spumarius* adults were collected from the almond crop, followed by olive and vineyard crops. *N. campestris* was more abundant in the SSUa collected from almond and vineyard, and less abundant in olive.

In November 2018 all SSU were sampled (Fig. 47). Adults of Aphrophoridae were found only in the SSUa. In this case, both adults of *P. spumarius* and *N. campestris* were more abundant in olive and vineyard crops compared to almond one.

In July 2018 and June 2019 and 2020, adults were collected from SSUt and SSUs since at that time of the year the cover herbaceous vegetation (SSUa) was no longer present due to high evapotranspiration (Fig. 47). In 2018, adults of *P. spumarius* were more abundant in olive trees compared to vineyard plants. No adults were collected from almond trees. The abundance of adults of *P. spumarius* was higher in the border vegetation (SSUs) compared to the crop plants (SSUt). The abundance of adults of *P. spumarius* was similar in the SSUs of all crops (Fig. 47). Results obtained in June 2019 were substantially different, in fact, adults of *P. spumarius* were collected only from SSUt of olive crops (Fig. 47). Abundance of adults in 2020 was also very low. Adults of *P. spumarius* and *N. campestris* were collected from olive and vineyard crops only. Adults of *N. campestris* were present only in the cover vegetation (SSUa), being more abundant in the olive and vineyard crops and less abundant in the almond one (Fig. 47).

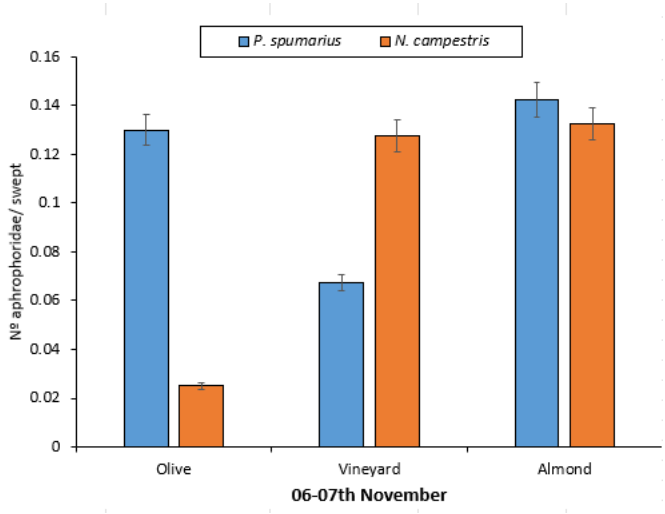

Figure 46. Abundance of *P. spumarius* and *N. campestris* adults in Ibiza in 2017 in SSUa of almond, olive and vineyard crops.

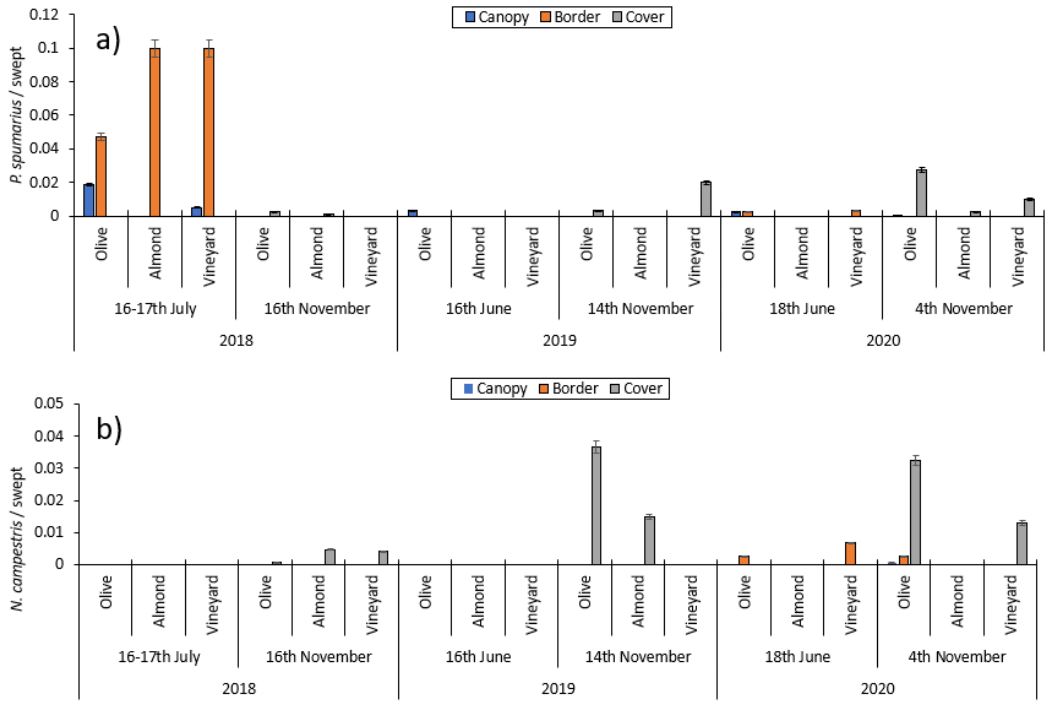

Figure 47. Abundance of adults of a) *P. spumarius* and b) *N. campestris* adults in Ibiza in July-November 2018 and June- November 2019 and 2020 in the SSU in almond, olive and vineyard crops.

Potential vectors in Formentera

Sampling in Formentera was conducted in June and November of 2019 and 2020 (Fig. 48). Adults of *P. spumarius* and *N. campestris* were found in very low abundance, and present in the SSUt and SSUa in olive and vineyard crops in both years of sampling. Adults of both species were also present in the SSUa of both crops in November, but not in the SSUs.

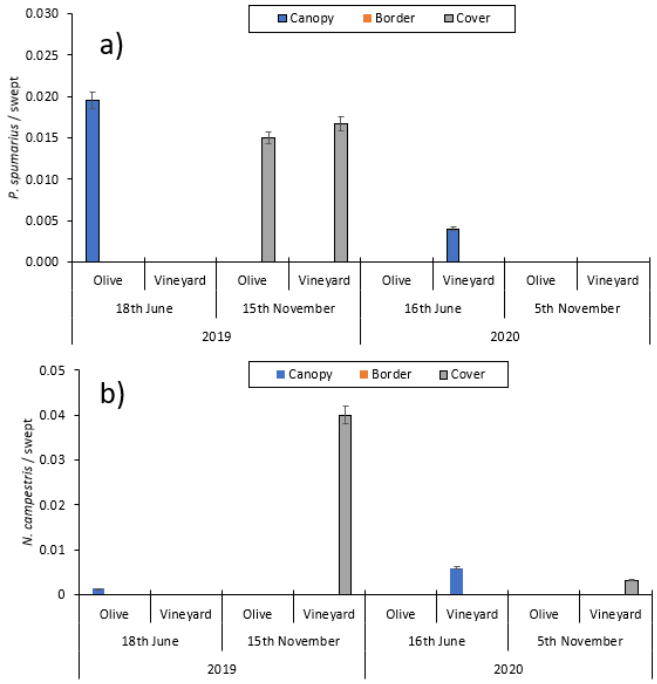

Figure 48. Abundance of *P. spumarius* (a) and *N. campestris* (b) adults in Formentera in June-November 2019 and 2020 in the different SSU of olive and vineyard crops.

Potential vectors in Minorca

In 2018, adults were sampled in July and November in all SSU of almond, olive and vineyard crops, while in 2019 adults were sampled in June and November and in 2020, in June and October (Fig. 49 a, b). In July 2018 adults of *P. spumarius* were detected only from SSUt in olive and vineyard while in November 2018 adults were detected only from the SSUa of olive and vineyard (Fig. 49 a). Abundance was higher in olive crops compared to vineyard ones. No adults of *N. campestris* were collected in July 2018 in any of the crops,

while in November 2018, adults were found only in SSUa of almond crops (Fig. 49 a). Differently, in June 2019 adults of *P. spumarius* were found in all SSU of vineyard, only SSUt of almond and SSUs of olive crops (Fig. 49 a). The highest abundance of adults was found in vineyard compared to the other crops. In November 2019 adults of *P. spumarius* were found in SSUt and SSUs of almond and SSUs of vineyard, while in November 2020 adults were found only in SSUa being more abundant in olive crop, followed by vineyard and almond orchards. Adults of *N. campestris* were very scarce in all SSU in June 2019 and 2020 (Fig. 49 b). In November 2019 and 2020 adults of *N. campestris* were present in the vineyard and olive crops, but again, showing very low abundance (less than 0.1 adult/sweep) (Fig. 49 b).

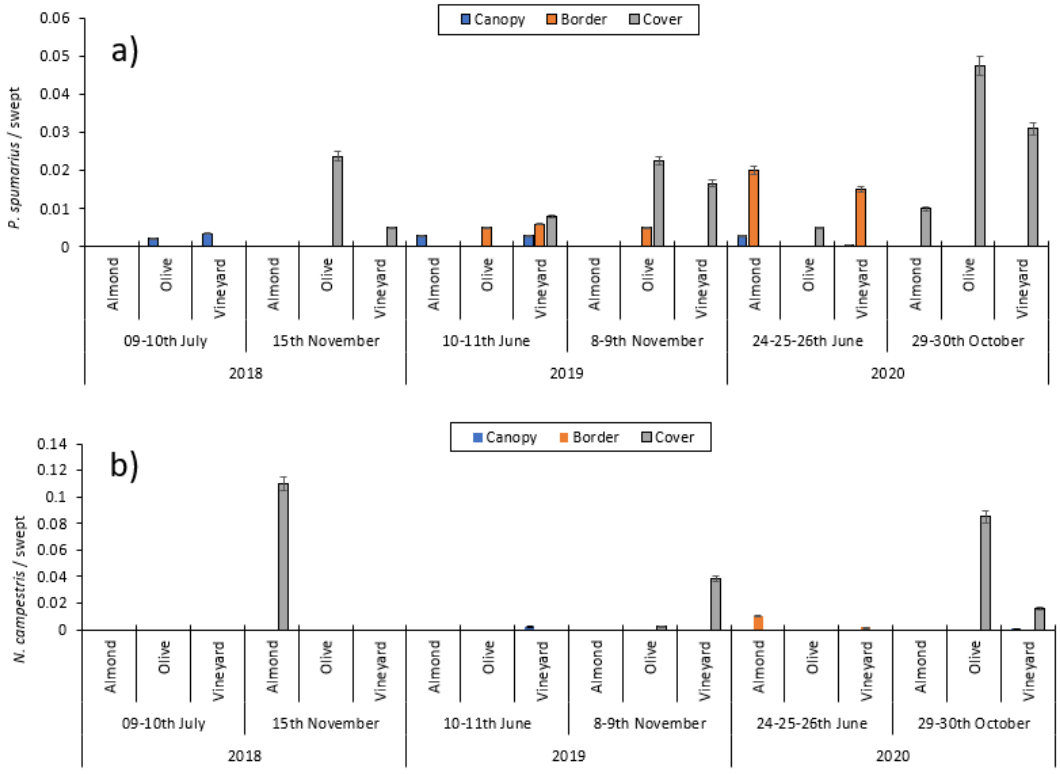

Figure 49. Abundance *P. spumarius* (a) and *N. campestris* (b) adults in Minorca in 2018, 2019 and 2020 in the different SSU of almond, olive and vineyard crops.

Discussion

This is the first European extensive surveillance on *X. fastidiosa* vectors to investigate their abundance and phenology in an affected area of olive, almond and vineyard crops during three consecutive years. In this study we confirmed the presence of *P. spumarius* and *N. campestris* in Majorca, Ibiza, Formentera and Minorca associated with the major crops of the region, almond, olive and vineyard. The spittlebug *P. spumarius* has been confirmed as the most abundant and frequent aphrophoridae species in the Balearic Islands, showing similar results to other areas of Europe such as Spain (Morente *et al.*, 2018), Italy (Ben Moussa *et al.*, 2016; Bodino *et al.*, 2017; Cornara *et al.*, 2017; Bodino *et al.*, 2019; Dongiovanni *et al.*, 2019; Cavalieri *et al.*, 2019; Bodino *et al.*, 2021; Avosani *et al.*, 2022), France (Albre *et al.*, 2021; Mesmin *et al*., 2021), Greece (Antonatos *et al.*, 2019; Antonatos *et al.*, 2021), Turkey (Yutserver, 2000; Zeybekoglu *et al.*, 2013), Belgium (Hasbroucq *et al*., 2020) and UK (Park *et al.*, 2018).

In general, two major peaks of adults were observed in the cover vegetation (SSUa) in April-May and October-November, coinciding with the adult emergence and mating, respectively. A minor peak of insects was observed from July to September in bordering woody shrubs (SSUs) such as oak, lentisk and wild olive. Similar seasonality was observed in the Iberian Peninsula (Morente *et al*., 2018), Italy (Bodino *et al*., 2019 and 2021) and Greece (Antonatos *et al*., 2021).

Being *P. spumarius* and *N. campestris* the major vectors of *X. fastidiosa*, its presence and abundance will determine the risk of transmission of the bacteria and its spreading. So, unveiling seasonal abundance of insect vectors in risked zones is a crucial component of disease epidemiology (Jeger and Bragard, 2019).

Nymphal abundance and seasonality

Nymphal seasonality in Majorca was recorded from March to early June in almond, olive and vineyard crops. *Philaenus spumarius* nymphs were more abundant than *N. campestris*, as it was observed in other Mediterranean regions in olives and vineyards crops in the Iberian Peninsula (Morente *et al*., 2018), Italy (Piedmont, Liguria and Apulia regions)

and Greece (Dongiovanni *et al*., 2019; Bodino *et al.*, 2019; Bodino *et al.*, 2021; Antonatos *et al.*, 2021). The nymphal density recorded in Majorca was 0.03 nymphs/m^2 for *P. spumarius* and 0.005 nymphs/m^2 for *N. campestris*, 2,000 times lower than the density (60 nymphs/m^2) recorded in Italy for *P. spumarius* in vineyards (Bodino *et al*., 2021) and 1,000 times lower than the density found in Greece, 10-40 nymphs/m^2 for *P. spumarius* and 2-25 nymphs/m^2 for *N. campestris* (Antonatos *et al*., 2021). The difference in the abundance between our areas and the above cited ones may be due to the particular mixed orchards present in the Balearics, in particular compared to the Italian areas affected by *X. fastidiosa*. In addition, high levels of water stress (very common in mediterranean areas) and poor understory in the area may hamper the development of nymphs (Cornara *et al*., 2021).

The peak of nymphs' abundance was located between end-March and early-April in Majorca, similarly to other reports from Italy, Greece and Portugal (Bodino *et al.*, 2019; Bodino *et al.*, 2020; Villa *et al*., 2020; Antonatos *et al.*, 2021; Bodino *et al.*, 2021;). Nevertheless, both species of Aphrophoridae had the same nymphal population dynamics, variation in time and space of spittlebugs is almost identical.

In our study, the instars development occurred in the cover vegetation with an overlapping of instars throughout the time that extends the nymphs seasonality for over three months, from March to April in all crops in Majorca.

Regarding nymphs' instars, *N. campestris* presented a trend for the bottom position in the herbaceous cover vegetation in comparison to *P. spumarius*, but un 2018 and 2020 nymphs were also found in medium and upper parts of the cover plants. In the case of *P. spumarius*, we did not observe a clear pattern for the position in the plant. Nymphs N1 are difficult to identify at the field due to its little size, their position at the bottom of the plants and the difficulty to identify from N2. The first extensive description on this species was reported by Weaver and King (1954), after this study, in Europe there has been other studies that have confirmed the same observations. In Portugal, Villa *et al*., (2020) found that foams were observed mainly in medium part of the plants. In Italy, first and second *P. spumarius* instars were mainly found in the basal part, whereas from third instar, they were found alongside the entire plant (Bodino *et al*., 2020). Our results for *N.*

campestris are in accordance with Bodino *et al*., (2020) that also found them mostly in the base of plants. When nymphs emerge, they are immobile and stay at the base and on low rosetting type plants to be protected from the sun, natural enemies and maintain their own microclimate to start feeding and produce the spittle (Weaver and King, 1954). The apparent trend for the basis of plants may be due to reduce loss of foam by evaporation (Whittaker, 1970; Bodino *et al*., 2020). Also, as season progresses and nymphs moult to N5, they move up on the plant seeking tender new growth parts (Weaver and King, 1954; Bodino *et al*., 2020). In addition, Weaver and King (1954) described that in early morning, foams may be found at the top of plants, but as temperature arises foams dry up and nymphs move down again to lower parts to avoid heat. The mobility of nymphs is not only alongside the same plant, but also later nymph instars can also move to other closer plants and led them to fed on wider range of plant species.

Aphrophoridae nymphs were observed feeding from 31 different plant species belonging to 17 genera and 12 plant families. *Philaenus spumarius* showed preference for Compositae (*Glebionis* spp., *Sonchus* spp., *Calendula* spp.) and Leguminosae (*Medicago* spp.) plants. This is in agreement with other investigations in Portugal (Villa *et al*., 2020), in Italy (DiSerio *et al.*, 2019; Dongiovanni *et al*., 2019; Bodino *et al*., 2020), in the Iberian Peninsula (Morente *et al*., 2018) and in Greece (Antonatos *et al*., 2021). In some cases, those previous studies reported Apiaceae as a high colonized plant family, but it was not our case neither in Bodino *et al*., (2020). Also, it is noteworthy that Leguminosae is one of the most infested plants in our study because other researchers reported that *P. spumarius* preferred nitrogen-fixing plants for nymphal development (Craig and Ohgushi, 2002; Wood and Jones, 2020). Legumes are self-supporters of nitrogen soil fertilization through atmospheric nitrogen fixation in root nodules in symbiosis with soil bacteria (Hasanuzzaman *et al*., 2020). Meanwhile, *N. campestris* was mainly found in Poaceae, being in line with other observations from the Iberian Peninsula (Morente *et al*., 2018), Italy (Dongiovanni *et al*., 2019; Bodino *et al*., 2020; Bodino *et al*., 2021) and Greece (Antonatos *et al*., 2021). Furthermore, the less preferred plant species in our study were the genera *Convolvulus* (Convolvulaceae), *Beta* (Amaranthaceae), *Anagallis* (Primulaceae), *Diplotaxis*

(Brassicaceae), *Euphorbia* (Euphorbiaceae), *Rubia* (Rubiaceae), *Avena* (Poaceae), *Sherardia* (Rubiaceae), *Reichardia* (Compositae), *Hypericum* (Guttiferae), *Sinapis* (Brassicaceae) and *Malva* (Malvaceae). Our results demonstrated that *P. spumarius* nymphs are generalist phytophagous on a wide range of plants and that prefers herbs other than grasses as reported previously in Italy (Weaver and King, 1954; Cornara *et al*., 2018; Dongiovanni *et al.,* 2019). Although, the reasons behind host preference remain unknown, further studies under controlled conditions are needed to know which factors drive nymphs to choose plant species for their development. Also, association of insects with plant genera or species may vary temporally and spatially (Halkka *et al*., 1967) due to variability of plant communities in each geographic region such as flowering timing, composition and plant species abundance because of soil composition, climatic conditions and agricultural management. For example, Bodino *et al*., (2020) observed that *P. spumarius* nymphs shifted during the season from Compositae plants with basal rossettes to Fabaceae or Plantaginaceae probably due to plant phenology and different availability of plant taxa during nymphal season.

Adult abundance and seasonality

The general seasonal pattern of Aphrophoridae adults in the Balearic Islands indicated an univoltine cycle. Adults emerged in spring in cover vegetation from they migrate to tree canopies and shrubs when grass dries. Then, in summer they are abundant in canopies and shrubs and were so difficult to detect. Finally, in autumn there is a second peak of adults in the cover vegetation, corresponding with mating. Only few females were detected overwintering.

The annual sampling in Majorca resulted in 1990 Aphrophoridae collected from 2018 to 2020, being 86.6 % *P. spumarius* and 13.4 % *N. campestris*. *Philaenus spumarius* was the most abundant species in all the crops sampled. In fact, this species is considered to be the widest distributed, and probably most abundant, aphrophoridae in Europe (Weaver and King, 1954; Cornara *et al.*, 2017; Antonatos *et al.*, 2019). In the 3-yr survey conducted in Majorca 767 *P. spumarius* and 95 *N. campestris* were collected from olive

orchards. Other similar number of captures were found in Apulia (Italy) were Ben Moussa *et al*., (2016) collected in a 2-yr sampling 955 *P. spumarius* and 207 *N. campestris,* and in Greece in a 13-mo survey Tsagkarakis *et al*., (2018) only collected 55 *P. spumarius* and 6 *N. campestris*. The number of captures is differing between places. So, detectability of vectors may be driven by other ecological factors such as climatic, presence of forests, coverage, sunlight hours and plant heigh (Avosani *et al.*, 2022). Those factors, apart of plant coverage, were not considering in the present study. This is in accordance with our modelling results, that the best fitting models for estimating the presence and abundance of spittlebugs include temperature, evapotranspiration, and precipitation. In fact, our results showed that *P. spumarius* presence, but not abundance, was positively affected by precipitation. Temperature and evapotranspiration had no effect for the presence of *P. spumarius,* while higher temperature and evapotranspiration, decreased abundance of *P. spumarius*. In the case of *N. campestris*, temperature showed a negative effect on the presence and abundance of the spittlebug. The effect of temperature and evapotranspiration help us to understand why in summer months spittlebugs are less presence and abundant in the cover vegetation, when evapotranspiration and temperature arises, herbaceous cover dries, and they move up to other compartments such as tree canopies and shrubs. The heatwave in Europe during July 2019 (Ma *et al.* 2020) probably affected the population dynamic of the adults, leading to a higher mortality and/or early movement to shelters, as this year presented the lowest density of insects in comparison to 2018 and 2020. Several studies have shown the effect of climate in the presence and abundance of populations of *P. spumarius*. Weaver and King (1954) showed that eggs were easy to desiccate and embryo may die due to high temperatures or low humidity. Halkka et al., (2006) correlated the North Atlantic Oscillation (NAO) to significant effects on the abundance of *P. spumarius* in Finland populations by reducing population. Beal *et al*., (2021) suggested that *P. spumarius* natural population declined over 30 yr in California due to shifts in temperature and humidity. We can assume that agricultural plots with irrigated plants may be less affected by changes in climatic variables (i.e., lower evapotranspiration),

then being optimal ecological shelters for *X. fastidiosa* vectors, which may increase the risk of transmission and spreading.

Spittlebug adults in Majorca showed a clear seasonality in all crops and in the different SSU surveyed. In our study, adult aphrophoridae showed the highest abundance in the SSU herbaceous (SSUa) cover vegetation in olive, followed by almond and vineyard crops. Adults were more frequent in the cover vegetation during spring, when they completed the development from the fifth nymphal instar. In autumn, females moved to the ground to lay eggs in dry straw near herbaceous plants that will be the source of food for newly emerged nymphs during the following spring. The current study showed that adults of *N*. campestris were abundant in cover vegetation in spring and autumn, but virtually absent from main crops within the rest of the year. This may have importance for the *X. fastidiosa* transmission role of *N. campestris* compared to *P. spumarius*. Conversely, *P. spumarius* was generally abundant in cover vegetation. Same pattern was also found in other regions such as Italy (Cavalieri *et al.*, 2019; Bodino *et al.*, 2019; Bodino *et al.*, 2021), Ajaccio (Corsica, France) (Albre and Gibernau, 2019; Albre *et al*., 2021), Greece (Tsagkarakis *et al*., 2018; Antonatos *et al*., 2019; Antonatos *et al*., 2021), California (Beal *et al*., 2021) and Spain (Morente *et al*., 2018).

When present, adults of *P. spumarius* showed an abundance in the cover vegetation between 0.0006 and 0.09 adults/sweeps and *N. campetris* values from 0.0006 to 0.018. adults/sweeps. Our densities were more than twice times lower than the abundance reported in other countries, such as Greece where adult density in olive crop was 0.2 to 15 adults/sample (Antonatos *et al*., 2021); in vineyards from northern Italy, *P. spumarius* densities reached 0.4-2 adult/sweeps and *N. campestris* 0.02-0.12 adult/sweeps (Bodino *et al*., 2021). Other samplings in southern Spain detected values of *P. spumarius* from 0.01 to 0.02 adult/sweeps (Morente *et al*., 2018).

For both species, the peak of spring was higher than in autumn, except in 2019 when the peak of autumn was higher than in spring for *N. campestris*. Similar results were shown by Bodino *et al*., (2021) in autumn in vineyards, with a maximum density of *N. campestris* of 0.12 adults/sweeps. In our study, last adults were collected from cover in

December, but some overwintering females were detected in January and February 2020. In olive plots from Liguria, last adults were captured in October-November and in Apulia in October (Bodino *et al*., 2019).

In spite of the extreme conditions found in summer months, spittlebugs survive by aestivating in shrubs and tree canopies (Drosopoulos *et al*., 2010). In our study, there was a peak of abundance in summer in the tree canopy and bordering woody shrubs (e.g., oak, wild olive and lentisk) of olive, almond and vineyard crops, probably because herbaceous plants were no longer suitable for spittlebugs due to high evapotranspiration that causes loss of plant turgor. The abundance of adults from the border vegetation (i.e., *P. lentiscus*) at that time was higher than the cover and tree canopy. Spittlebugs were recorded in borders from June to November in 2018, from April to December in 2019 and from March to December in 2020. The presence of adults in woody plants from June to October is crucial for the secondary transmission of *X. fastidiosa* to crops. *Philaenus spumarius* was found in low abundance, from 0.0025 to 0.02 adults/sweeps and *N. campestris* from 0.001 to 0.003 adult/sweeps. There was no difference of abundances among crops. Regarding the tree canopy we observed that *P. spumarius* was more abundant in olive plots than in almond and vineyard. Adults were present in canopies from April to November in 2018 and 2019, and from March to November in 2020. In 2018 and 2020 densities of *P. spumarius* were from 0.005 to 0.06 adult/sweeps, observing a higher peak in June and another one in October. In 2019, abundance was from 0.0005 to 0.005 adult/sweeps and no peaks were observed maybe because of these low densities recorded. In other countries such as France (Cruaud *et al*., 2018; Albre and Gibernau, 2019; Albre *et al*., 2021; Mesmin *et al*., 2021), Italy (Cornara *et al*., 2017; Bodino *et al*., 2019 and 2021), Greece (Drosopoulos, 2003; Antonatos *et al*., 2021), Turkey (Yurtsever, 2001) and in the Iberian Peninsula (Morente *et al*., 2018), spittlebugs were also often captured in shrubs and tree canopies in summer. Even so, our densities were lower than in those places, for example, Bodino *et al*., (2021) found in vineyard canopies 0.43 ± 0.07 *P. spumarius*/sweeps and in border 0.48 ± 0.06 *P. spumarius*/sweeps, being oaks the preferred bordering plant. In olive canopies Bodino et al., (2019) detected 0.5-1.55 *P. spumarius*/sweeps and up to 1.6 *P. spumarius*/sweeps in

wild woody shrubs. Nevertheless, in the southern Spain, Morente *et al*., (2018) recorded similar densities in olive tree canopies (0.002 to 0.007 *P. spumarius*/sweeps) as in our study.

It is important to identify the plant species where vectors remain in the border of crops because it represents a reservoir of *X. fastidiosa* and therefore, may increase the risk of transmission and persistence. We collected *P. spumarius* in *Olea europaea* var. *sylvestris*, *Ceratonia siliqua*, *P. lentiscus*, *Quercus ilex*, *Q. coccifera*, *Foeniculum vulgare* and *Pinus halepensis*. While *N. campestris* was only found in *Quercus* spp., *Olea europaea* var. *sylvestris* and *Pinus halepensis*. For both species, the preferred woody plants were *Quercus* spp., *P. lentiscus* and *Olea europaea* var. *sylvestris* in almond, olive and vineyard crops. The most dominant forests in the Balearic Islands are pinewoods and holm oaks (Larrucea, 2008; GOIB, 2011), then spreading of *X. fastidiosa* puts those habitats at risk, in fact, *X. fastidiosa* positive insects were collected from wild woody shrubs (see Chapter 3). Furthermore, in Majorca, Minorca and Ibiza, a high number of *X. fastidiosa* positive plants were found in *O. europaea* var. *sylvestris* masses (Olmo *et al*., 2021). Same results were described in other areas of the Mediterranean. Mazzoni *et al*., (2005) cited the presence of *P. spumarius* in the following trees and shrubs from Tuscany region (Italy): *Acer campestre*, *Crataegus* spp., *Prunus domestica*, *Arbutus unedo*, *Myrtus communis*, *Phyllirea* spp., *P. lentiscus*, *Ostrya carpinifolia*, Pomaceae, *Quercus ilex*, *Q. suber*, *Q. petrea*, *Q. pubescens*, *Q. robur*, *Salix alba*, *S. babylonica* and *Vitis vinifera*. In the case of *N. campestris*, they have been commonly found in cypress in Tuscany (Mazzoni *et al*., 2005) and *P. halepensis* in the Iberian Peninsula (Morente *et al*., 2018). Bodino *et al*., 2019 in olive plots of Italy found a preference of *P. spumarius* for *Quercus* spp., *P. lentiscus* and *P. terebinthus* and *Myrtus communis*. Finally, in Italian vineyards *Quercus* spp. was the preferred woody plant for *P. spumarius* and *N. campestris*, and some *N. campestris* were also recorded in *Robinia pseudoacacia* (Mazzoni *et al*., 2005).

However, the movement of insects from crops to border plants during summer is still unclear, since in places where plant cover persist evergreen during summer (i.e., north Italy), adults still move from land cover to trees (Bodino *et al*., 2019). Apparently moving from cover plants to trees is related to the need of adults to explore further plant food

sources and not merely the lack of herbaceous plants during summer. This mechanism of aestivation has been also described in the Iberian Peninsula, in the Mediterranean basin and northern Africa for other species such as *P. signatus*, *P. italosignus, P. maghresignus* and *P. tesselatus* (Drosopoulos *et al.*, 2010). This fact is also related with the morphotypes of *Philaenus* spp. that provide a cryptic coloration for surviving during aestivation (Drosopoulos *et al.*, 2010).

Abundance in Ibiza, Minorca and Formentera

In the samplings in Ibiza, Minorca and Formentera we detected the same vector species as Majorca, *N. campestris* and *P. spumarius*, being the last the most abundant. Aphrophoridae were found in the border (SSUs) and canopy (SSUt) in June and in the cover plants (SSUa) in October-November, as observed in the seasonality Majorca and in other regions such as Italy (Cavalieri *et al.*, 2019; Bodino *et al.*, 2019; Bodino *et al.*, 2021), Ajaccio (Corsica, France) (Albre and Gibernau, 2019; Albre *et al*., 2021), Greece (Tsagkarakis *et al*., 2018; Antonatos *et al*., 2019; Antonatos *et al*., 2021) and Spain (Morente *et al*., 2018).

Philaenus spumarius abundance ranged from 0.005 to 0.13 adult/sweeps in Ibiza, Formentera and Minorca in all kind of crops, being similarly abundant to *N. campestris* that ranged 0.002 to 0.13 adult/sweeps. Densities were similar to those recorded in Majorca and the frequency of sampling carried out did not allow to observe any crop preference.

In general, Ibiza and Minorca are characterized for having only 20 % of the territory devoted to crops (IBESTAT, 2021). Forest masses in Minorca, Ibiza and Formentera may have an effect on vector distribution compared to Majorca, and therefore, insects may be more difficult to be collected. In addition, plant diversity associated to crop areas (i.e., border vegetation) was also different among islands. In Majorca, orchards were surrounded by mixed shrubs of *P. lenticus* (Anacardiaceae) and *O. europaea* var. *silvetris* (Oleaceae), while in Minorca the main border vegetation (SSUs) was *O. europaea* var. *silvestris* and in Ibiza, *Juniperus oxycedrus* (Cupressaceae) mixed with *Pinus halepensis* (Pinaceae). Since species of Aphrophoridae such as *P. spumarius* are highly polyphagous, plant species composition may drive the distribution of the insect in

the orchard and surroundings, considering not only plants as a source of food, but also as a protection against environmental threats such as high temperature and low humidity in summer (Drosopoulos *et al*., 2010).

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CHAPTER 2

Life cycle of *Philaenus spumarius* and *Neophilaenus campestris* under controlled conditions

Abstract

The pathogenic bacteria *Xylella fastidiosa* (Proteobacteria: Xanthomonadaceae) was first detected in the Balearic Islands in October 2016. A microcosm study was conducted to observe the biology, ecology and monitor the life cycle of *X. fastidiosa* vectors under controlled conditions. It was set up during September and June 2019-2020 and 2020-2021. For this purpose, 50 cages containing one male and one female insect vector and one host plant were placed in semi-field conditions in September 2019 and 2020. Straw was also used as oviposition substrate and grass (Poaceae) was planted. The plants species selected were *Rosmarinus officinalis*, *Mentha x piperita*, *Ocimum basilicum*, *Pistacia lentiscus* and *Lavandula dentata*. The presence of egg batches was checked, and countered once adults were not recorded alive. When nymphs emerged, bionomic of the vectors was observed every two days until the end of the life cycle. Microcosm results showed that vector eggs were found in *R. officinalis*, *L. dentata* and *O.basilicum*. Nymphs emerged in January until April and first adults were observed in March until June in all the plant species tested. With this experiment we were able to simulate *P. spumarius* and *N. campestris* life cycle and confirm the field data (Chapter 1).

Introduction

Microcosms, or also named model systems, are small ecosystems in containers designed in ecology to analyse the growth and development of insects, as well as population dynamics. This permits to simplify and simulate processes occurring in natural ecosystems (Stevenson and Dindal, 1985; Lawton et al., 1996; Fraser and Keddy, 1997).

These model systems are of interest to understand the fundamental principles of ecology, such as demographic parameters, predator-prey population dynamics, food-web structure and multi-trophic interactions, competition, and predation, invasibility and community complexity, species coexistence and community stability and persistence (Benton et al., 2007).

Advantages of using microcosms include ease of replication, precise control over environmental variables (if needed) and the power of manipulate the parameters and treatments under investigations (Fraser and Keddy, 1997).

Auchenorrhyncha is the hemipteran suborder that includes cicadas, leafhoppers, froghoppers or spittlebugs, planthoppers and treehoppers (Bostanian *et al.*, 2012). Vectors of the plant pathogen *Xylella fastidiosa* belong to Auchenorrhyncha suborder and different families such as Aphrophoridae, Cicadellidae or Cercopidae. Spittlebugs (Aphrophoridae) are the widespread vectors in the European Union known to transmit *Xylella fastidiosa* (EFSA, 2021). Currently, *Philaenus spumarius*, *Neophilaenus campestris* and *P. italosignus* are the species known as vectors, being *P. spumarius* the most important. This species is the most common homopteran occurring in terrestrial habitats throughout the Holarctic region (Yurtsever, 2001). It is one of less 10 % of herbivorous insects that feed on more than 3 families of host plants (Bernays and Graham, 1988; Wood and Jones, 2020).

Philaenus spumarius and *N. campestris* are hemimetabolous insects with an univoltine cycle. In early-March nymphs start to emerge in the cover vegetation until May, throughout five nymphal stages. Nymphs can aggregate themselves within their self-produced spittle masses. The spittle are excretions of surplus water from the large amount of xylem sap ingested enriched with mucopolysaccharides and proteins from the Malpighian tubules (Biedermann, 2003). Even so, host plant quality, plant species and plant

nutrient content may affect insect mortality, fecundity, and fitness. The preference-performance hypothesis (PPH) suggests that females select oviposition sites in order to optimize offspring fitness and this choice is likely to be especially important for species with immobile or slow-moving nymphal stages such as spittlebugs (Wood and Jones, 2020).

Around end of April, first adults move from the cover plants to woody shrubs and tree canopies. By autumn, adults return to cover plants to mate and lay the eggs. *Philaenus spumarius* has two obligate separate ovarian and overwintering diapauses (Avosani *et al.*, 2022; Morente *et al.*, 2021).

An extensive knowledge of the vector species life cycle is needed to improve and implement control management actions against *X. fastidiosa*. A way to study this is using the microcosm technique that consists of the confinement of insects in controlled conditions that allow to study the bionomics of a particular species (Stevenson and Dindal, 1985; Benton et al., 2007).

The aim of this study was to increase the knowledge in the biology of *P. spumarius* and *N. campestris*. With that purpose we set up a microcosm study in Majorca by simulating their natural habitat in cages and tested five different plant species as host plant. Also, this information would be useful to explore possibilities of rearing for future experiments such as vector competence (Bodino *et al.*, 2021).

Materials and methods

In order to study the biological cycle in microcosm conditions of the potential vectors, a total of 50 cages (50 cm x 45 cm, 79 L) were placed in the experimental plot of Ca's Valencià at the main Campus of the University of the Balearic Islands (Palma, Majorca) (Fig. 50 a).

We set up 10 cages for each of the following plants species placed in plastic pots placed into fibre bags in order to facilitate movement of the insects in the cage: *Rosmarinus officinalis* (Lamiaceae), *Mentha x piperita* (Lamiaceae), *Ocimum basilicum* (Lamiaceae), *Pistacia lentiscus* (Anacardiaceae) and *Lavandula dentata* (Lamiaceae). Then a mix of grass

seeds were planted in the same soil (*Festuca arundinacea terrano*, *F. arrundinacea merida*, *F. arrundinacea fesnova*, *F. arrundinacea bizem*, *Poa pratensis*, *Lolium perenne*) (Fig. 50 b). Furthermore, we added straw for the egg laying of Aphrophoridae adults.

In each cage, we left one male and one female of Aphrophoridae field collected from September to December. Adult mortality was checked every week and adults ere replaced if not found. In December, egg batches were examined and left for emerging. When nymphs emerged, its bionomy was observed until the end of the life cycle. The bionomy study consisted in collecting data about eclosion rate, development periods and adult emergence.

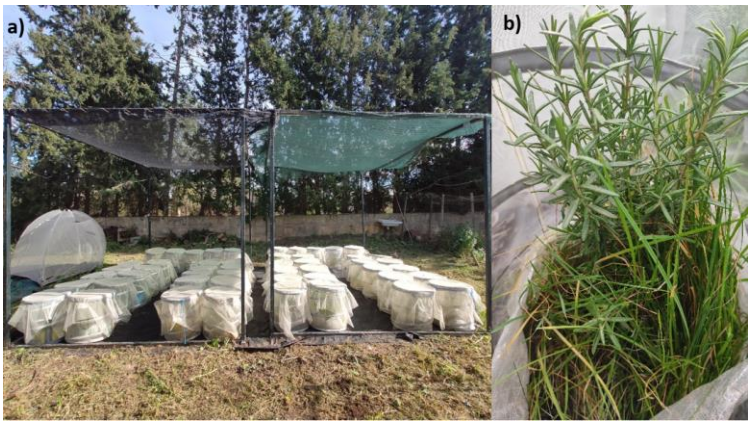

Figure 50. a): Microcosm placement in Ca's Valencià located at the University of the Balearic Islands and b): Example of a microcosm cage including *Rosmarinus officinalis*. © J. López- Mercadal.

In 2019, only *P. spumarius* adults were used. The couples were added between 25/09/2019 and 17/10/2019. The insect presence was inspected every two or three weeks. Then additional males and/or females were added into the cages if dead of initial adults was confirmed. The insects were not removed from the cages and observation under binocular about oviposition in the straw pieces was carried out between 31/01/2020 and 04/02/2020. At this time, first nymphs were detected, and bionomics were checked every two days (Fig. 51).

In 2020, both *P. spumarius* and *N. campestris* species were used for the microcosm trials (Fig. 51). Eight plants of each plant species contained *P. spumarius* and two plants *N.*

campestris. Then, the procedure was the same as in 2019 trials. Observation about oviposition in the straw pieces was carried out the 21/12/2020.

Figure 51. Microcosm set up in Majorca where *P. spumarius* and *N. campestris* bionomics were studied from egg until adult F1. a): checking microcosm cage. b): *P. spumarius* egg batch. c): *P. spumarius* nymphs. d): *P. spumarius* adult. © J. López-Mercadal.

Results

2019-2020 trial

From the 50 cages, eggs batches were detected from *P. spumarius* placed in *R. officinalis* and in *L. dentata* plants (Table 7). We obtained 8 % of cages with oviposition in the microcosm environment units and 30 % of females succeeded in ovipositing (assuming one egg batch/female).

Table 7. Summary table of microcosm trials 2019 to 2020: Host plant, number of egg batches, number of eggs and number of nymphs emerged.

Cage	Plant sp.	Vector	Oviposition Yes/No	Nº egg batches	Nº Eggs/batch	Nº nymphs emerged
1 - 10	*R. officinalis*	*P. spumarius*	Yes	1	11	12
11 - 20	*P. lentiscus*	*P. spumarius*	Yes	Not found	Not found	1
21 - 30	*O. basilicum*	*P. spumarius*	Yes	Not found	Not found	7
31 - 40	*Mentha x piperita*	*P. spumarius*	Yes	Not found	Not found	2
41 - 50	*L. dentata*	*P. spumarius*	Yes	14	3.21 (45 eggs)	38

Nymphs of *P. spumarius* were observed in *Rosmarinus officinalis*, *Ocimum basilicum*, *Menta x piperita* and *Pistacia lentiscus* cages. *Philaenus spumarius* nymphs developed to adult both in the plant and in the grass (Poaceae).

First nymphs were detected the 31st of January 2020 in *L. dentata* until 18th May 2020. First adults of *P. spumarius* adults were observed the 21st of March (Fig. 52).

Timing for development and seasonal dynamics found in the microcosm trials were similar to the one observed in the field conditions, but nymphs emerged before of what observed in the field (see Chapter 1).

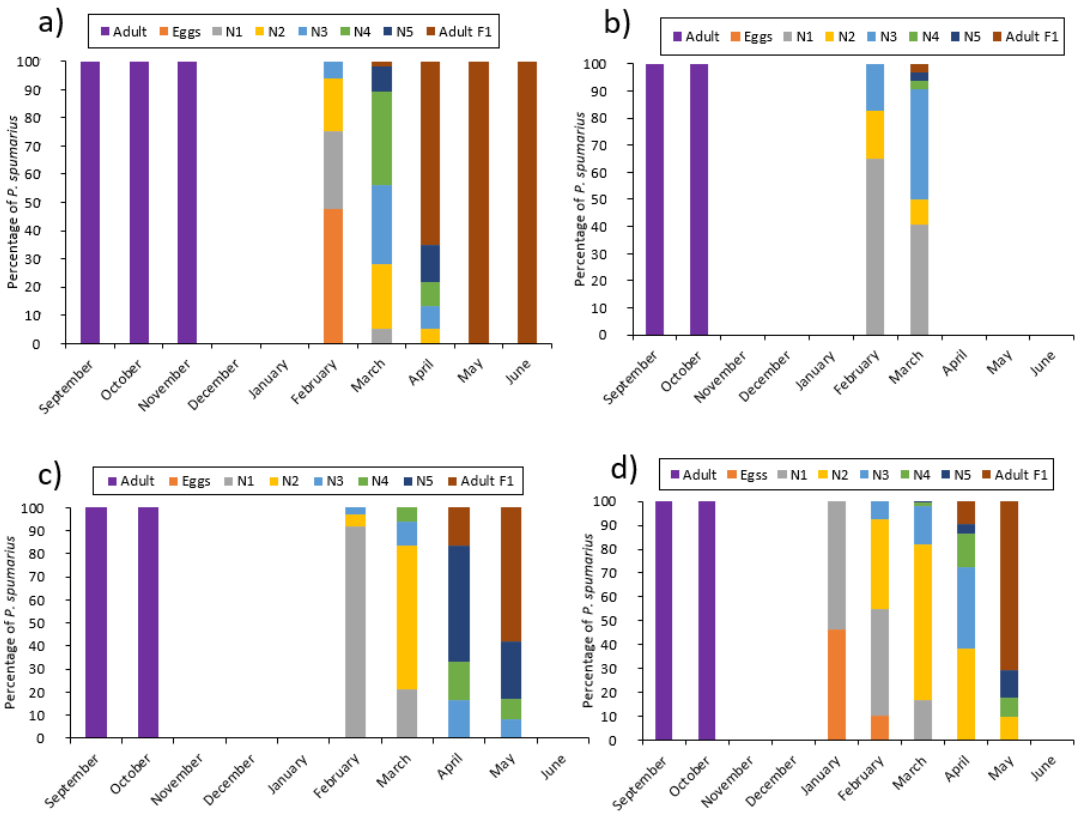

Figure 52. Development of *P. spumarius* in plants of *Rosmarinus officinalis* (a), *Pistacia lentiscus* (b), *Mentha piperitha* (c) and *Lavandula dentata* (d) in microcosm trials from September 2019 to June 2020.

2020-2021 trial

From the 50 cages, eggs batches were only detected from cages with *P. spumarius* placed in *R. officinalis,* in *L. dentata* and in *O. basilicum* plants (Table 8). We obtained 23 % of cages with oviposition in the microcosm environment units and 10 % of females succeeded in ovipositing (assuming one egg batch/female).

Table 8. Summary table of microcosm trials 2020 to 2021: Host plant, number of egg batches, number of eggs and number of nymphs emerged.

Cage	Plant sp.	Vector	Oviposition Yes/No	Nº egg batches	Nº Eggs/batch	Nº nymphs emerged
1 - 8	*R. officinalis*	*P. spumarius*	Yes	9	0.70 (7 eggs)	110
9 - 10		*N. campestris*	Yes	Not found	Not found	5
11 - 18	*P. lentiscus*	*P. spumarius*	Yes	Not found	Not found	13
19 - 20		*N. campestris*	Yes	Not found	Not found	2
21 - 28	*O. basilicum*	*P. spumarius*	Yes	10	0.8 (8 eggs)	34
29 - 30		*N. campestris*	Yes	Not found	Not found	37
31 - 38	*Mentha x piperita*	*P. spumarius*	Yes	Not found	Not found	22
39 - 40		*N. campestris*	Yes	Not found	Not found	5
41 - 48	*L. dentata*	*P. spumarius*	Yes	12	0.75 (9 eggs)	200
49 - 50		*N. campestris*	No	Not found	Not found	0

Adults were caged in October 2020 and were checked weekly until end-November. In December, when adults were no present, hay was checked to see the presence of eggs. In January there was no activity in any of the cages, while the first nymphs emerged in February.

First *P. spumarius* nymphs were detected the 14th of February 2021 in *R. officinalis*, *L. dentata*, *P. lentiscus*, *M. piperita* and *O. basilicum* (Fig. 53). Meanwhile, *N. campestris*

nymphs emerged differently for every plant species (Fig. 54). In *R. officinalis* nymphs were detected the 17th of February 2021; in *L. dentata* did not emerge; in *P. lentiscus* nymphs were detected the 3rd of March 2021; in *M. piperita* were detected the 19th of March 2021 and in *O. basilicum* were detected the 14th of February 2021.

Philaenus spumarius nymphs were observed either in the plant and in the grass, while *N. campestris* nymphs were only observed developing in the grass placed around the plants.

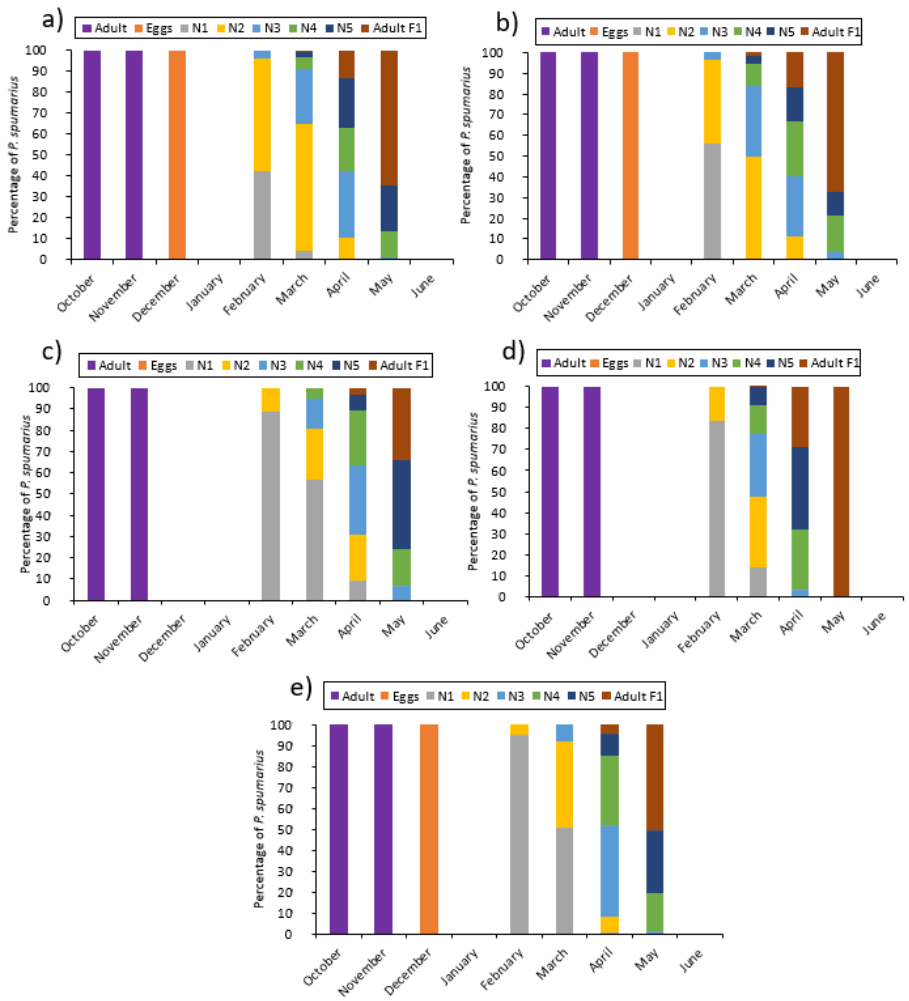

Figure 53. Development of *P. spumarius* in plants of *Rosmarinus officinalis* (a), *Lavandula dentata* (b), *Pistacia lentiscus* (c), *Mentha piperitha* (d) and *Ocimum basilicum* (e) in microcosm trials from October 2020 to June 2021.

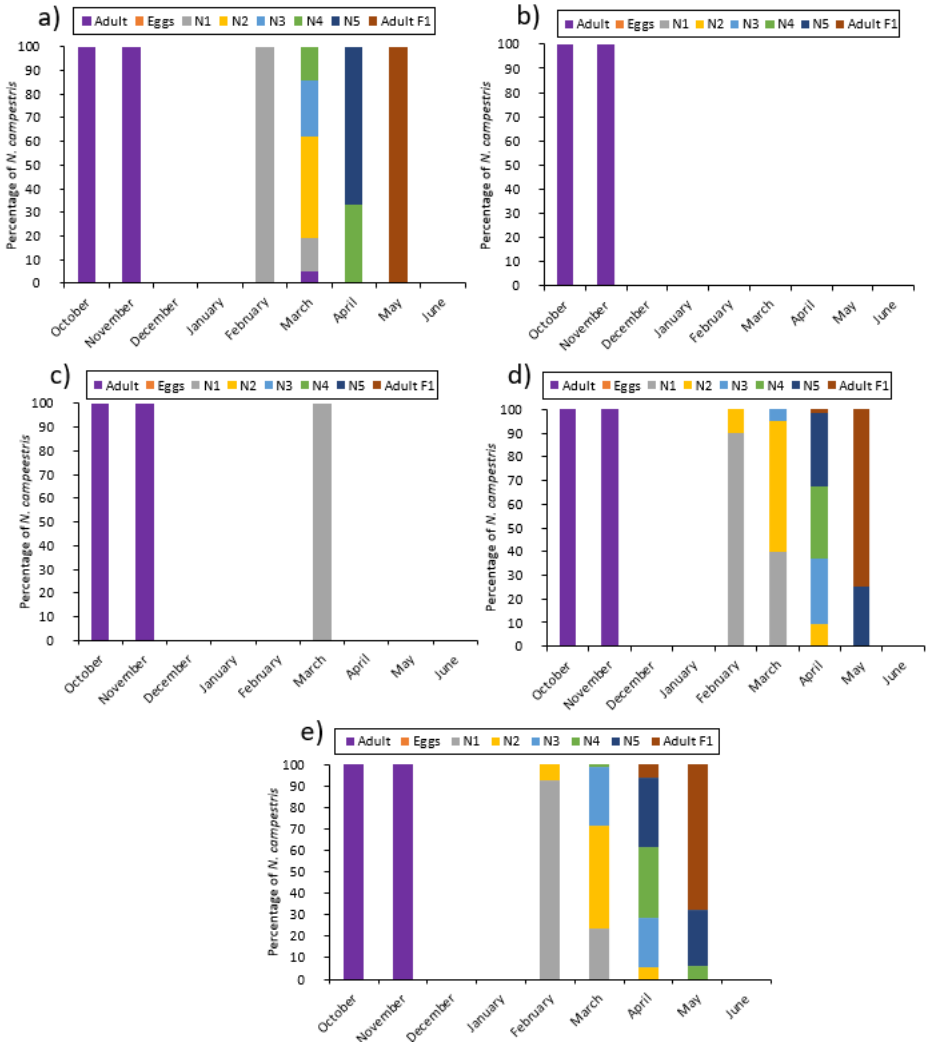

Figure 54. Development of *N. campestris* in plants of *Rosmarinus officinalis* (a), *Lavandula dentata* (b), *Pistacia lentiscus* (c), *Mentha piperitha* (d) and *Ocimum basilicum* (e) in microcosm trials from October 2020 to June 2021.

Discussion

The microcosm technique is an interesting tool to explore ecological threats with a controlled or semi-controlled environment (Stevenson and Dindal, 1985). Our microcosms study in Majorca showed that *R. officinalis*, *L. dentata*, *M. piperita*, *O. basilicum* and *P. lentiscus* may be suitable plants for adults of *P. spumarius* and *N. campestris*. Nymphs of *P. spumarius* were able to develop in the five plant species tested, but in rosemary and

lavender abundance were highest than in other plants suggesting more affinity and demonstrating their capacity of polyphagia. The case of *N. campestris* was the same as in the field observations, nymphal development was conducted only in the grass. Some species of plants (i.e., *R. officinalis*) seemed to be more appropriate for the development of adults than others (i.e., *O. basilicum*). Even so, Markheiser *et al*., (2020) suggested that *R. officinalis* was the less suitable for *P. spumarius* adult survival in comparison with other plants.

Philaenus spumarius oviposition rate was detected in 8 % of cages in 2019-2020 microcosm trials with three to five eggs per female, and in the 23 % of cages in 2020-2021 microcosm trials with 5 to 13 eggs per female. In our study we recorded from one to four egg masses in the five cages where eggs were found. *Neophilaenus campestris* eggs were not found but nymphs and F1 adults were recorded. Similar studies such as Di Serio *et al.*, (2019) recorded an average of 20 egg masses per cage, concluding that a single female could produce an average of 90-110 eggs in Torino (Italy) and 18-20 eggs in Bari (Italy).

In this study, insects were collected from herbaceous cover vegetation and caged in microcosm units from September to November. At that time, adults are known to mate and subsequently, to lay eggs, as explained in the Chapter 1. Females have eggs or oocytes under maturation in their ovaries and start to emit calling signals to males (Avosani *et al*., 2022). Nevertheless, *P. spumarius* offspring was not recorded in all the cages. In 2019-2020, *P. spumarius* nymphs were observed in 66 % of cages and adults in 16 %. In 2020-2021 trails, *P. spumarius* nymphs were in 87 % of cages and adults in 60 %, while *N. campestris* nymphs were in 60 % of cages and adults in 20 %.

These results may suggest that the selected plants are not optimal for *P. spumarius* and *N. campestris* full development, because they may have important mechanical limitations on food resources such as presence of trichomes in leaves or stems and hardness of stems (Hoffman and Mcevoy, 1986). According to Yurtsever (2000), nymphs and adults of spittlebugs feed not only on stems of plant, but on leaves, flowers and fruits. In addition, females may have impotent matings or inadequate sperm for fertilisation from

the first mating due to sperm depletion, as *P. spumarius* is a polyandrous species that presents multiple mating strategy to enhance fertility (Yurtsever, 2001). Thus, females should be exposed to more males to ensure proficient mating.

In our study, egg masses were found in the straw placed in the pots. Straw was considered a good oviposition substrate as Weaver and King (1954) described that the presence of straw increases in 65 % the oviposition rate in *P. spumarius*. Futher, it is known that the type of oviposition site influences the number of eggs per mass and also the total number of eggs laid (Weaver and King, 1954).

In our two years of study, egg hatching was observed between January and February, earlier than what may occur in field conditions as we observed N1 and N2 nymphs in March. At this moment in 2020 mean temperature inside the microcosm cages was 15 ± 3 °C (Annex III), while in the macrocosm (field) temperatures were about 10 °C. For example, egg hatching in early-March was recorded in olive groves from Italy with a mean temperature about 12 °C (Bodino *et al*., 2020) and in Greece with a mean temperature between 13 and 16 °C (Antonatos *et al*., 2021. This difference in timing among our cages and field studies may be due to greenhouse effect in cages as egg winter diapause is broken when nymphs are exposed to a chill period of less than 5 °C in 100 days as described by West and Lees (1988).

After hibernation of eggs, first instars were observed between January and February in both years of trials, while in field conditions few first instars were observed at that time (Chapter 1), probably because as the already mentioned effect of temperature on nymphal development. In addition, N1 and N2 nymphs are highly difficulty to detect them due to its small size and tendency to locate at the base of plants and the lack of foam in the early nymphal stages. In the microcosm, *Philaenus spumarius* showed preferences for the top of the plants and *N. campestris* for the base of grass. Even so, all instars of *P. spumarius* were found randomly in all the aerial part of the plant, probably because of the limited movement in the microcosm plant. In the field, N1-N2 nymphs are usually found at the base of plants and basal rosettes and more developed instars move to upper parts of

plants, as described firstly by Weaver and King (1954) Bodino *et al*., (2020) and Bodino *et al*., (2021) in olive and vineyard plots in Italy respectively. This behaviour is related with the need of nymph for self-protection and to avoid desiccation (e.g., sun and drying wind) and predators. We did not observe this trend maybe because caged nymphs had no threats and nymphs were frequently found without the spittle (author's observation).

In our study, first *P. spumarius* adults were detected in early-April 2020, 64 days after nymphal emergence. In 2021, *P.spumarius* adults were observed in mid-March in micrososms cages 31 days after nymphal emergence and 68 days for *N. campestris* in end-April. Those results are in accordance with the data under field conditions, Yurtsever (2002) explained that *P. spumarius* adult emergence occured after 50 days from egg hatching and Weaver and King (1954) after 58 days. On the contrary, in olives groves from Liguria (northern Italy) *P. spumarius* adults were collected 71 days after nymphal emergence (Bodino *et al*., 2019). Those differences in timing may be due to weather variation among places and years sampled (Kingsolver, 1989).

With this microcosms trials we accomplished to generate offspring of *P. spumarius* and *N. campestris* under controlled conditions in *R. officinalis*, *L. dentata*, *P. lentiscus*, *O. basilicum* and *Mentha piperita* with 8 to 23 % oviposition rate. We observed that first nymphs emerged in January-February, while in macrocosms we detected them from March to May. Finally, adults were detected from April as occurs in the field.

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CHAPTER 3

Xylella fastidiosa prevalence of the potential insect vectors in the Balearic Islands

Abstract

The bacterium *Xylella fastidiosa* (*Xanthomonadaceae*) is a xylem-sap limited pathogen that can affect more than 600 plant species worldwide, causing economical losses to farmers. The pathogen is transmitted by Cicadomorpha (Hemiptera) xylem feeder insects. In Europe, two species of Aphrophoridae insects are considered as major vectors: *Philaenus spumarius* and *Neophilaenus campestris*. The bacterium was detected in Majorca Island (Balearic Islands, Spain) in October 2016. The main objective of this study was to assess the prevalence of the pathogen in vectors collected from olive, almond and vine crops. For this, samples were collected from 2017 to 2020 in Majorca, Minorca, Ibiza and Formentera. Sampling of vectors was conducted by using sweep net, then insects were identified and preserved in ethanol at –20 °C for qPCR analysis. Both *P. spumarius* and *N. campestris* adults were present in all crops, with 2751 Aphrophoridae collected throughout the years. The general prevalence of *X. fastidiosa* in the vectors was 22.8 %, being 23.6 % for *P. spumarius* and 20.8 % for *N. campestris*. Highest prevalence was reached in Majorca Island with 24 % of insects collected positive, followed by Menorca (21.5 %) and Ibiza (21 %). Formentera remained free of *X. fastidiosa*. Analysis per crops showed that in Majorca, the highest prevalence was registered in olive, in Ibiza in almond and in Minorca in olive.

Introduction

Xylella fastidiosa is known as an important plant pathogen in grape, citrus, olive, almond, coffee and many other species in horticulture, ornamental, and wild plants (Chatterjee *et al*., 2008; ESFA, 2013). The Pierce Disease (PD) was first described by Newton Pierce in Southern California in the 1980s (Pierce, 1892). There were three periods in the research of *X. fastidiosa* in the XX century (Almeida *et al*., 2016). The first belongs to the epidemic PD of grapevine in USA in the 1930s and 1940s that led to characterization of the disease and the knowledge of sap feeders as vectors in California's San Joaquin Valley and North Coast (Severin, 1949). Second period was in the 1960s were Japanese researchers (Doi *et al*., 1967; Ishiie *et al*., 1967) described the causal agent as "mycoplasma-like organisms" that causes PD and other yellow diseases, and then, after culturing in vitro it was identified as a bacterium called *X. fastidiosa* (Davis *et al*., 1978; Wells *et al*., 1987). The third period was provoked by two epidemics, PD in California and CVC in Brazil, and the urge of molecular tools that led to new findings in the 2000, as well as study of the transmission biology especially using grapevines as model systems.

It was originally restrained to the Americas but at the beginning of the XXI century it emerged in Europe and Asia (Denancé *et al*., 2017). Firstly, it was isolated from pear trees (*Pyrus pyrifolia*) in Taiwan at the end of 90s (Leu and Su, 1993) and then it was reported in grapevines in 2013 (Su *et al*., 2013). The same year, *X. fastidiosa* subsp. *pauca* (ST53) was extensively detected in olives trees in southern Italy, being the first outbreak in Europe (Cariddi *et al*. 2014; Saponari *et al*. 2014; Loconsole *et al*. 2016). A year later, in 2015, the pathogen was isolated from almond and grapevine in Iran (Amanifar *et al*., 2014), and until then it was also reported in France *X. fastidiosa* subsp. *Multiplex* (ST6, ST7 and ST88) and subsp. *Pauca* (ST53) in Corsica and PACA regions (Denancé *et al*., 2017; Cunty *et al*., 2022). In 2016, *X. fastidiosa* was detected in the Balearic Islands during a government surveillance (Olmo *et al*., 2017). Since then, the archipelago become in a special situation as each island presents different subspecies of the bacteria. The subspecies *pauca* was identified in Ibiza (ST80), while the subspecies *fastidiosa* was detected in Majorca and Minorca (ST81) (Olmo

et al., 2021). And at the same time, in Majorca it was detected the subspecies *multiplex* (ST7 and ST1) (Olmo *et al*., 2021).

Afterwards, new *X. fastidiosa* infected spots appeared in other regions of Europe such as Germany (EPPO, 2016), Alicante (Giampetruzzi *et al*., 2019), Madrid (Giampetruzzi *et al*., 2019), Tuscany (Saponari *et al*., 2019) and Porto (EPPO, 2019).

Only Hemiptera insects possesses sucking mouthparts that are highly modified for piercing tissues and extracting the fluid contents of plants (ITIS, 2022). Xylem-feeders need to pump and to ingest a high amount of fluid sap to obtain sufficient food energy. In consequence, they have a very specialized mouthpart. There are approximately 30,000 potential vectors species belonging to Cicadomorpha out of Europe, among this 49 were confirmed as vectors (e.g., *Homalodisca vitripennis* and *Graphocephala atropunctata*) (EFSA PLH, 2019). In Europe, there are three species of Aphrophoridae known to be vectors of *X. fastidiosa*: *Philaenus spumarius*, *P. italosignus* and *Neophilaenus campestris* (EFSA, 2021). *Philaenus spumarius* is a polyphagous insect that feeds on many dicotyledons through rarely on monocotyledons (Weaver and King 1954). Their sucking stylet-like mouthparts (mandibular and maxillary stylets) allow them to reach the xylem of plants, from which they ingest sap (Wiegert, 1964; Horsfield, 1978; EFSA, 2013). Winged adults are mostly responsible of *X. fastidiosa* spread due to their high mobility, so sensitive diagnostic tools are needed to detect the bacterium in the vector insects (EFSA, 2013).

Prevalence of *X. fastidiosa* in potential vectors is a potential tool to avoid the disease spreading and enhance the probabilities to detect it in new areas. Also, it is useful to detect the bacterium in buffer zones and symptom-less areas. This concept is known as "spy insects" and is widely used in *X. fastidiosa* infected regions (Cruaud *et al*. 2018).

The goal of this study was to assess the prevalence of *X. fastidiosa* in field collected insects from the Balearic Islands to assess risk infectivity in each studied region.

Materials and methods

Insect collection

Insects were collected biweekly from olive, almond and vineyard orchards using a sweep net during the surveillance program from November 2017 to December 2020.

Sampling on cover vegetation consisted in five repetitions of 20 m transect line in all crops (20 sweeps/transect). Almond and olive canopies are sweeps surrounding tree canopy in 18 trees (20 sweeps/tree). In vineyard, sampling consisted in a 100 m transect line repeated five times (100 sweeps/transect). Finally, bordering woody shrubs were sampled by sweeping randomly five individuals (20 sweeps/shrub). From the overall content of the sweeping, only 1/3 of potential vectors were collected, following a conservative sampling procedure. In the laboratory, the aphrophoridae were identified (Bieman *et al*., 2011; Kunz *et al*., 2011; Mozaffarian and Wilson, 2015; Wilson *et al*., 2015), counted and the relative abundance in each sampling site was calculated by dividing the total number of insects from the total number of sweeps in the sampling unit. Then, insects were preserved in ethanol 96° and frozen at -20 °C for molecular analysis.

In order to assess infection prevalence in insects collected, the percentage of insects harboring the bacterium versus the total number of collected insects on different hosts during the season was assessed (Cornara *et al.*, 2017).

qPCR analysis

Molecular analysis for the diagnosis of *X. fastidiosa* was performed from the heads of the vectors with eyes previously removed. The analysis was performed by the Laboratory of Microbiology at UIB. qPCR protocol in the Annex IV.

Samples with the three triplicates with a Ct value lower than 35 were considered positive. Ct values higher than 35 or without the three triplicates positives were considered unclear results, and the analysis was repeated to confirm the result.

Statistical analysis

Generalized Mixed Linear Models (GLMMs) with binomial error and logit link function were used to assess if *X. fastidiosa* infection in insects was affected by vector species (*P. spumarius / N. campestris*), sex (female / male) and crop (olive, almond, vineyard) (Fixed factors). Also, a Pearson correlation test was performed to assess the dependency between the vector abundance with the *X. fastidiosa* prevalence in the insects. Statistical analyses were performed in R software 3.2.5 (R Core Development Team, 2017) with the packages "lme4" and "lmer" (Bates *et al*. 2014) and "performanceAnalytics" (Peterson *et al*., 2018).

Results

Prevalence per vector species

In general, we detected a prevalence of 23 % of positive insects to *X. fastidiosa* in all the Balearic Islands except in Formentera (Table 9). From 1059 *P. spumarius* analysed in the Balearics, the 23.8 % were positive for *X. fastidiosa*. Also, from 488 *N. campestris* analysed, the 21.3 % resulted positive for the bacterium. The prevalence of *X. fastidiosa* was significantly higher in *P. spumarius* than in *N. campestris* taking into account inconclusive insects as positive for the model (Estimate: 0.2950; Std. Error: 0.0984; P- value = 0.0119) and without the inconclusive insects (Estimate: 0.2604; Std. Error: 0.1360; P- value = 0.0555). Furthermore, the number of infected adults was independent from species abundance (*P. spumarius:* r = 0.28, P-value = 0.003; *N. campestris:* r = 0.20, P-value = 0.04).

There were not significant differences in prevalence by sex in *P. spumarius* (24.2 % in females and 23.1 % in males; Estimate: 0.0937; Std. Error: 0.1312; P- value = 0.475). In the case of *N. campestris*, there were neither significant difference among sex (20.8 % in females and 19.6 % in males; Estimate: -0.0586; Std. Error: 0.205; P- value = 0.775).

Prevalence per crops

The highest prevalence of *X. fastidiosa* in insects per crops was observed in almond with 25.7 %, followed by 22.8 % in olive and 21 % in vineyard. There was a high percentage of inconclusive results in qPCR analysis reaching a maximum of 13.2 % in olive, 11.6 % in vineyard and 10.6 % in almond. No effect of the type of crop in the prevalence was observed in the models (olive-almond: Estimate: -0.01623; Std. Error: 0.13347; P- value = 0.992; vineyard-almond: Estimate: -0.17185; Std. Error: 0.14924; P- value = 0.481; vineyard-olive: Estimate: -0. 15562; Std. Error: 0. 13098; P- value = 0.459).

The prevalence of *X. fastidiosa* detected from *P. spumarius* was not significantly different among crops (olive: Estimate: 0.15648; Std. Error: 0.16010; P- value = 0.328; vineyard: Estimate: -0.01309; Std. Error: 0.18654; P- value = 0.944).

In the case of *N. campestris,* the model indicated significant differences between prevalence of *X. fastidiosa* in crops (olive: Estimate: -0.5384; Std. Error: 0.2536; P-value = 0.0337; vineyard: Estimate: -0.4430; Std. Error: 0.2499; P-value = 0.0763). But due to low captures of the species, no significant differences were observed in the post-hoc analysis (olive-almond: Estimate: -0.53841; Std. Error: 0.25355; P- value = 0.0851; vineyard-almond: Estimate: -0.44303; Std. Error: 0.24993; P- value = 0.1788; vineyard-olive: Estimate: 0.09538; Std. Error: 0.24555; P- value = 0.9202).

Prevalence per islands

In Majorca, a total of 25.4 % of the analysed insects were positive (25 % of *P. spumarius* and 27.1 % of *N. campestris*). In Ibiza, the total prevalence of *X. fastidiosa* reached the 21.3 % of all analysed insects (20.8 % of *P. spumarius* and 21.7 % of *N. campestris*). Finally, in Minorca the 21.4 % of the analysed insects were infected with *X. fastidiosa* (27.3 % of *P. spumarius* and 10.4 % *of N. campestris*).

In Majorca, the year showing the highest *X. fastidiosa* prevalence was 2018 for *P. spumarius* and *N. campestris* (Table 9), as well for Ibiza, while in Minorca, insects collected in 2019 showed the highest prevalence (Table 9). In general, in Ibiza and Minorca, we

observed that *X. fastidiosa* prevalence was lower in June-July than in October-November (Table 9).

Table 9. Number and percentage of *X. fastidiosa* positive (+) insects analysed by qPCR from the total number of individuals of *P. spumarius* and *N. campestris* collected in Majorca, Ibiza, Minorca and Formentera from 2017 to 2020.

Island	Month/Year	*P. spumarius* +/total (%)	*N. campestris* +/total (%)
Majorca	Feb – Dec/2018	102/354 (28.8 %)	32/74 (43.2 %)
	Jan – Dec/2019	11/83 (13.3 %)	5/51 (9.8 %)
	Jan – Dec/2020	48/208 (23.1 %)	5/30 (16.7 %)
Ibiza	Nov/2017	17/93 (18.3 %)	13/86 (15.3 %)
	Jul/2018	1/16 (6.3 %)	-
	Nov/2018	26/61 (42.6 %)	31/86 (36 %)
	Jun/2019	0/4	-
	Nov/2019	1/7 (14.3 %)	0/19
	Jun/2020	0/8	1/5 (20 %)
	Nov/2020	1/32 (3.1 %)	3/25 (12 %)
Minorca	Jul/2018	1/33 (3 %)	-
	Nov/2018	8/33 (24.2 %)	3/23 (13 %)
	Jun/2019	0/15	0/2
	Nov/2019	11/21 (52.4 %)	1/14 (7.1 %)
	Jun/2020	4/15 (26.7 %)	0/2
	Oct/2020	21/48 (43.8 %)	5/48 (10.4 %)
Formentera	Jun/2019	0/14	0/1
	Nov/2019	0/8	0/12
	Jun/2020	0/6	0/9
	Nov/2020	-	0/1

Prevalence in Majorca

For the seasonal prevalence of *X. fastidiosa* during 2018, the number of *P. spumarius* that resulted positive of *X. fastidiosa* were mainly in the months of April, May and June (Fig. 55 a). For *N. campestris*, positive insects were detected earlier than *P. spumarius*, from March to June (Fig. 55 b). During 2019, there were less insects that resulted positive for *X. fastidiosa* in Majorca than in 2018, but there was a peak of *P. spumarius* positives from April to July and another one in October and November (Fig. 55 c). For *N. campestris*, *X. fastidiosa* positive individuals were detected in August, October, and December (Fig. 55 d). In 2020, positive insects were detected during all the year (Fig. 55 e). Also, *X. fastidiosa* positive *P. spumarius* were collected during summer and autumn when they were in the canopy. In the case of *N. campestris*, only few insects were captured during February and August (Fig. 55 f).

The highest prevalence of *X. fastidiosa* in *P. spumarius* was in almond (27.6 %), followed by vineyard (25.6 %) and olive (22 %). The same occurred with *N. campestris*, 36.1 % of positives were from almond, followed by 29.3 % from vineyard and 19.4 % from olive crops.

The analysis of *X. fastidiosa* positive vectors per crop in 2018 (Fig. 56 a) showed that the highest prevalence for *P. spumarius* was found in the almond crop (32.6 %), followed by vineyard (32 %) and olive (25.7 %). For *N. campestris* was similar, the highest prevalence was recorded in almond (43.5 %), followed closely by vineyard (42.9 %) and olive (36.8 %).

In 2019 (Fig. 56 b), the highest prevalence in *P. spumarius* was in olive (22.5 %), followed by vineyard (7.7 %), while no positive adults were detected in almond crops. In the case of *N. campestris*, 25 % of the insects collected from almond crop were positive followed by olive (9.1 %) and vineyard (4.7 %) crops.

Finally, in 2020 (Fig. 56 c) the highest prevalence of *X. fastidiosa* in *P. spumarius* was recorded in almond (30.3 %), followed by vineyard (24

%) and olive (18.3 %). In the case of *N. campestris*, the highest prevalence found was in vineyard (25 %), then almond (16.7 %) and olive (15 %).

In spite of these results, there was no differences among the prevalence of *X. fastidiosa* in *P. spumarius* (Olive: Estimate: 0.05319, Std. Error: 0.19251, P- value = 0.782328; Vineyard: Estimate: 0.13305, Std. Error: 0.23557, P- value = 0.572211) and neither in *N. campestris* (Olive: Estimate: -0.7015, Std. Error: 0.4372, P- value = 0.109; Vineyard: Estimate: -0.1924, Std. Error: 0.4291, P- value = 0.654).

The subspecies of *X. fastidiosa* was detected in some of the analysed insects. From the *N. campestris* positive for *X. fastidiosa* in Majorca 2018, it was possible to determine one specimen as infected with *X. fastidiosa* subsp. *fastidiosa*. In the case of *P. spumarius*, six specimens were infected with *X. fastidiosa* subsp. *fastidiosa* and three with *X. fastidiosa* subsp. *multiplex.* In 2019, one *N. campestris* and two *P. spumarius* were infected with *X. fastidiosa* subsp. *multiplex.* Then in 2020, it was possible to determine *X. fastidiosa* subspecies in 17 insects, from these one *N. campestris* and ten *P. spumarius* were infected with *X. fastidiosa* subsp. *fastidiosa,* and six *P. spumarius* were infected with *X. fastidiosa* subsp. *multiplex.*

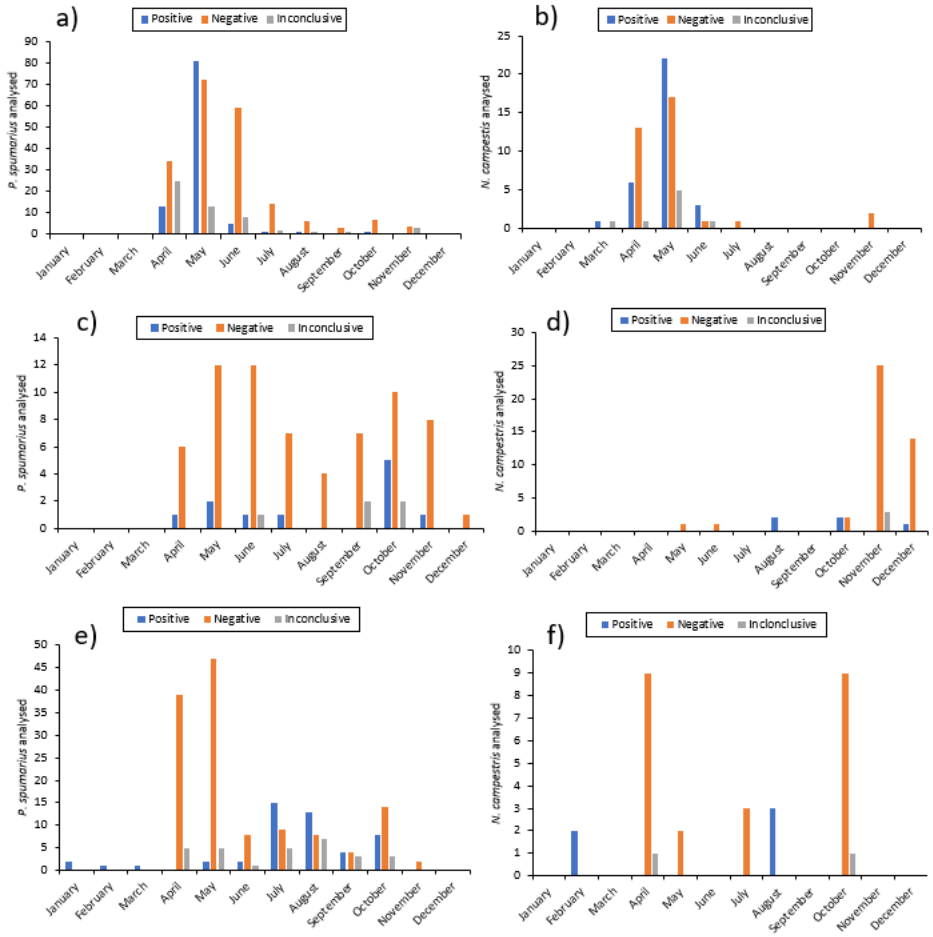

Figure 55. Detection of *X. fastidiosa* in field collected Aphrophoridae from Majorca during 2018 (a, b), 2019 (c, d) and 2020 (e, f). Positive means a Ct<35; Negative Ct>35; Inconclusive means Ct values Ct>35 or without the three triplicates positives for molecular detection of *X. fastidiosa*.

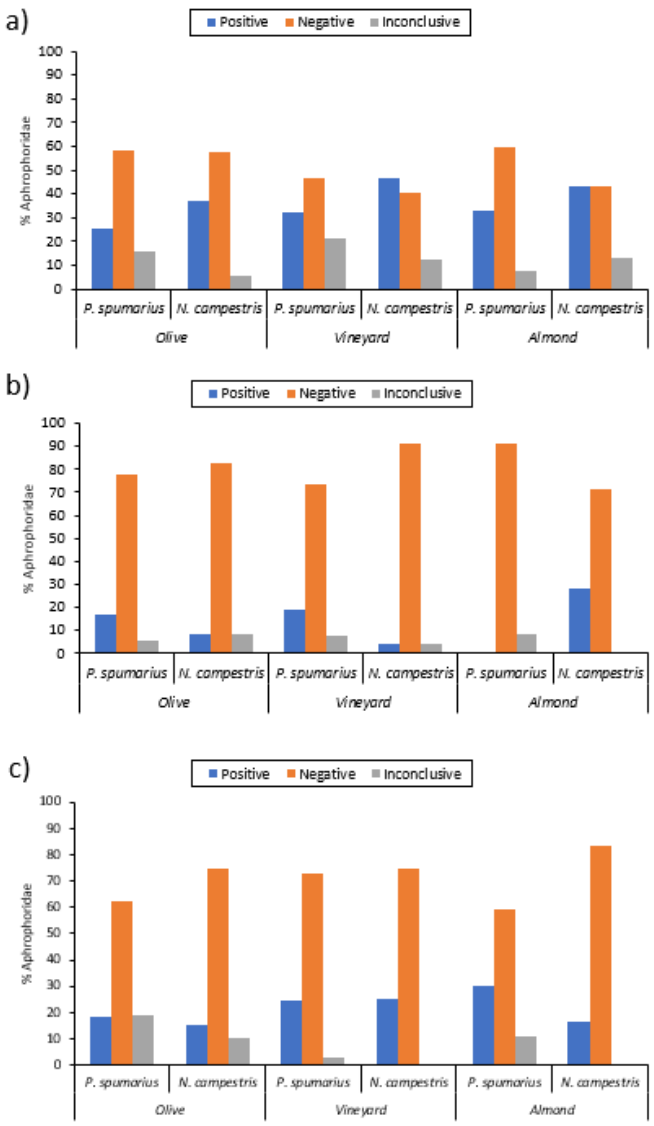

Figure 56. Detection of *X. fastidiosa* in field collected Aphrophoridae from Majorca in 2018 (a), 2019 (b) and 2020 (c). Positive means a Ct<35; Negative Ct>35; Inconclusive means Ct>35 or without the three triplicates positives for molecular detection of *X. fastidiosa*.

Prevalence in Minorca, Ibiza and Formentera

Analysis of vectors from Minorca, Ibiza and Formentera (Fig. 57) showed that adults positive for *X. fastidiosa* were found in all crops in Ibiza, none in Formentera and only in olive and vineyard in Minorca. In Ibiza, the highest prevalence of *X. fastidiosa* in insects

from 2017 to 2020 was detected in *N. campestris* in olive crop with a 31.7 %, and for *P. spumarius* in vineyard with the 26.8 % of positive insects analysed positive. In the case of Minorca, highest prevalence was detected in olive for *P. spumarius* with 37.5 % of positive insects and for *N. campestris* with 11.7 % of positives insects.

In Minorca, it was possible to determine the subspecies of *X. fastidiosa* subsp. *multiplex* in 26 insects (1 *N. campestris* and 25 *P. spumarius*). In Ibiza, *X. fastidiosa* subsp. *pauca* was detected in two *N. campestris* and two *P. spumarius*.

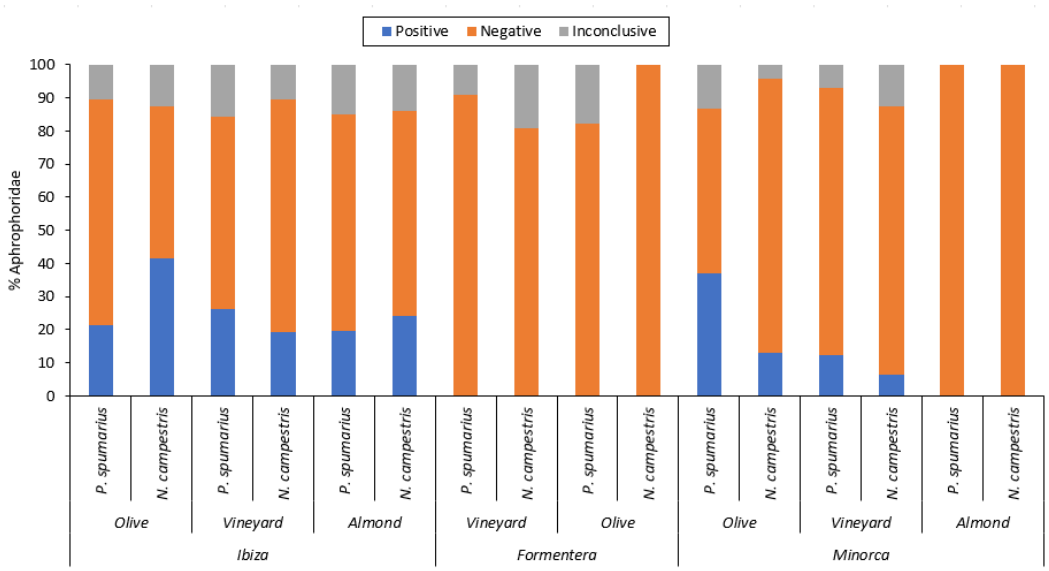

Figure 57. Detection of *X. fastidiosa* in field collected Aphrophoridae from Ibiza, Formentera and Minorca from 2017 to 2020. Positive means Ct<35; Negative Ct>35; Inconclusive means Ct values Ct>35 or without the three triplicates positives for molecular detection of *X. fastidiosa*.

Discussion

Results of this study provided new insights to the *X. fastidiosa* epidemiology in the Balearic Islands to assess the risk of transmission and improve the early detection of the disease (EFSA PLH, 2019). There were analysed 1,547 insects by qPCR to detect the presence of *X. fastidiosa* from olive, almond and vineyard crops by analysing *P. spumarius* and *N. campestris* foreguts. From these, 23 % resulted positive for *X. fastidiosa* (23.8 % *P.*

spumarius and 21.3 % *N. campestris*) collected from infected areas of Majorca, Minorca and Ibiza, while Formentera insects were free of harbouring the bacteria. These two vector species, along with *P. italosignus*, are considered the main potential vectors of *X. fastidiosa* in EU (Cavalieri *et al*., 2018; EFSA, 2021). Our results indicated that *P. spumarius* showed higher prevalence of *X. fastidiosa* infection in comparison to *N. campestris*, that would confirm the major role of *P. spumarius* on the transmission of the bacterium in the Balearic Islands. These outcomes are in line with the observations of Saponari *et al*., (2014) and Cornara *et al*., (2016) in Italy, that firstly demonstrated *P. spumarius* as an effective vector under natural conditions by infecting plants with field collected insects. Although *X. fastidiosa* prevalence in insects was around 20 %, *N. campestris* reached 40 % of positivity in 2018, similar to results of Elbiano *et al*., (2014) that found in Gallipoli (Lecce, Italy) a total of 45 % of positive insects from an infected area, but in the same province Yaseen *et al.*, (2015) found that where infections rates were 23.7 % in *N. campestris*, 16.3 % in *E. lineolatus* and 14.7 % in *P. spumarius*.

In addition, the prevalence obtained in our study was similar to the one obtained in Alicante (Spain) where 327 Aphrophoridae collected form almond crop were positive for *X. fastidiosa* (27 % *P. spumarius* and 21 % *N. campestris*) (EFSA, 2018). Even so, the prevalence obtained in our study is far from those obtained in other regions of Europe such as Italy. For example, Cornara *et al*. (2017) captured adults of *P. spumarius* regularly in the olive tree canopy giving values of 90 % positivity to *X. fastidiosa*.

In the other *X. fastidiosa* affected area in Spain, Alicante, the prevalence of the bacteria in the foregut of insects is five times lower than in our study with a 4.2 % (N = 9,467) of positivity, being 1.4 % *N. campestris* and 7.2 % *P. spumarius* (Generalitat Valenciana, 2022).

Vector foregut microbiome communities vary among the vector geographical distribution (Backus and Morgan, 2011), that would explain the difference of prevalence between different sites. It is unknown if the *X. fastidiosa* subspecies and ST may affect its capacity to colonize the insect foregut, as is the case of Italy (subspecies *pauca* ST53) and Majorca (subspecies *fastidiosa* ST1 and, *multiplex* ST81 and ST7). Furthermore, in the

Balearic Islands there are no extensive crops, while landscape is a mosaic of different crops (abandoned and cultivated) with diverse plant species that may cause a dilution effect diminishing the probability of detecting insects and the bacteria. Recently, Capellari *et al*., (2022) showed the potential key role of non-managed grasslands in the spread of *X. fastidiosa* and the effect of mosaic habitats of arable lands, grasslands, olive groves vineyards and woodlands.

In our study there was no correlation between the number of insects infected with its abundance in the field. Contrary to Ben Moussa *et al*., (2016) that found a higher correlation of *P. spumarius* and *E. lineolatus* abundance with *X. fastidiosa* incidence in insects, in exception with *N. campestris*. The lack of correlation may be due to the low occurrence of insects during our 3-yr survey as reported in the Chapter 1, and higher abundance of vectors may be needed to observe the trend.

As well as there was no effect of gender on the number of *X. fastidiosa* positive insects neither in *P. spumarius* and *N. campestris.* Similar results were reported in *H. vitripennis*, an american *X. fastidiosa* vector, reporting that gender did not affect acquisition and retention of the bacteria in the insect (Krugner *et al*., 2021). Also, Yaseen *et al*., (2015) did not revealed correlation between the infection rate to the gender of *P. spumarius*, *N. campestris* and *E. lineolatus* in Apulia.

Philaenus spumarius and *N. campestris* were infected with *X. fastidiosa* subsp *multiplex* and *fastidiosa* in Majorca from olive, vineyard and almond crops. In Minorca, *X. fastidiosa* subsp *multiplex* and in Ibiza *X. fastidiosa* subsp *pauca* were detected in *P. spumarius* and *N. campestris* from olive and vineyard crops. These results are in accordance with the epidemiology and description of the disease in plants in the Balearic Islands previously described by Olmo *et al*., (2021) where three subspecies and four Sequence Types (STs) are distributed across the islands. Nowadays in the Balearic Islands, the bacterium has been detected in 28 plant species, but the disease is widely spread throughout the islands in cultivars and wild plants. *Xylella fastidiosa* subsp. *fastidiosa* (ST1) is found only in Majorca, *X. fastidiosa* subsp. *pauca* (ST80) in Ibiza, *X. fastidiosa* subsp. *multiplex* (ST81) in Majorca and Minorca, and *X. fastidiosa* subsp. *multiplex* (ST7) in Majorca

(Olmo *et al*., 2017). Previously, *X. fastidiosa* subsp *fastidiosa* was detected in cherry and *P. myrtifolia* (Olmo *et al*., 2017), in *P. avium* (Landa *et al*., 2018) and in vineyards (Gomila *et al*., 2019; Moralejo *et al.*, 2019). In other regions of Spain such as Alicante, *X. fastidiosa* subsp. *multiplex* (ST6) was detected in almond (Giampetruzzi *et al*., 2018). The characteristic situation in our territory indicates that there were several introductions and establishment of *X. fastidiosa* decades before (Moralejo *et al*., 2020). Each of these subspecies and ST are susceptible to infect different kind of plant species but they have no specificity for vectors. Usually, infected insects did not harbour different subspecies and/or ST of *X. fastidiosa* as in our study, but there was observed in *H. vitripennis* from California two strains (*multiplex* and *fastidiosa*) of the bacteria in the same individual and Cruaud *et al*., (2018) found in Corsica 6 % of *P. spumarius* captured carrying two subspecies of bacterium. Also, microbial colonies in the foregut of insects may influence *X. fastidiosa* infection in vectors (Baccus and Morgan, 2011).

The prevalence of *X. fastidiosa* in vectors per crops was around 20 to 40 % of positivity. The highest prevalence in the Balearic Islands of infected vectors was observed in almond (25.7 %), followed by olive (22.8 %) and vineyard (21 %). In fact, almond is the crop with the major area covered (165.3 km^2), followed by olive (48.6 km^2) and vineyard (17.3 km^2).

In Minorca the highest prevalence was recorded in *P. spumarius* in olive crop (37.5 %), in Majorca in *N. campestris* in almond (35.1 %) and in Ibiza in *N. campestris* in olive crop (31.7 %). From plants analysed in Olmo *et al*., (2021), the highest prevalence of *X. fastidiosa* was detected in almond (24.8 %), followed by vineyard (14.1 %) and olive (9.8 %), this comprised the 60.8 % of total positives that they detected. The same happens in Alicante where from the prospections from 2017 to 2020, the 90.3 % of positive plants were from almond (Generalitat Valenciana, 2022). In both aforementioned Spain provinces, the major spread subspecies of *X. fastidiosa* are *multiplex* and *fastidiosa*, that would explain the impact on almond. Our results also indicate that almond is being more affected than the other crops in the Balearics and explain the higher prevalence of *X. fastidiosa* from the vectors captured in almond crops. But Almeida and

Purcell (2003a, b) in California found that in almond the transmission efficiency was lower than in vineyards with *H. coagulata*. Vector efficiency seems to be different depending on the vector species and host plant, among other ecological factors (Almeida *et al*., 2005).

In Ibiza and Minorca, the insects captured from olive crops had higher prevalence of *X. fastidiosa* than almond and vineyard. Subspecies *pauca* and *multiplex* have been mainly found in olive and wild-olive trees. The subspecies *pauca* ST80 detected in Ibiza seems less virulent that ST53 that causes deadly dieback in Italy. The analysis of the *X. fastidiosa* prevalence in vectors provide a useful tool for pathogen surveillance, as proposed by other authors such as Yaseen *et al*., (2015), Ben Moussa *et al*., (2015), D'onghia (2017) and Cruaud *et al*., (2018) using the "spy insect" approach for the case of *X. fastidiosa* surveillance in Italy and France. Insects are easy to be captured and the analysis targets the mouth parts. On the contrary, the bacteria infecting plants may be absent from plant tissues from where samples are collected (e.g., leaves of twigs).

Regarding the seasonality of infection rate in the vectors, positive insects in Majorca were detected from March to January, similarly to the results obtained from Apulia in Italy (Ben Moussa *et al*., 2016), and *P. spumarius* had the highest prevalence also. In May-June the percentage of positive insects ranged from 15-50 % in *P. spumarius* an *N. campestris*, and in October-November there were 10-50 % of *P. spumarius* and 20 % of *N. campestris* positive. These results are in line with the prevalence recorded in Corsica (France) in *P. spumarius* ranging from 0-43.7 % in June and 12.5-34.4 % in October (Cruaud *et al*., 2018). Higher percentage of positive *P. spumarius* were recorded between May and June in Salento (Italy) observing 50 % to 82 % of infective insects (Ben Moussa *et al*., 2015), and 60 % to 70 % from November (Saponari *et al*., 2014). On the contrary, in Tuscany (Italy) only 1.5 % (N=662) of insects were infected and all of them were collected between September and November (Gargani *et al*., 2021). In addition, Cornara *et al*., (2016) found the highest infectivity values in *P. spumarius* from June to August in Gallipoli (Italy). For other species different from spittlebugs, in California vineyards Beal *et al*., (2021) revealed that the naturally infected spittlebugs were found 9-11 weeks after adult emergence and coincided with the adult peak in July-August. For the sharpshooter *Oncometopia nigricans*

(Cicadellidae) in Florida vineyards naturally infected individuals were detected 6-10 weeks after adult emergence (April and May) (Alderz and Hopkins, 1979). The timing to become infective is driven by the availability of infected host plants with enough bacterial load for vector acquisition and transmission (Beal *et al*., 2021). With other *X. fastidiosa* vectors such as *H. coagulata* and *G. atropunctata* it was demonstrated that they can transmit the bacteria in a persistent manner, becoming very efficient vectors (Almeida and Purcell, 2003a and b).

Adults move from herbaceous cover vegetation in late spring to trees and woody shrubs to stay for summer, from which acquisition of *X. fastidiosa* occurs and persist until insect death (Hill and Purcell, 1995; Moussa *et al*., 2016). Therefore, incidence of positive insects under field conditions was directly influenced by the life cycle of each species (Moussa *et al*., 2016; Bodino *et al*., 2021). Even so, in EFSA PLH (2015) reported that in Italy *P. spumarius* from winter and spring never tested positive for *X. fastidiosa*. Unless the seasonality of infective observed in our study and in other literature, there are vineyards in California with chronical Pierce Disease infections that indicates insect vectors resist to mild winters and keep infecting next year, even so to dormant plants (Feil *et al*., 2003; Almeida *et al*., 2005; Beal *et al*., 2021).

There are available serological and molecular methods to detect *X. fastidiosa* (ESFA, 2021). The methodology used for the detection in the vectors is described in the EPPO diagnostic standard PM 7/24 (4) (EPPO, 2019). It recommends conventional PCR, real-time PCRs and loop-mediated isothermal amplification (LAMP) due to *X. fastidiosa* is usually present in low numbers (EFSA, 2021). This would lead to lack of sensitivity in terms of detection, finding inconclusive results, like for example we found a percentage of inconclusive insects between 5 % and 20 %.

Clear positive insects can be easily identified when the bacterial load is high as described in Cruaud *et al*., (2018). In Corsica, 73 % of vectors analyzed had almost one undetermined result with the qPCR (Cruaud *et al*., 2018). If the bacterial load is high enough to be detected by the recommended methods, we hypothesized that routinary *X. fastidiosa*

surveys by putative vectors analysis is the key for the epidemiology and transmission of the disease into new areas such as the case of Formentera, still free of the disease.

In conclusion, our survey provided the first information on seasonal abundance of *X. fastidiosa*-infected *P. spumarius* and *N. campestris* in the Balearic Islands in olive, almond and vineyard crops. However, we also suggest that both species do not play the same role as vectors, as *N. campestris* abundance and presence in crops is significantly different and lower than *P. spumarius*.

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CHAPTER 4

Vectorial capacity of *Philaenus spumarius* and *Neophilaenus campestris*

Abstract

Xylella fastidiosa is a pathogen xylem-limited bacterium detected in the Balearic Islands (Spain) in 2016 and transmitted by xylem-sap feeders insect vectors. The major potential vectors described in the Balearics are the spittlebugs *Philaenus spumarius* and *Neophilaenus campestris* (Aphrophoridae). In order to assess the potential transmission efficiency of these vectors, two types of transmission tests were conducted. In the first type, we assessed the natural infectivity of the vectors. For this, *P. spumarius* and *N. campestris* adults were collected from the field and kept in groups of three to five insects on alfalfa (*Medicago sativa*) for 96 hours for the inoculation access period (IAP). The infection status of alfalfa plants was checked at 15, 30, 45 and 60 days after IAP. In the second type of experiment, uninfected adults (only *P. spumarius*) were caged with *X. fastidiosa* infected grapevine and almond plants for 96 hours of acquisition access period (AAP) and then transferred onto healthy plants of alfalfa as described for the first type of experiment. In both experiments, the presence of *X. fastidiosa* in plants and insects was determined by qPCR analysis. In the first experiment, 21.7 % of *P. spumarius* and 15.6 % of *N. campestris* (field collected) tested were positive for *X. fastidiosa*; 34.8 % of the plants exposed to *P. spumarius* and 42.9 % of those exposed to *N. campestris* were infected. In the second experiment, 3.8 % of *P. spumarius* acquired the bacteria from infected vine plants and 14.3 % of the alfalfa plants resulted positive to *X. fastidiosa* after the IAP. In conclusion, both species of spittlebugs found in the Balearics are able to transmit *X. fastidiosa* in laboratory trials. However, its comparative epidemiological role could be different due to host preference, distribution, and abundance.

Introduction

The first identification of the vectors of the etiological agent of Pierce's disease was made by Hewitt *et al*., (1942), suggesting that they were sharpshooters and leafhoppers (Hemiptera: Cicadellidae). Then, several species belonging to Auchenorrhyncha has been described as potential vectors (Redak *et al*., 2004). *Philaenus spumarius*, *P. italosignus* and *Neophilaenus campestris* (Hemiptera:Aphrophoridae) are the insect vectors proven to be able to transmit *Xylella fastidiosa* in Europe (EFSA, 2021). Among them, *P. spumarius* has revealed to be the dominant and widespread xylem-sap feeder in vineyard and olive agrosystems in the Mediterranean basin (Morente *et al.*, 2018; Antonatos *et al.*, 2019; Bodino *et al.*, 2019; Bodino *et al.*, 2021a; Bodino *et al.*, 2021b). The risk of transmission of *X. fastidiosa* is due to the feeding activity of adults in tree canopies and bordering woody shrubs (Cornara *et al.*, 2017; Cavalieri *et al.*, 2019; Bodino *et al.*, 2019). The presence of *X. fastidiosa* in suitable areas with the occurrence of competent vectors is a serious threat to cropping systems and landscape (Cavalieri *et al.*, 2019), in particular because vectors are able to handle with a diversified range of resources across multiple habitats, as it is demonstrated by the complexity of their life cycle (Cappellari *et al*., 2022). *Xylella fastidiosa* does not colonize the haemolymph or internal organs of insects, and transmission by adults is persistent and without transovarial transmission (Severin, 1949; Freitag, 1951; Purcell *et al*., 1979). In fact, it has been quantified that the foregut of vectors may house up to ~50,000-100,000 cells of the bacteria (Almeida *et al.,* 2016).

First transmission tests with spittlebugs were carried out by Severin (1950) after the epidemic outbreaks of Pierce disease in vineyards in California's central valleys (Almeida *et al*., 2016). The transmission efficiency depends on the vector species and the plant recipient species (Daugherty *et al*., 2010), but it is not affected by gender (Krugner *et al*., 2019). The bacterium forms a biofilm on portions of the vector foregut, which is the source for inoculated cells (Baccari *et al*., 2013). *Xylella fastidiosa* expresses context-dependent behaviours that enable its efficient colonization of both host plant as well as obligatory transmission by insects that is coordinated by cell-cell signalling (Baccari *et al.*, 2013). In the case of sharpshooters, inoculation of *X. fastidiosa* to plants is originated from

the precibarium, caused by salivation and rinsing egestion, which are performed before the onset of sustained ingestion. Adult may acquire and inoculate the bacteria within a few hours after initiating feeding (Krugner *et al*., 2019). The list of plant host species is large, more than 600 can be infected by *X. fastidiosa* (EFSA, 2021), so it is necessary to confirm the association between insect vectors and different plant species under controlled conditions by transmission assays. The general guidelines for the identification of new *X. fastidiosa* vectors in Europe proposed by EFSA PLH (2015) described the procedures to be considered. Firstly, to know the vector status of field-collected insects by the confinement of field-collected insects on uninfected plants and secondly using systematic testing to determine vector status by plant-to-plant transmission tests.

The aim of this work was to assess the potential transmission efficiency of *P. spumarius* and *N. campestris*, by conducting two types of transmission tests. We investigated i) the pathogen transmission success of natural infected vectors to *Medicago sativa* seedlings and ii) the efficiency of acquisition from natural infected vineyard and almond trees and transmission to *M. sativa* seedlings.

Materials and methods

Study site

In order to assess the vector role of the different Aphrophoridae species found in the Balearic Islands, vector competence test under controlled conditions were performed. All vector competence test were conducted in insect-proof cages placed at the biosecurity greenhouse in the experimental plot of Ca's Valencià at the Campus of UIB, as well as in the biosecurity (BSL2) insectary at UIB.

Insect vector collection and rearing

Two types of experiments (A and B) were carried out to assess the vectorial capacity of *P. spumarius* and *N. campestris*. For the transmission test A, adults of *P. spumarius* and *N. campestris* (Hemiptera:Aphrophoridae) were field collected from September-October 2019 and 2020 using a sweep net from the herbaceous cover vegetation from areas where plants had been confirmed to be positive to *X. fastidiosa*.

Consequently, insects were kept alive in groups of three to five in alfalfa plants and caged in polypropylene fabric mesh cages (30x30x30 cm) in the insectary (25 °C / 70-80 % HR) for transmission test.

For the transmission test B, a hundred *P. spumarius* N2-N3 nymphs were field collected between March and April 2019 and 2020 in different orchards of Majorca. Nymphs were identified (Zenner *et al*., 2005) and transferred using a brush to alfalfa in groups of 25 caged in polypropylene fabric mesh cages (30x30x30 cm) in the insectary (25 °C / 70-80 % RH). Adult insects free of *X. fastidiosa* were maintained in the same plants until the start of the tests.

Transmission efficiency of *X. fastidiosa*

Plants used for the tests were *X. fastidiosa* free alfalfa seedlings produced in the biosecurity 2 greenhouse. In the experiment A (Table 10), the insects were caged in groups of at least five individuals in alfalfa plants for 96 hours of inoculation access period (IAP) to test the natural infectivity of the vectors (Fig. 58) (Newmann *et al*., 2003, 2004; Saponari *et al*., 2014; Cornara *et al.*, 2016). Only insects alive at the end of the experiment were analysed. After the IAP period, head of insects were dissected, and eyes removed to determine bacteria presence in the vector by qPCR (EPPO, 2017). Plant samples were analysed to confirm the bacteria acquisition at the 15, 30, 45 and 60 days after the IAP. The transmission tests A were carried out between September and December 2019 and 2020.

Figure 58. a) and b): Inoculation cages used to maintain the vectors in contact with the plants in the insectary. c): *Philaenus spumarius* during the inoculation access period in alfalfa. © J. López-Mercadal.

Table 10. Number of plant (*M. sativa*) replications and insects (*P. spumarius* and *N. campestris*) used for the transmission test type A in 2019 and 2020.

Year	Date	*M. sativa*	*P. spumarius*	*N. campestris*
2019	24/09/2019 to 20/11/2019	27	75	39
2020	15/09/2020 to 27/12/2020	30	116	34

In the Transmission Test B, three to five free *X. fastidiosa* vectors were caged with nets (Figure 59) allowed to feed on a branch of a source plant infected of *X. fastidiosa* for 96 hours for acquisition access period (AAP). Three to five almond trees and vineyard plants were selected for the AAP from two organic orchards of Majorca following the methodology described in Cornara *et al.*, (2017), depending on the number of vectors available. After AAP, vectors were transferred to alfalfa plants for inoculation access period (IAP) following the same procedures as transmission test A. Also, leaves that were in contact with the insects were analysed by qPCR to confirm *X. fastidiosa* infection in the branch (EPPO, 2017). The Transmission Test B was carried out between May and July of 2019 and 2020.

Figure 59. Transmission nets used for the acquisition access period for *P. spumarius* in Transmission Test B in vineyard (a) and almond (b). Transferring *P. spumarius* into the transmission nets in an almond tree for the transmission trials type B (c). © J. López-Mercadal.

qPCR analysis

The Laboratory of Microbiology at UIB carried out the molecular analysis of *X. fastidiosa* in both insects and plants. Before analysis by qPCR, the head of the insect was detached, and the eyes were removed using sterilized pins. qPCR protocol is in the Annex IV.

Samples with the three triplicates with a Ct value lower than 35 were considered positive. Ct values higher than 35 or without the three triplicates positives were considered unclear results, and the analysis was repeated to confirm the result.

Statistical analysis

Generalized Mixed Linear Models (GLMMs) with binomial error and logit link function were used to assess the effect of gender and species (fixed factors) on the status of *X. fastidiosa* infection (infected / no infected). The year was included as random term. Also, a Pearson correlation test was performed to assess the dependency between the *X. fastidiosa* prevalence of vector and the gender (male / female), vector species (*P. spumarius* / *N. campestris*) and year of trial (2019 / 2020). Statistical analyses were

performed in R software 3.2.5 (R Core Development Team, 2017) with the packages "lme4" and "lmer" (Bates *et al*. 2014) and "performanceAnalytics" (Peterson *et al*., 2018).

Results

Results trials Transmission Test A:

2019 transmission trials

A total of 114 field collected adults (75 *P. spumarius* and 39 *N. campestris*) were used for the transmission test in 2019 (Fig. 58). From those adults, 62 *P. spumarius* and 22 *N. campestris* were analysed for the detection of *X. fastidiosa* (Table 11), meaning a survival rate of 82.7 % for *P. spumarius* and 56.4 % for *N. campestris*. After qPCR analysis, the 21 % and 8 % of *P. spumarius* were positive and Inconclusive, respectively. About *N. campestris*, 9.1 % were positive for *X. fastidiosa*, while 4.6 % were inconclusive.

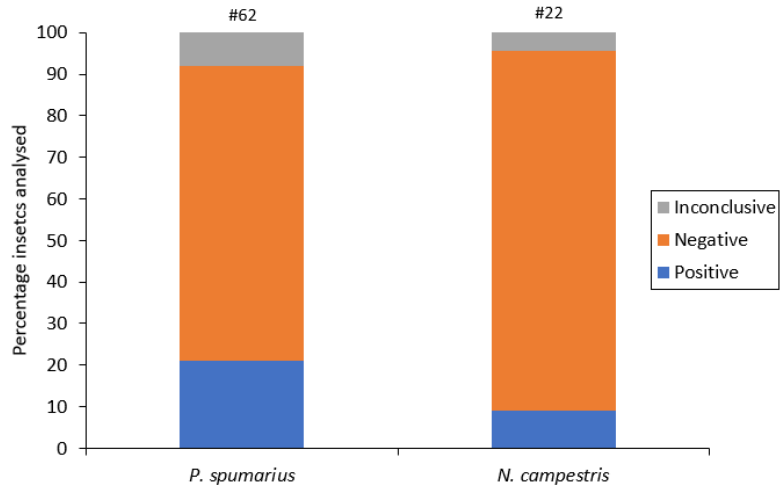

Figure 60. Detection of *X. fastidiosa* analysis in the field collected adults of Aphrophoridae used for the Transmission Tests A in 2019.

Table 11. Gender of the Aphrophoridae employed for the Transmission Tests A in 2019.

	Female	Male
P. spumarius	56	6
N. campestris	18	4

Inoculation to plants (*M. sativa*) of *X. fastidiosa* by field collected Aphrophoridae adults was confirmed 15 days after inoculation for *P. spumarius* and 30 and 45 days after inoculation for *N. campestris* (Fig. 61).

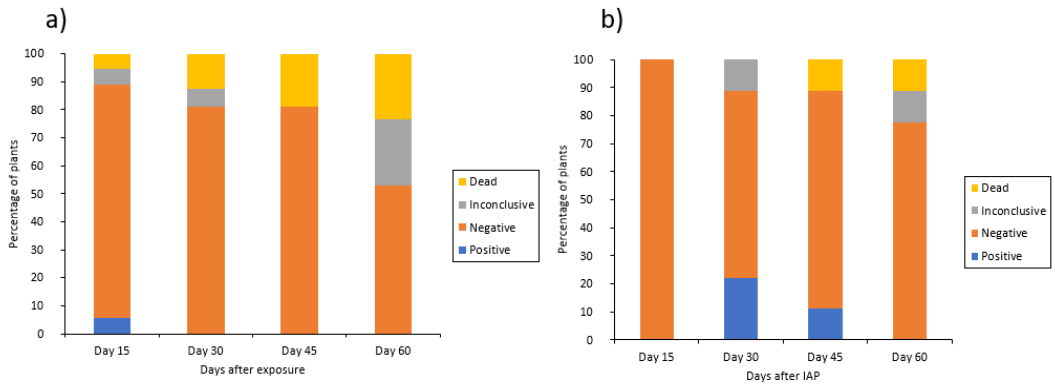

Figure 61. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to field collected adults of *P. spumarius* (a) and *N. campestris* (b) in the Transmission Test in 2019.

2020 transmission trials

A total of 150 vectors (116 *P. spumarius* and 34 *N. campestris*) were used for transmission test in 2020 (Fig. 62). From these, 106 *P. spumarius* and 32 *N. campestris* were finally analysed (Table 12), meaning a survival rate of 92.2 % for *P. spumarius* and 91.4 % for *N. campestris*. The 21.7 % and 26.4 % of *P. spumarius* were positive and inconclusive respectively. About *N. campestris*, 15.6 % were positive for *X. fastidiosa,* while 18.8 % were inconclusive.

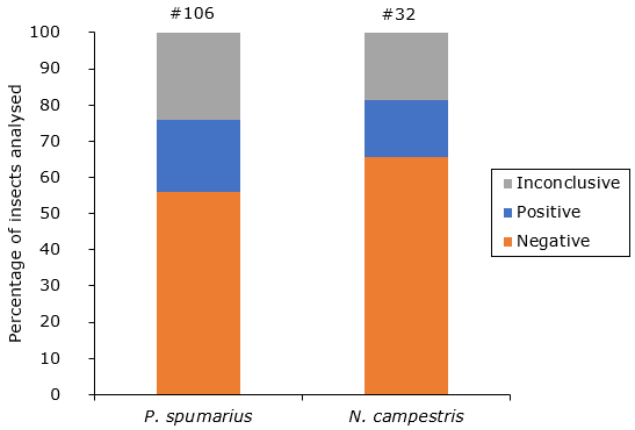

Figure 62. Detection of *X. fastidiosa* analysis in the field collected adults of Aphrophoridae used for the Transmission Test A in 2020.

Table 12. Gender of the Aphrophoridae employed for the Transmission Tests A in 2020.

	Female	Male
P. spumarius	64	43
N. campestris	18	13

Inoculation to plants (*M. sativa*) of *X. fastidiosa* by field collected insects was confirmed for days 15 to 60 days (end of trial) after inoculation for *P. spumarius* and for days 15, 45 and 60 for *N. campestris* (Fig. 63).

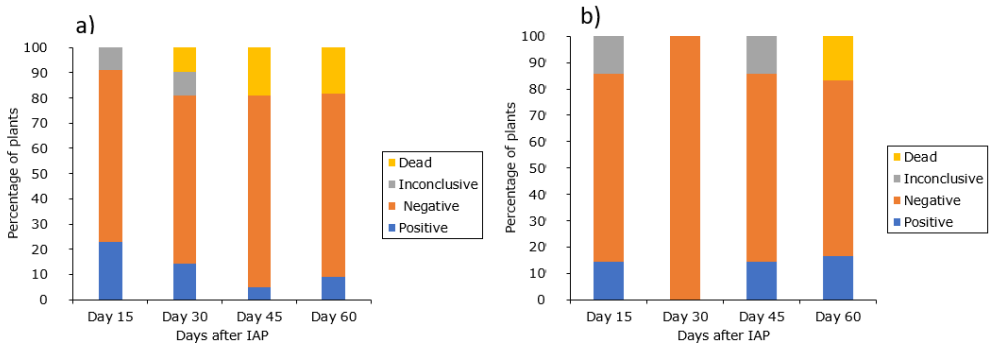

Figure 63. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to field collected adults of *P. spumarius* (a) and *N. campestris* (b) in the Transmission Test A in 2020.

Assessing vector inoculation

According to the GLMM, the prevalence of *X. fastidiosa* in *P. spumarius* and *N. campestris* during all the transmission trials did not depend on the vector species (Estimate: 0.01531, Std. Error: 0.35469, P-value = 0.966), gender (Estimate: -0.75622, Std. Error: 0.65733, P-value = 0.250) or year of the trial. Both species were infected equally even though prevalence in *P. spumarius* showed to be higher when compared to *N. campestris*.

Furthermore, the prevalence of *X. fastidiosa* in *M. sativa* after IAP did not depend on the vector species used (Estimate: 0.6348, Std. Error: 0.7072, P-value = 0.3693) neither on how many of them were infected (Estimate: 0.4626, Std. Error: 0.2465, P-value = 0.0605).

Finally, there was no correlation between the number of positive insects and the plants infected at the end of the trials (r = 0.25; P-value = 0.05207).

Results Transmission Test B:

2019 transmission trials

A total of 23 *X. fastidiosa*-free *P. spumarius* were used for the transmission test in almond and 14 *P. spumarius* in vineyard (Fig. 64) (Table 13). In almond, the 34.43 % of insects acquired the bacteria from trees, the 26.09 % were inconclusive and the 43.48 % were negative (Fig. 64). In the case of vineyard, the 87.71 % of insects acquired the bacteria from trees, the 7.14 % were inconclusive and the 7.14 % were negative (Fig. 64).

Table 13. Gender of *Philaenus spumarius* employed in the Transmission Tests B in 2019.

	Female	Male
Almond Transmission Tests	13	10
Vineyard Transmission Tests	7	7

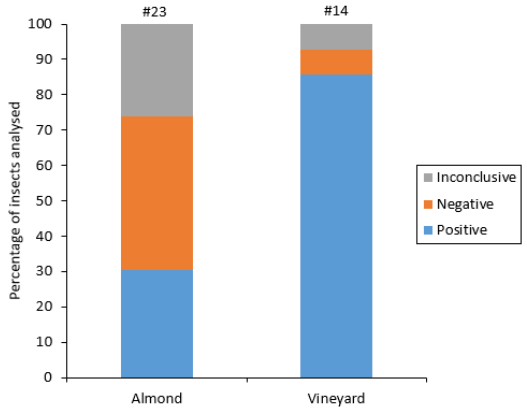

Figure 64. Detection of *X. fastidiosa* in *P. spumarius* that acquired the bacteria from almond and vineyard in the Transmission Test B in 2019.

Leaves of branches from naturally infected plants that were exposed to *P. spumarius* resulted positive to *X. fastidiosa* in almond and vineyard (Fig. 65).

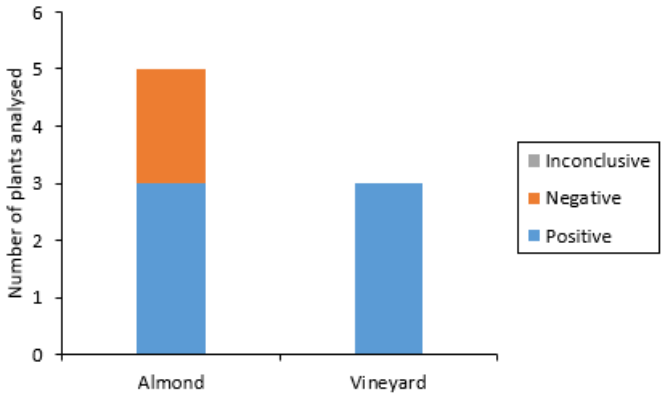

Figure 65. Detection of *X. fastidiosa* in the plants where *P. spumarius* were exposed in the Transmission Tests B in 2019.

Inoculation of *X. fastidiosa* to *M. sativa* plants by *P. spumarius* adults that acquired the bacteria from naturally infected almonds trees was confirmed 15 days after inoculation for (Fig. 66).

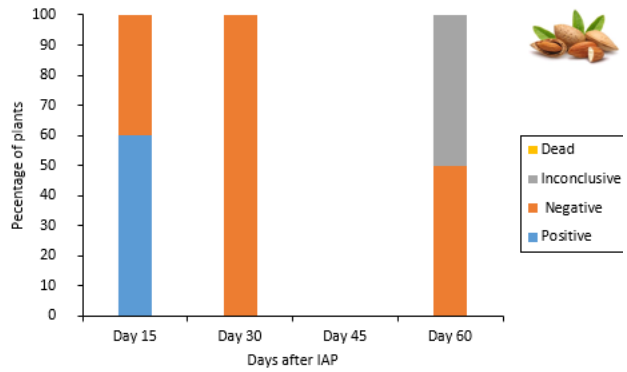

Figure 66. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to *P. spumarius* adults that acquired the bacteria from naturally infected almonds trees in the Transmission Tests B in 2019.

Inoculation of *X. fastidiosa* to *M. sativa* plants by *P. spumarius* adults that acquired the bacteria from vineyard plants was not confirmed after inoculation (Fig. 67).

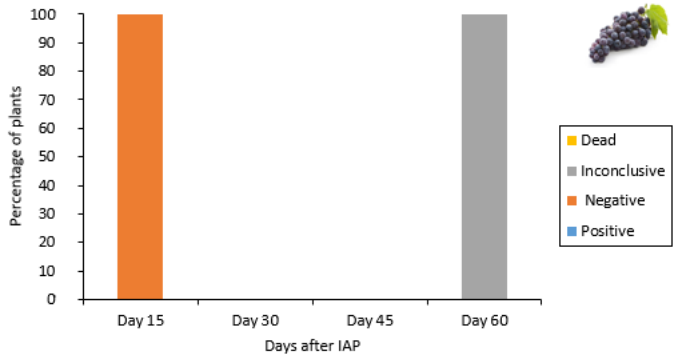

Figure 67. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to *P. spumarius* adults that acquired the bacteria from infected vineyard plants in the Transmission Test B in 2019.

2020 transmission trials

A total of 14 *X. fastidiosa*-free *P. spumarius* were used for the transmission test in almond and 12 *P. spumarius* in vineyard (Fig. 68) (Table 14). In almond, the 100 % of insects resulted negative to *X. fastidiosa* after AAP (Fig. 66). In the case of vineyard, the 8.3 % were inconclusive and 91.7 % negative (Fig. 66).

Table 14. Gender of *Philaenus spumarius* employed in the Transmission Tests B in 2020.

	Female	Male
Almond Transmission Tests	5	9
Vineyard Transmission Tests	8	4

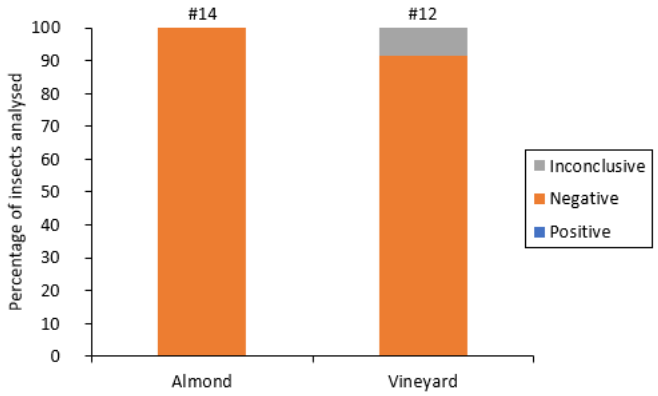

Figure 66. Detection of *X. fastidiosa* in *P. spumarius* that acquired the bacteria from naturally infected almond and vineyard in the Transmission Test B in 2020.

Leaves of branches exposed to *P. spumarius* resulted positive of *X. fastidiosa* in almond, while samples collected from vineyards resulted negative to *X. fastidiosa* infection (Fig. 67).

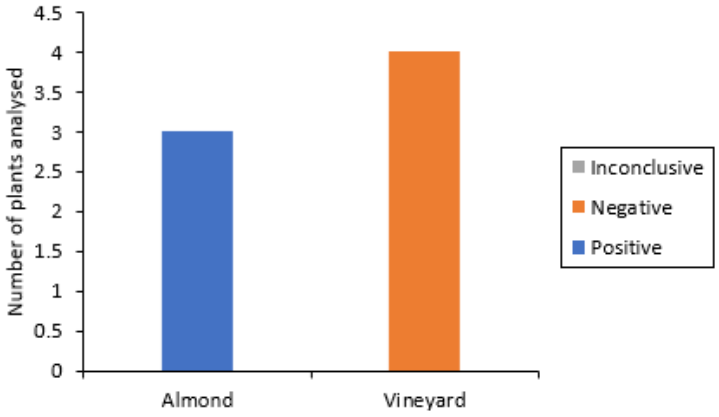

Figure 67. Detection of *X. fastidiosa* in the plants where *P. spumarius* were in the Transmission Test in 2020.

Inoculation of *X. fastidiosa* to *M. sativa* plants by *P. spumarius* that acquired the bacteria from naturally infected almonds was not confirmed (Fig. 68).

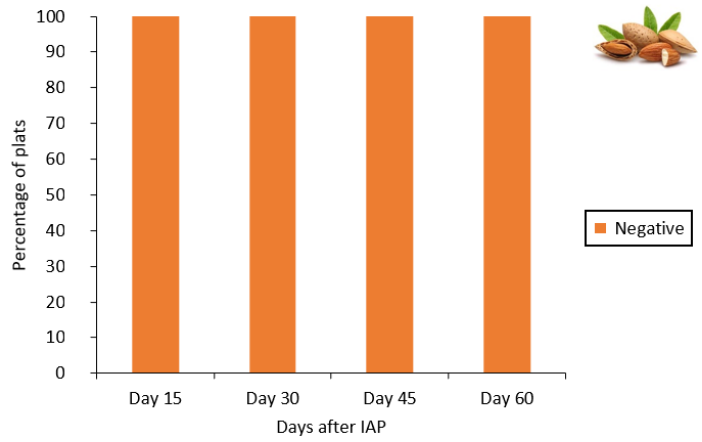

Figure 68. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to *P. spumarius* adults that acquired the bacteria from infected almonds trees in the Transmission Test B in 2020.

Inoculation of *X. fastidiosa* to *M. sativa* plants by *P. spumarius* that acquired the bacteria from naturally infected vineyards was not confirmed after inoculation (Fig. 69).

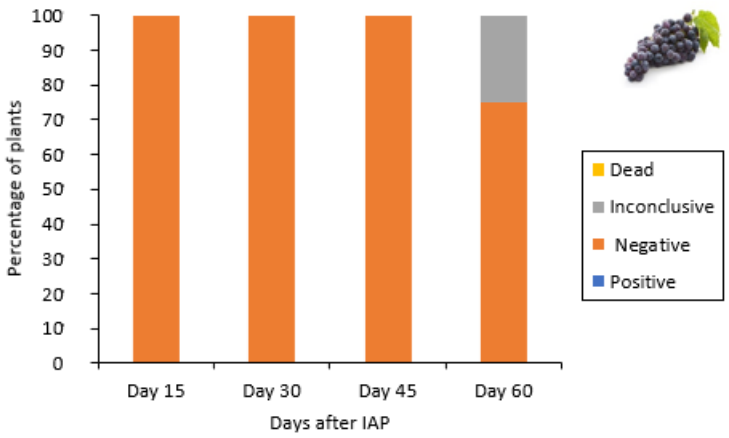

Figure 69. Detection of *X. fastidiosa* on the *Medicago sativa* plants exposed to *P. spumarius* adults that acquired the bacteria from infected vineyard plants in the Transmission Test in 2020.

Discussion

Currently, in the EU three species of Aphrophoridae: *P. spumarius*, *P. italosignus* and *N. campestris* are known to transmit *X. fastidiosa*, being *P. spumarius* the major vector (EFSA, 2021). The transmission of *X. fastidiosa* is a complex process due to many factors that influence the acquisition and the retention of the pathogen in the vectors (Killiny and Almeida, 2009; Baccari *et al.*, 2013). So, once new vector species are identified, there is crucial to provide information on the efficacy of the transmission process (EFSA PLH, 2015).

We demonstrated that either field infected *P. spumarius* and *N. campestris* from Majorca were able to transmit *X. fastidiosa* to pathogen free *M. sativa* plants. *Philaenus spumarius* showed a natural prevalence of *X. fastidiosa* between 21-21.7 % and inoculated the bacteria to 5.5-23 % of alfalfa plants, while 9.1-15.6 % of *N. campestris* were positive to *X. fastidiosa* and inoculated the pathogen to 4.7-22 % of alfalfa plants. These results suggest that transmission efficacy was the same for both species. Even so, insect prevalence in this work (21 % for *P. spumarius*) was 1.5 times lower than in the transmission tests carried out in Italy where *P. spumarius* were 30.8 % infected (Saponari *et al*., 2014). Other authors encountered different results with *N. campestris*, such as Cavalieri *et al*., (2019) that categorized *N. campestris* a less efficient vector with an acquisition percentage of 5.6 % (three times lower than in our study) and 22.2 % of *P. italosignus* that acquired *X. fastidiosa* from olives, the last with similar our results with *Philaenus* species.

After IAP, survival rate of *P. spumarius* was 18 % higher than *N. campestris* in 2019 trials, while in 2020 both species surpass the 90 % of survival. Even so, survival rate will be driven by different factors such as vector species, host recipient plant, and temperature. In transmission tests from Apulia (Italy), *P. spumarius* showed more than 80 % of survival when inoculating in cherry, almond, olive, myrtile-leaf milkwort and periwinkle, while *N. campestris* survival was 51 – 87 % in olive, myrtile-leaf milkwort and periwinkle (Cavalieri *et al*., 2019). Also, Cornara *et al*., (2016) found high mortality in oleander, a non host plant for vectors, but transmission efficiency was not modified. In other vector species such as the glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera:Cicadellidae), survival rate was 96 % in grape plants (Daugherty *et al*., 2009b). Bodino *et al*., (2021b)

demonstrated with microcosm experiments that insect survival was influenced by age, season and climatic conditions that may affect transmission outcome.

Once we knew *X. fastidiosa* prevalence in vectors collected from the field (as reported in the Chapter 3) and that they were able to transmit *X. fastidiosa* to pathogen free alfalfa plants when exposed to naturally infected vectors, acquisition was carried out with almond and vine plants, and inoculation to alfalfa plants. In 2019 tests, from naturally infected almonds and vine plants, 34.4 % and 87.7 % of *P. spumarius* adults acquired *X. fastidiosa*, respectively. However, subsequent transmission of *X. fastidiosa* to pathogen free alfalfa plants only succeeded with insects that acquired the bacterium from naturally infected almond plants (60 % of alfalfa plants after 15d IAP). Further, in 2020 tests, there was no successful acquisition by *P. spumarius* from almond and vine plants and neither transmission to alfalfa plants. Same results were observed by Borras *et al*., (2021) were the 27.5 % and 72.5 % of *P. spumarius* acquired the bacteria from naturally infected almonds and vine plants, respectively, but only the 37.5 % of insects transmitted *X. fastidiosa*. The highest acquisition rates observed from vineyard may be due to vine plants usually harbouring higher populations of *X. fastidiosa* compared to almond trees, that is reflected in higher acquisition rates (Almeida and Purcell 2003a, b and c). Cornara *et al*., (2016) described in grapevine that *P. spumarius* was able to acquire and inoculate with an efficiency of 15 % per individual per day, but they succeed to inoculate to *V. sativa* with a 44-56 % of plants harbouring *X. fastidiosa* after IAP.

Our models systems (both types of transmission tests) were previously tested by Severin (1950) in California, were the natural transmission efficiency for *P. spumarius* was 65 % and successfully transmitted the bacterium from alfalfa to alfalfa, vine to alfalfa and alfalfa to vine plants.

When comparing the transmission ability between Aphrophoridae and Cicadellidae, a major part of the literature about transmission of *X. fastidiosa* has been carried out with the American vector species that are different from Europe ones (EFSA PLH, 2015; Krugner *et al.,* 2019). Redak *et al.*, (2004) described that spittlebug have a lack of ability to spread *X. fastidiosa* in comparison to leafhoppers due to the lower capacity of

dispersion by the insect. Even inside the same group, for example Cicadellidae, difference between species was found. The species, *G. atropunctata* has been shown to be a more efficient vector than *H. vitripennis* according to transmission tests (Almeida and Purcell, 2003b; Daugherty and Almeida, 2009). Baccus and Morgan (2011) explained that *G. atropunctata* and *H. vitripennis* showed different microbial colonies in the foregut that could explain the difference of vectorial capacity between different vector species because of competitive binding of *X. fastidiosa* in the foregut of vectors.

Another factor that influences transmission success is vector density, Dhaugerthy and Almeida (2009) observed an accelerated of Pierce Disease symptoms when increasing vector abundance with *G. atropunctata*, hence increasing initial plant infection levels. With a higher number of vectors, the probability of probing increases. However, this do not imply a more supplying pathogen inoculum (Dhaugerthy and Almeida, 2009).

Also, temperature can affect inoculation efficiency as reported with *H. vitripennis* and *G. atropunctata*, with more proportion of infected plants in higher temperatures (Daugherty and Almeida, 2009b), in our case the inoculation was under the same temperature in the insectary (25 °C).

We led the insects moving free around the entire plant during the inoculation process to avoid stress, but it was observed that *G. atropunctata* and *D. minerva* increased acquisition rates by 20-50 % in alfalfa by confinement at the base of the plant than in the upper part (Daugerthy *et al*., 2010). That means that insect behavior will affect disease incidence as for example Daugherty *et al*., (2011) observed sharpshooters (*G. atropunctata* and *H. vitripennis*) were able to discriminate against infected vineyards avoiding them for feeding, using visual and/or olfactory cues for host selection (Fereres and Moreno, 2009; Daugherty *et al*., 2011).

Acquisition efficiency could differ depending on host plant and vector species interaction or *X. fastidiosa* subspecies (Almeida *et al*., 2016). For example, *G. atropunctata* was a better vector of *X. fastidiosa* in gape plants than *D. minerva*, but with alfalfa plants happened the opposite (Frazier and Freitag, 1946; Hewitt *et al*., 1946). Also, it is correlated

with the bacterial load, higher populations yield higher acquisition efficiency (Hill and Purcell, 1997).

Another factor that will affect acquisition and inoculation efficiency of *X. fastidiosa* is the times of plant access/inoculate periods. In our experiments we used AAP and IAP of 96 hours, which is the time widely used (Saponi *et al*., 2014) and is the general procedure recommended by EFSA (EFSA PLH, 2015). More than four days would lead to major number of inoculation events as reported by Almeida and Purcell (2003a) and Cornara *et al*., (2016). But in fact, to accomplish *X. fastidiosa* transmission, only small number of bacteria cells must be attached in the precibarium as no latent period is needed (Almeida and Purcell, 2006).

Unveiling knowledge on the vector species that transmit *X. fastidiosa* is crucial for understanding its epidemiology and develop adequate control strategies to reduce the impact of the disease (Cavalieri *et al.*, 2019). With our study we conclude that *P. spumarius* and *N. campestris* can acquire and inoculate *X. fastidiosa* to *M. sativa* plants under controlled conditions, confirming its role as vector.

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CHAPTER 5

Mechanical control methods against *Xylella fastidiosa* vectors in Majorca

Abstract

Xylella fastidiosa (Proteobacteria:Xanthomonadaceae) is a xylem pathogen bacterium transmitted by xylem feeder insects that causes several important plant diseases such as Pierce's disease in grapes or leaf scorch in almond and olives trees. The bacterium was detected in the Balearic Islands in October 2016, including three subspecies: *fastidiosa*, *multiplex* and *pauca.* The major potential vectors described in the Balearics are *Philaenus spumarius* and *Neophilaenus campestris*. To interfere the life cycle of vectors, we tested the effect of mechanical control of the plant cover on the most vulnerable phases, such as nymphs and/or newly emerged adults. For this, we selected four organic orchards in Mallorca, three olive and one vineyard plot. Owners of each selected plot conducted mechanical control according to their common procedures and their own machinery, which in general included cut and tillage of the plant cover during March-April. Nymph abundance per surface (30 sampling points/treatment/orchard x 0,25 m^2) was measured in each plot in a weekly basis before and after mechanical control. Our results indicated that either tillage and mowing decreased nymphal density of *Xylella fastidiosa* vectors in both types of crops. These results contribute to the integrated pest management of vectors by conducting feasible farm-based management of the regular plant cover.

Introduction

Land-management strategies with environmental, social and economic benefits also include to maintain local biodiversity and associated ecosystem services such as pollination and pest control (Foley *et al*., 2009). An effect of cover cropping is the increasing of biodiversity, to reduce number of specialized parasites and to increase ecological stability (Koike *et al*., 1997; Daane and Costello, 1998; Jutzi, 1997; Pardini *et al*., 2002). Intensive tillage has been shown to decrease plant and animal species diversity for some taxa (Paoletti *et al*., 1998; Kazakou *et al*., 2016; Winter *et al*., 2017), while the use of cover crops in vineyard inter-rows has showed to have positive effects on pest control (Berndt *et al*., 2006; Sanguankeo and León, 2011), such as increasing food web complexity and intraguild predation (Finke and Denno, 2004). On the contrary, certain plant species may also increase potential pest species by acting as a host plant (Begum *et al*., 2006), by providing resources or shelter (Danne *et al*., 2010). There are different ways to manage weeds by the farmer such as flaming, mowing or tillage as an alternative to herbicides that are producing resistance in weed communities (Mainardis *et al*., 2020; Mia *et al*., 2020).

Cover plants are essential for the development of groups of insects such as Cicadomorpha (Hemiptera) (Evans, 1947; Carpio *et al*., 2020), which are vectors of important pathogens such as *Xylella fastidiosa* (Wells et al., 1987). This bacterium is a pathogen of plants limited to the xylem and capable of infecting more than 600 plant species (EFSA 2015, 2018, 2020). This species has great number of genotypic and phenotypic diversity, that allows the bacterium to have a wide host range (Schuenzel *et al*., 2005; Nunney *et al*., 2013; EFSA PLH 2015a; EFSA 2018). Transmission of *X. fastidiosa* is conducted by the xylem feeding activity of Cicadomorpha adults (Purcell and Finlay, 1979; Hill and Purcell, 1995; Redak *et al*., 2004; Almeida *et al*., 2005; Chatterjee *et al*., 2008). Within Cicadomorpha, Aphrophoridae are the major vectors of *X. fastidiosa* in Europe (ESFA, 2021). In the Balearic Islands (Spain), they overwinter as egg form until March when nymphs start to emerge in the cover vegetation. Adults start to appear in end-April and remain in the cover until it dries in summer to migrate to tree canopies and bordering woody shrubs (Miranda *et al*., 2017, López-Mercadal *et al*., 2021). Then, adults return to

cover in autumn for mating, completing their univoltine life cycle (López-Mercadal *et al*., 2021). The bacterium is restricted to their alimentary canal, where they adhere to, multiply and persist in the precibarium and cibarium foregut parts of the insect (Almeida *et al*., 2005; EFSA 2018).

The bacterium is associated with important diseases in a wide range of plants, being an important emerging pathogen (Redak *et al*., 2004; EFSA, 2013). Each subspecies and genetic type (ST) have different host range causing diseases such as the Pierce's disease in grapevine (*Vitis vinifera*), citrus variegated chlorosis, leaf scorch (almond, elm, oak, oleander, American sycamore, mulberry and maple), alfalfa dwarf, olive quick decline, plum leaf scald and peach phony rickettsia (Hopkins and Purcell, 2002; Chatterjee *et al*., 2008; Janse and Obradovic 2010; Krugner et al., 2019; EFSA 2021). Nevertheless, many species of plants may remain symptomless (EFSA PLH 2018; EFSA, 2013). In Europe, there are hosts with a high economic value such as *Olea europaea*, *Prunus dulcis*, *Vitis vinifera*, *Prunus avium*, *Prunus domestica*, *Prunus salicina* or *Citrus* spp. (EFSA PLH, 2019), being olive and vineyard crops the largest cultivation in the Mediterranean basin (Pardini *et al*., 2002).

Xylella fastidiosa vectors do not act as insect pest and usually they cause little damage to plants (Almeida *et al*., 2005). Nevertheless, if *X. fastidiosa* was fully spread, it would cause an annual production loss of 5.5 billion euros that affects the 70 % of older olive trees (over 30 years old) and the 35 % of younger olive production; 13 % of almond, 11 % of citrus and 1-2 % of grapevine (European Commission, 2021). In Italy, it was estimated that olive producers have already lost between 0.2 and 0.6 billion euros in investments, and it could increase until 1.9 to 5.5 billion of euros over the next 50 years (Schneider *et al*., 2020; Albre *et al*., 2021).

Since *X. fastidiosa* was first detected in Europe in 2013 (Saponari *et al*., 2013), a huge effort was made to avoid the spread of the disease. Chemical curative control against the bacterium is still unknown, otherwise prevention by use of resistant varieties, hygienic and cultural measures (i.e., cover plant management), and biological (i.e., parasitoids or spiders) and chemical (neonicotinoids and pyrethroids) vector control are the pathways to

achieve it (Janse and Obradovic, 2010; EFSA PLH, 2019). Combining multiple of these control strategies is considered as the best management strategy (Almeida et al., 2005). The aim of this study is to assess the efficacy of mechanical control methods against *X. fastidiosa* vectors in olive and vine organic orchards in Majorca.

Material and Methods

Study site

Three olive and one vineyard orchards were selected from Majorca (Balearic Islands), all of them under official organic farming management. The climate in the Balearics in the Mediterranean type, characterized by dry and hot summers and wet mild winters. The annual mean temperature is 21.8 °C and the annual mean precipitation 456 mm (AEMET, 2018).

Cover vegetation assay

The same methodology was used for olive and vineyard crops in 2020 and 2021. Nymphs of the vectors of *X. fastidiosa* were surveyed from end-March in each plot. Three samplings were made to each plot every year. If nymphs were present, total density was determined and rows in the crop were marked as control or treatment (Fig. 70). Density was determined by 30 randomly woody rectangles (0.25 m^2) on the control and in the treatment rows selected in each orchard. The position of each rectangle was marked with a rope to assess the density in the same place every time. After first measurement, cover vegetation was cut by farmers using regular equipment in the treatment rows and nymph density was checked after 2 and 3 weeks from the tillage date in both treatment and control rows. Weed control in olive orchards were by mowing and in vineyard orchards by tillage.

Figure 70. Treatment row (T) where cover plants were mowed and control (C) rows where no tillage was conducted in a vineyard.

Statistical analysis

Zero-inflated models were used to assess the influence of tillage or mowing on the cover herbaceous vegetation on nymphal density with the package pscl (Jackman et al., 2015). Week (1, 2 or 3), treatment (control or treatment) and time (pre-treatment or post-treatment) were included as fixed factors. Post-hoc analysis with Tukey adjustment were performed with emmeans (Searle et al., 1980) and multcomp packages (Hothorn et al., 2016). We accepted as significant the p-values below 0.05. Statistical analyses were performed in R software 3.2.5 (R Core Team, 2019).

Results

2020 trials

The trial was done in one plot of olive and one of vineyard. In both plots there were nymphs of *P. spumarius* and *N. campestris.* In the case of the vineyard crop, initial density was the same for both zones with 0.2 nymphs/m^2 (Estimate: 0.5839, Std. Error: 0.6865, P- value = 0.395) (Fig. 71 a). Due to zero density in the treatment zone in the first week post-treatment, data was not able to be analysed statistically. In the second week post-

treatment we observed significantly differences among control and treatment (Estimate: -2.6135, Std. Error: 0.7119, P- value <0.05). Otherwise, since densities were too low, post-hoc analysis did not show differences among treatment neither week. In the case of olive (Fig. 71 b), initial density was the same for both zones with 0.35 nymphs/m^2 (Estimate: -0.4170, Std. Error: 0.3844, P- value = 0.395). In the second week, we observed statistically differences among control and treatment (Estimate: 3.262, Std. Error: 1.302, P- value = <0.05). Finally, in the third week nymph density was not significantly different (Estimate: -0.3860, Std. Error: 0.5156, P- value = 0.4541).

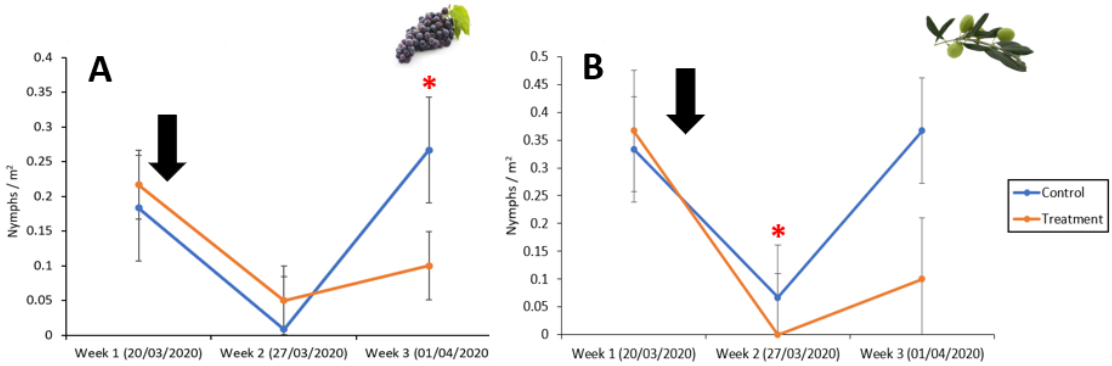

Figure 71. Nymph density per m^2 in vineyard (A) and in olive (B) crops. Pre-treatment corresponds to week 1 and post-treatment to week 2 and 3. The black arrow indicates the of moment tillage or mowing. The red asterisk represents P-value<0.05.

2021 trials

The trial was done in two organic olive orchards. In both plots there were nymphs of *P. spumarius* and *N. campestris.* Due to the difference of the dynamics of both plots, we decided to analyse them for separate. Initial density before tillage was statistically the same for treatment and control in the plot A (Estimate: 0.3622; Std. Error: 0.2587; P-value = 0.1615) (Fig. 72 a) and in the plot B (Estimate: 0.3622; Std. Error: 0.2587; P-value= 0.1615) (Fig. 72 b).

In the plot A, after the tillage, there was no overall effect of the factor treatment, but there was a crossover interaction. Density was significantly different in week one (1

nymphs/m^2) against week two (0.2 nymphs/m^2) (Estimate: -2.8875; Std. Error: 0.6285; P-value<0.05) and week three (Estimate: 1.2033; Std. Error: 0.3744; P-value<0.05). Nymph abundance decreased after tillage showing statistically differences among treatments. Treatments did not differ among them in the second week (Estimate:1.3836; Std. Error: 1.1610; P-value=0.233), but they did in the third week (Estimate: -2.0871; Std. Error: 0.7814; P-value<0.05) being lower the density in treatment than in control. In plot B, initial density in control and treatment was the same (0.6 nymphs/m^2) (Estimate: 0.3242; Std. Error: 0.4771; P-value= 0.497). After tillage, there were statistically differences among the treated cover plant rows in the second and third week (Estimate: -2.4563; Std. Error: 1.1022; P-value<0.05).

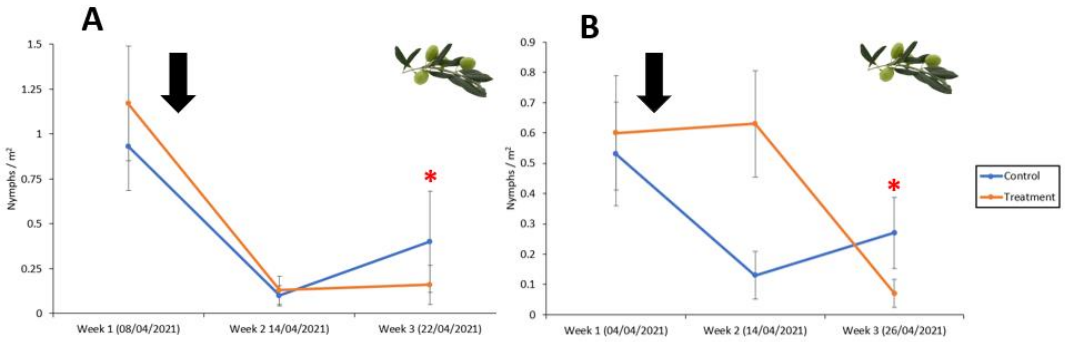

Figure 72. Nymph density per m^2 in two different olive orchards (A and B). Pre-treatment corresponds to week 1 and post-treatment to week 2 and 3. The black arrow indicates the moment of tillage or mowing. The red asterisk represents P-value<0.05.

Discussion

In this study we assessed the management of the cover vegetation in olive and vineyard organic orchards to decrease *X. fastidiosa* vector density. Our results suggested that could be an efficient method because tillage and mowing decreased the nymphal density between the 10 to 50 % in comparison with the untreated rows. The experiment was carried out according to nymphal seasonality described in Chapter 1, coinciding with the peak of nymphs. Also, nymphs were between N2 to N5 stage at the time of performing the treatment.

Management of the first stages of juvenile vector species would be more efficient due to their low movement (Bodino *et al*., 2020; Sanna *et al*., 2021). In addition, mechanical weed control methods are considered to also reduce adult vector population (Buri *et al*., 2016; Cornara *et al*., 2018; Sanna *et al*., 2021; Capellari *et al*., 2022). Cover cropping management has also further advantages, for example, it can contribute to reduce and even to eliminate the use of herbicides, fungicides and pesticides (Pardini *et al*., 2002; Berg *et al*., 2018; Theodorou *et al*., 2021). This guarantees a sustainable production through the maintenance of soil fertility in organic orchards (Porter, 1998; Pardini *et al*., 2002).

Aim of controlling *X. fastidiosa* spreading is currently ongoing in the EU, considering that the management should be based on a combination of multiple tactics (Almeida *et al*., 2005, Cornara *et al*., 2018; Morelli *et al*., 2021). Several protocols were developed to avoid short and long-range spreading (EFSA PLH, 2019). One of the methods to decrease short-range spreading is the control of *X. fastidiosa* insect vectors such as nymphs or newly emerged adults (e.g., removal of ground vegetation) (Cornara *et al*., 2018; EFSA PLH, 2019; Santoiemma *et al*., 2019; Sanna *et al*., 2021).

In olive plots it was usual to use mowing techniques, otherwise, tillage was the preferent in vine. Such techniques were demonstrated to have different effect on *P. spumarius* populations, and tillage can have a stronger effect than mowing (Sanna *et al*., 2021). Previous studies in Apulia showed that tillage performed in winter and spring reduced the abundance of *P. spumarius* and *N. campestris* on the cover vegetation and olive canopies (EFSA PLH, 2019). In fact, according to Nickel and Hildebrandt (2003), mowing regime in grasslands would have a severe impact causing a long-term exclusion of Auchenorrhyncha species. Nevertheless, plants remaining in the border of orchards and near the tree that may also hold important number of nymphs and its management would be also required (Sanna *et al*., 2021).

Permanent removal of ground vegetation is very common in traditional agricultural management in the Mediterranean Basin and would imply to serious ecological problems and enhance pests in the crop (Altieri *et al*., 2005; Nicholls *et al*., 2008) and soil loss (Sastre

et al., 2017). Therefore, in the case of *X. fastidiosa* vectors management, specific tillage or mowing of cover plants is advised in specific period of time to eliminate development and feeding sites for vectors (Kamas *et al*., 2000). It is important to point out that *P. spumarius* and *N. campestris* do not act as pest in crops, but their population is conditioned by the management intensity of each plot (Santoiemma *et al*., 2019) because they have two ground-dependent stages: nymphs and egg laying.

Nevertheless, nymphs from N3 to N5 gain mobility and can reach other plants when cover is mowed or tillage. Due to this, mechanical control methods are limited to weeks where nymphs are N1 and N2, and not all of them are going to be killed.

The plant cover management has long-termed effects on the composition and structure of the vegetation by excluding and promoting certain species of plants, thus strongly affecting host availability and habitat conditions (Nickel and Hildebrandt, 2003). Taking this into account, the management prevents the immigration of shrubs and trees and decreases phytophagous activity in the crop (Nickel and Hildebrandt, 2003).

In conclusion, mowing and tilling significantly reduce the nymphal density of Aphrophoridae and repeating the treatment by the time may reduce the adult vector population and thus the *X. fastidiosa* transmission risk.

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3. General discussion

Global trade routes have increased the risk of new disease outbreaks and enhance the invasive species spreading worldwide (Hulme, 2009; Markheiser *et al.*, 2020). When an insect-borne pathogen is introduced in an environment with suitable climatic conditions and efficient vectors, its spread and establishment have higher probabilities to occur (Fereres 2015; Sicard *et al.* 2018; Markheiser *et al.*, 2020). This is the case of *X. fastidiosa*, since its description in the Americas in the XIX century it has spread throughout the continents where insect vectors were present and capable to transmit the bacteria. It has been reported in 16 countries according to EPPO distribution (https://gd.eppo.int/taxon/XYLEFA/distribution). Nowadays, it is listed as a "priority pest" in Europe due to the several diseases that causes the bacteria in important crops such as olive or vine (Landa *et al*., 2022).

The present study shows the results of five different studies conducted in the Balearic Islands aiming to describe the abundance, seasonality, transmission efficiency and control strategies of the *X. fastidiosa* vectors. This is the first work at European level that addresses the vector bioecology and species composition in major crops such as olive, vineyard and almond in a *X. fastidiosa* containment area.

Abundance and seasonality of vectors is the first step to study the epidemiology of *X. fastidiosa* and to understand future trends of spreading. The study of macrocosm conducted in this thesis included three years of sampling (2017-2020) to deeply investigate the bioecology of *X. fastidiosa* vectors in olive, vineyard and almond crops in the Balearic Islands and simultaneously check *X. fastidiosa* prevalence in those vectors (Chapter 1). Results showed that, similarly to other regions affected by *X. fastidiosa* in Europe, the main vectors are the spittlebugs: *Philaenus spumarius* and *Neophilaenus campestris*. Spittlebugs are widely distributed in Europe, contrary to America where sharpshooters are the major vectors (EFSA, 2021). Both species observed have shown to be present in all the main crops of the Balearic Islands, olive, almond and vine, as well as in all the sampled islands (Majorca, Minorca, Ibiza and Formentera). Therefore, the risk of long-term transmission of *X.*

fastidiosa in the Balearic Islands can not be underestimated. The presence of the bacteria in wild plant species (Olmo *et al*., 2021) makes also difficult to predict a scenario where *X. fastidiosa* is not threatening crops. Unless this, Formentera remains free of *X. fastidiosa* and it should be recommended to keep analysing vectors and potential vectors to detect earlier the disease. Using insects to monitor the disease has been previously proven in Italy and France (Ben Moussa *et al*., 2015; D'Onghia *et al*., 2017; Cruaud *et al*., 2018).

Not only adults, but pre-imago instars were highly present in the selected crops. Spittle bugs have been traditionally not controlled due to its little impact on agriculture, however, its transition to highly important vectors has forced the development of control methods, frequently lacking evidence on their efficacy. In this thesis we observed nymphs from early March to end May in the cover vegetation of all crops. The species *P. spumarius* was mainly found in Compositae and Leguminosae while *N. campestris* nymphs were only observed in Poaceae plants (Chapter 1). These results led us to develop and implement a control method based on the tillage/mowing of herbaceous cover vegetation in olive and vineyard plots to decrease vector density (Chapter 5). Both techniques demonstrated to influence the nymphal density of *X. fastidiosa* vectors, by dropping abundance as already reported in Italian vineyards (Bodino *et al*., 2021). Also, it was demonstrated that adult population dropped halved when mowing in comparison to years with no cover management (Bodino *et al*., 2021). Using cultural control by farmers would require to precisely know the moment when nymphal stages are more vulnerable to tillage/mowing, and therefore, systematic sampling of nymphs should be conducted to determine the best moment.

Control of vectors is also limited due to the crop landscape structure in the Balearic Islands. Crops are in general of small size plots, combining different types of plant crops in a relatively small area. This makes that the spread of the bacteria by vector movements from crop to crop to be highly frequent (Cornara *et al*., 2018). In fact, we showed how insects are present and move from one habitat to another (cover vegetation, trees and border plants), making difficult to target them for control (i.e., using pesticides). This migration behaviour is complex and may be influenced by multiple factors (i.e., biotic, and

abiotic) and it has profound implications for the spread of the disease (Minter *et al*., 2018). Despite the large number of potential vectors, only few species may play a significant role in a particular crop and region (Almeida *et al*., 2005). In fact, Lago *et al*., (2021a, b) showed that *N. campestris* can fly more than 2 km in five weeks, and *P. spumarius* 500 m in 30 min. Another factor to take into account for estimating the risk of transmission and persistence of *X. fastidiosa* in the Balearic Islands is the prevalence of *X. fastidiosa* in the insects. In general, we detected an infection rate of 20-40 % in *P. spumarius* and *N. campestris* and transmission tests carried out with alfalfa plants concluded that both species have the same role as vectors (Chapter 3 and 4). Unless this, *P. spumarius* has shown higher abundance in the field than *N. campestris*, and the last remains negligible in tree canopies and shrubs. So, *P. spumarius* may be considered as the principal vector in the Balearic Islands.

We obtained the complete cycle of *P. spumarius* and *N. campestris*, observing nymphs and F1 adults in all the five species of plants tested (*Rosmarinus officinalis*, *Lavandula dentata*, *Pistacia lentiscus*, *Mentha piperitha* and *Ocimum basilicum*) (Chapter 2). It is highlighted the development success of *P. spumarius* in *R. officinalis* and *L. dentata* and that *N. campestris* developed exclusively in the grass. Wise *et al*., (2006) observed that as number of nymphs increase per plant, also does the mortality. Nymphs usually migrate from crowded plants to other plants in the field to avoid competition (Wise *et al*., 2006). Spittlebugs have ovarial and overwintering diapause dependent to ecological factors such as climatology and vegetation cycle. This, under laboratory conditions has been possible to rear *P. spumarius* continuously (Morente *et al*., 2018b, 2021).

4. Conclusions

1. Two species of Aphrophoridae have been detected in Majorca, Minorca, Ibiza and Formentera in almond, olive and vineyard crops: *Philaenus spumarius* and *Neophilaenus campestris*, being *Philaenus spumarius* the most abundant.
2. Nymphs of spittlebugs were present in the cover vegetation of all crops (almond, olive and vineyard) from early March to end May. Nymphs were more abundant in the cover vegetation of olive, followed by vineyard and almond.
3. Nymphs of *Philaenus spumarius* were found in a wide variety of plant species, mainly from the family Compositae (i.e., *Glebionis* spp. And *Sonchus* spp.), while *Neophilaenus campestris* nymphs were found exclusively in Poaceae species.
4. Adult seasonal pattern of both species showed two peaks of abundance in the cover vegetation, one in May and another in October-November. Presence of *Philaenus spumarius* increased in tree canopy and bordering woody shrubs in June until September. *Neophilaenus campestris* presence in trees and shrubs was negligible.
5. We obtained an oviposition rate between 8 and 23 % in the microcosm trials with *Philaenus spumarius*.
6. *Philaenus spumarius* was able to complete the life cycle in the five plant species tested (*Rosmarinus officinalis*, *Lavandula dentata*, *Pistacia lentiscus*, *Mentha piperitha* and *Ocimum basilicum*), while *Neophilaenus campestris* developed only in Poaceae.
7. The general prevalence of *Xylella fastidiosa* in the vectors was 22.8 %, being 23.6 % for *Philaenus spumarius* and 20.8 % for *Neophilaenus campestris.*
8. Highest prevalence was reached in Majorca Island with 24 % of insects collected positive, followed by Minorca (21.5 %) and Ibiza (21 %). Formentera remained free of *Xylella fastidiosa*.
9. The highest amount of positive insects were from almond (25.7 %), followed by olive (22.8 %) and vineyard (21 %).

10. Adults of *Philaenus spumarius* and *Neophilaenus campestris* collected from the field in infected areas of Majorca succeed in inoculating *Xylella fastidiosa* to uninfected plants of *Medicago sativa* that were positive to *Xylella fastidiosa,* detected by qPCR 15, 30, 45 and 60 days after inoculation.
11. *Philaenus spumarius* only succeed in one year of the experiments in acquiring the bacteria from vine and almond plants and then inoculating the bacteria to alfalfa plants.
12. The role of *P. spumarius* and *N. campestris* as vectors of *X. fastidiosa* has been confirmed in Majorca.
13. Mechanical control methods, such as tillage or mowing, significantly decreased aphrophoridae nymphal density in vineyard and olive crops.

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Annex I

Coordinates of the municipalities sampled in the Balearic Islands during the macrocosm observations from 2017 to 2020.

Island	Municipality	Coordinates
Majorca	Algaida	39° 33’ 36.7’’ N, 2° 53’ 30.7’’ E
	Manacor	39° 34’ 11’’ N, 3° 12’ 34.3’’ E
	Inca	39° 43’ 4’’ N, 2° 54’ 27.2’’ E
	Felanitx	39°29'51.81"N, 3°11'47.26"E
Ibiza	Santa Eulària des Riu	38° 59’ 7’’ N, 1° 32’ 6.3’’ E
	Sant Joan de Labritja	39° 4’ 43.5’’ N, 1° 30’ 47.7’’ E
Formentera	Sant Francisco	38° 42’ 19.6’’ N, 1° 25’ 42.6’’ E
	Sant Ferran de ses Roques	38° 42’ 26.9’’ N, 1° 27’ 26.5’’ E
	Es Pujols	38° 43’ 15.2’’ N, 1° 27’ 24.5’’ E
	Es Caló de Sant Agustí	38° 40’ 37’’ N, 1° 30’ 58.8’’ E
	Pilar de la Mola	38° 40’ 9.2’’ N, 1° 33’ 16.1’’ E
Minorca	Ciutadella	39° 59’ 56.7’’ N, 3° 50 20.2 E
	Ferreries	39° 58’ 59.4’’ N, 4° 00’ 45.8’’ E
	Es Mercadal	39° 59’ 14.6’’ N, 4° 5’ 37.8’’ E
	Alaior	39° 56’ 4.1’’ N, 4° 8’ 23.5’’ E
	Maó	39° 54’ 34.1’’ N, 4° 13’ 39.3’’ E
	Sant Lluís	39° 51’ 2.5’’ N, 4° 15’ 27.7’’ E

Annex II

Model selection for *P. spumarius* adults:

Model	AICc
Density ~ vegetation + crop + season + year + precipitation + locality | crop + temperature + year + locality + season + Et0	3756.97
Density ~ vegetation + crop + season + year + precipitation + locality | crop + season + temperature + year + locality	3757.70
Density ~ vegetation + crop + season + temperature + Et0 + year + precipitation + locality | vegetation + crop + season + temperature + Et0 + year + precipitation + locality	3757.82
Density ~ plot | Crop	4243.08
Density ~ season | Crop	4254.52
Density ~ vegetation | crop	4385.36
Density ~ locality | Crop	4418.79
Density ~ year | crop	4419.46
Density ~ crop | vegetation	4518.27
Density ~ temperature | Crop	4544.74

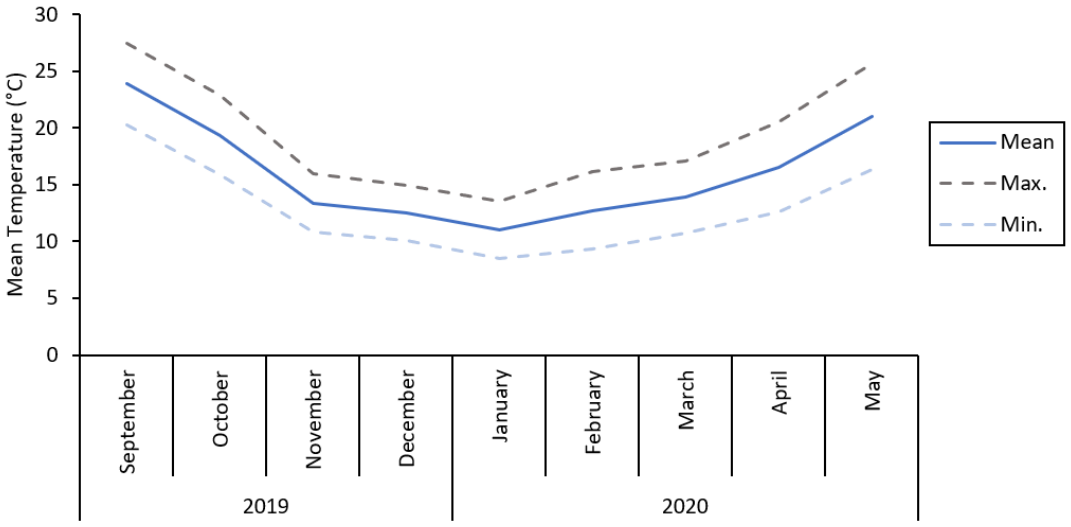
Model selection for *N. campestris* adults:

Model	AICc
Density ~ Vegetation + Season + Temperature + Year + Locality + Et0 + Crop + precipitation | Vegetation + Crop + Season + Temperature + Year + Locality	933.85
Density ~ Vegetation + Season + Temperature + Year + Locality| Vegetation + Crop + Season + Temperature + Year + Locality	934.52
Density ~ Vegetation + Season + Temperature + Year + Locality + Et0 + Crop + precipitation | Vegetation + Crop +	936.73

Season + Temperature + Year + Locality + precipitation + Et0	
Density ~ Vegetation + Season + Temperature + Year + Locality + Et0 + Crop + precipitation | crop	942.29
Density ~ season | crop	1043.89
Density ~ vegetation + dia^2 | crop	1049.21
Density ~ plot | crop	1066.19
Density ~ crop | vegetation	1068.41
Density ~ locality | crop	1081.54
Density ~ temperature | crop	1096.69

Annex III

Mean temperature registered with a HOBO device during 2019 and 2020 in the microcosm trials.



Annex IV

qPCR protocol for *X. fastidiosa* detection

DNA was extracted using the standard procedure based on CTAB DNA extraction by PCR to assess infection prevalence (EPPO 2016), using glass beads (710-1,180mm) instead of tungsten beads for the disruption of the head. DNA obtained for each vector was resuspended in 30 µl of water milliQ. The presence of *X. fastidiosa* was assessed by real time PCR following the EPPO procedures (EPPO 2019). A gene coding for the 16S rRNA processing RimM protein was amplified in triplicate by real-time PCR, following the Harper *et al.* 2010, erratum 2013, test. Controls were included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. Controls included were the negative isolation control (NIC) to monitor contamination during nucleic acid extraction, the negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix and positive amplification control (PAC) to monitor the efficiency of the amplification.

Samples with the three triplicates with a Ct value lower than 35 were considered positive. Ct values higher than 35 or without the three triplicates positives were considered unclear results, and the analysis was repeated to confirm the result.

rpoD gene by conventional PCR (Minsavage *et al.* 1994) was amplified from the positive *X. fastidiosa* samples and amplicons obtained were sequenced to determine the subspecies of *X. fastidiosa*. In order to determine the sequence type a nested MLST PCR based was used (Cesbron *et al.* 2020) increasing sensitivity and/or specificity of Yuan's PCR (Yuan *et al.* 2010). Anyhow, only two housekeeping genes are required for an assignment of *X. fastidiosa* subspecies, and only full MLST is compulsory if it is a new outbreak or new hosts (EPPO 2019). Therefore, due to the small amount of DNA obtained from positive samples, we amplified by nested PCR the *cysG* gen (sirohaem synthase), *leuA* gen (2-isopropylmalate synthase) and *malF* gen (ABC transporter sugar permease), these genes will help to differentiate between the sequence types recently described in Balearic Islands (ST1, ST80 and ST81). PCR conditions were defined in the EPPO procedure (EPPO 2016).

Furthermore, a nested PCR protocol for the *rpoD* gene (unpublished paper) was developed in the laboratory in order to increase the pitfall that suppose the lower concentration of bacteria in some samples (samples with Ct values >32), following the same criteria as nested MLST-PCR.

All the amplified samples were checked by 1.5% (p/v) agarose gel, purified by Multiscreen filter plates PCR (MSNU03010 Merck Millipore) and sequenced using the Sequencer 3130 of Applied Biosystems.

It is worth to mention that the positive amplification from the *rpoD* gene or the MSLT genes failed on some occasions, either by using the conventional PCR or the nested-PCR, although the samples were clearly positive to *X. fastidiosa*, probably due to the presence of inhibitors or the low pathogen-DNA concentration.

6. Predoctoral training

6.1. Publications related to this thesis

Casarin, N., Hasbroucq, S., Pesenti, L., Geradin, A., Emond, A., **López-Mercadal, J.**, Miranda, M. A., Grégoire, J-C. and Bragard, C. (2022). Salicaceae as potential host plants of Xylella fastidiosa in European temperate regions. *bioRxiv*.

López-Mercadal, J., Delgado, S., Mercadal, P., Seguí, G., Lalucat, J., Busquets, A., Gomila, M., Lester, K., Kenyon, D., Ruiz-Pérez, M., Paredes-Esquivel, C., and Miranda, M. A. (2021). Collection of data and information in Balearic Islands on biology of vectors and potential vectors of *Xylella fastidiosa* (GP/EFSA/ALPHA/017/01). *EFSA Supporting Publications*, *18*(10), 6925E.

Delgado-Serra, S., Miranda, M. A., Tugores, M. A., **López, J.,** Barceló, C., Paredes-Esquivel, C., Gomila, M., Lester, K., and Kenyon, D. (2018). Caracterización molecular de los vectores potenciales de *Xylella fastidiosa* en las Islas Baleares empleando el código de barras de ADN. *Phytoma España: La revista profesional de sanidad vegetal*, (304), 126-129.

Tugores, M. A., Seguí, G., Menéndez-Muntaner, A., **López, J.**, Barceló, C., Delgado, S., Paredes, C., Lester,K., Keyton, D., Lalucat, J., Gomila, M., Ruiz, M., Miranda, M. A. (2018). Especies de vectores potenciales de *Xylella fastidiosa* en las Islas Baleares: resultados de 2018. *Phytoma España: La revista profesional de sanidad vegetal*, (304), 124-125.

6.2. Other publications

López-Mercadal, J., Barretto Bruno Wilke, A., Barceló, C., and Miranda, M. A. (2021). Evidence of Wing Shape Sexual Dimorphism in Aedes (Stegomyia) albopictus in Mallorca, Spain. *Frontiers in Ecology and Evolution*, *9*, 369.

6.3. Contribution to conferences

6.3.1. Oral communications

Miranda, M.A., Mercadal, P., Delgado-Serra, S., Paredes-Esquivel, C., **López-Mercadal, J.** 2021. Understanding the epidemiological role of the vectors of *Xylella fastidiosa* in the Balearic Islands (Spain) by long-term macrocosm and microcosm studies 3rd European Conference on *Xylella fastidiosa*, Online.

López-Mercadal, J., Mercadal-Frontera, P., Delgado-Serra, S., Seguí, G., Busquets, A., Gomila, M., Paredes-Esquivel, C., Miranda, M. A. 2021. Prevalence of *Xylella fastidiosa* in vectors collected in olive, almond and vine crops of the Balearic Islands. XIX Congresso Ibérico de Entomología, Online.

Casarin, N., Hasbroucq, S., Emond, A., **López**, J., Tugores, M. A., Miranda, M. A., Bragard, C., Grégoire, J-C. 2018. Establishment of a Belgian Sentinel Plantation in Palma de Mallorca to Investigate the Susceptibility of Belgian Potential Host Plants to the Phytopathogenic Bacterium Xylella fastidiosa. COST Global Warning Final Action Meeting, Sursee (Switzerland).

Hasbroucq, S., Casarin, N., Emond, A., **López-Mercadal, J.**, Tugores, M.A., Miranda, M.A., Bragard, C., Grégoire, J-C. 2018. Etablissement d'une plantation sentinelle à Majorque pour l'évaluation de la susceptibilité de plantes belges face à Xylella fastidiosa, Houffalize (Belgium).

6.3.2. Posters

Casarin, N., Hasbroucq, S., **López-Mercadal, J.,** Bragard, C., Grégoire, J-C, Miranda, M. A.. 2021. Measuring the threat from a distance: a sentinel plantation in Palma de Mallorca to test the susceptibility of Belgian trees to several subspecies of *Xylella fastidiosa*. 3rd European Conference on *Xylella fastidiosa*, Online.

Delgado-Serra, S., **López-Mercadal, J**., Lester, K., Miranda-Chueca, M. A., Jurado-Rivera, J., Paredes-Esquivel, C. 2021. DNA-barcoding and assessment of the genetic diversity of the *Xylella fastidiosa* vectors in the Balearic Islands. 3rd European Conference on *Xylella fastidiosa*, Online.

López-Mercadal, J., Delgado-Serra, S., Mercadal, P., Seguí, G., Busquets, A., Gomila, M., Paredes-Esquivel, C., Miranda, M. A. 2021. *Philaenus spumarius* and *Neophilaenus campestris* as efficient insect vectors for *Xylella fastidiosa* in Majorca (Spain). 3rd European Conference on *Xylella fastidiosa*, Online.

Mercadal, P., **López-Mercadal, J.**, Miranda, M. A. 2021. Relative efficacy of different colour sticky traps for the capture of vectors of *Xylella fastidiosa*. 3rd European Conference on *Xylella fastidiosa*, Online.

López-Mercadal, J., Tugores, MA., Delgado-Serra, S., Mercadal, P., Forteza, P. A., Barceló, C., Paredes-Esquivel, C., Miranda, M. A. 2019. Effect of mechanical control on *Xylella fastidiosa* vector population in Mallorca (Balearic Islands, Spain). 2nd European conference on *Xylella fastidiosa*: how research can support solutions, Corsica (France).

Casarin, N., Hasbroucq, S., Emond, A., **López, J**., Miranda, M. A., Grégoire, J-C. Bragard, C. 2019. Investigations on Belgian flora and on xylem-feeding insects to evaluate the risk of introduction, establishment and spread of *Xylella fastidiosa* in Belgium. Third Annual Conference of the COST Action EuroXanth: Integrating science on Xanthomonadaceae for integrated plant disease management in Europe, Lednice (Czech Republic).

Casarin, N., Hasbroucq, S., Emond, A., **López, J.**, Miranda, M. A., Bragard, C., Grégoire, J-C. 2019. Current investigations on the susceptibility of potential host plants to *Xylella fastidiosa*, to evaluate the risk of introduction, establishment and spread in Belgium. 2nd

European conference on *Xylella fastidiosa*: how research can support solutions, Corsica (France).

Delgado-Serra, S., Tugores, M. A., **López-Mercadal, J.,** Lester, K., Kenyon, D., Miranda, M. A., Paredes-Esquivel, C. 2019. Characterization of *Xylella fastidiosa* vectors in the Balearic Islands using the DNA barcoding approach. 2nd European conference on *Xylella fastidiosa*: how research can support solutions, Corsica (France).

Delgado-Serra, S., Miranda, M. A., Tugores, M. A., **López-Mercadal, J.,** Barceló, C., Gomila, M., Lester, K., Kenyon, D., Paredes-Esquivel, C. 2018. Caracterización molecular de los vectores de *Xylella fastidiosa* en las Islas Baleares empleando el código de barras de DNA. *Xylella fastidiosa*, ¿una amenaza imprevisible? Avances técnicos y científicos para el control de las enfermedades, Valencia (Spain).

Tugores, M. A., **López**, J., Seguí, G., Menéndez-Muntaner, A., Ruiz, M., Barceló, C., Delgado, S., Paredes, C., Lester, K., Kenyon, D., Lalucat, J., Gomila, M., Miranda, M. A. 2018 Especies potenciales de vectores de *Xylella fastidiosa* en las Islas Baleares: resultados de 2018. *Xylella fastidiosa*, ¿una amenaza imprevisible? Avances técnicos y científicos para el control de las enfermedades, Valencia (Spain).

6.4. Stays in researcher groups

Earth and Life Institute (Catholic University of Louvain), Belgium: April 2019.

Centro do Investigaçao de Montanha (Instituto Politécnico Bragança), Portugal: October-November 2021.

6.5. Courses recieved

Course “Qualificat d’usuari profesional de productes fitosanitaris”, March 2019, ASAJA.

Course “Bioindicadors i mètodes de seguiment d’espècies en ecosistemes terrestres”, May 2021, UIMIR.

Course “Gestión de enfermedades y plagas agrícolas en el contexto Farm to Fork”, October 2021, COIAL.

Course “Teoría y práctica de modelos mixtos de efectos fijos y aleatorios. Aplicación a Ciencias Naturales usando R”, February-March 2022, Museo Nacional de Ciencias Naturales y CSIC.

6.6. Courses taken

Insectes vectors en el sector agrícola. Cas de *Xylella fastidiosa* a les Illes Balears. Part of the course for the Faculty of Science (UIB) “'Zoologia Aplicada: plagues, vectors de malalties, fauna invasora en illes i zoonosis”. March 2021

Profundizando en la biología de los insectos vectores de *Xylella fastidiosa*: transmisión y propuestas de control. Seminars “Investigamos para conocer y combatir las enfermedades causadas por Xylella fastidiosa”. June 2021. Link: https://youtu.be/VYj5Ldd8AKM