Final Report

Project title (Acronym)
The insect vectors of *Xylella fastidiosa* (Xf vectors)

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Contents

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2. Short project report

2.1. Executive summary

Xylella fastidiosa is a bacterial pathogen responsible for several serious plant diseases across the world such as Pierce disease within grapevine in California and Citrus Variegated Chlorosis in Brazil. In 2013 *X. fastidiosa* was detected in Europe, associated with Olive Quick Decline Syndrome in olive trees in Apulia, Southern Italy. Since, the presence of the bacterium has also been confirmed in France, Spain, and Portugal (Denance *et al*., 2012[1]; Saponari *et al*., 2013[2]; Olmo *et al*., 2017[3]). Although the initial introduction of *X. fastidiosa* in Europe was through movement of infected plant material, the natural spread of the bacteria from plant to plant occurs via xylem feeding insects belonging to the Order Hemiptera (Redak *et al*., 2004[4]). In the Americas, the primary vectors are the glassy-winged and blue-green sharpshooters of the Cicadellinae sub-family however within Europe, it is the common meadow spittlebug or froghopper *Philaenus spumarius* of the Aphrophoridae family that has been identified as the main vector (Cornara *et al.*, 2017[5]). However, all sharpshooters and spittlebug species should be considered potential vectors of *Xylella* (Almeida *et al*., 2005[6]). Further research is required on the biology and population levels of other potential vectors and should include wherever possible transmission studies on the efficiency of any vector to transmit the bacterium to a range of relevant host plant species. For example, *Cicadella viridis* is the most common and wide-spread sharpshooter of the Cicadellinae sub-family in Europe requiring further study on its behaviour and nymphal stages.

The movement of vectors between crops and wild plants is essential to understand the epidemiology of *Xylella.* Seasonal movement and abundance of vectors is well-studied in vineyards and citrus groves in the Americas and more recently within olive groves (Ringenberg *et al*., 2014[7]; Bodino *et al*., 2019[8]), however additional research into other agroecosystems would be of benefit.

Routine surveillance for *X. fastidiosa* is carried out on symptomatic plants however it is also possible to detect the bacterium within the foregut of insects. Recent studies have indicated that, in conjunction with plant surveys, testing vectors for *X. fastidiosa* could be an important tool for monitoring the bacteria within the wider environment (Yaseen *et al*., 2015[9]; Craud *et al*., 2018[10]). Within vineyards, the collection and monitoring of sharpshooters is carried out

[1] Denance *et al.* (2017). Several subspecies and sequence types are associated with the emergence of Xylella fastidiosa in natural settings in France. https://doi.org/10.1111/ppa.12695

[2] Saponari *et al.* (2013). Identification of DNA sequences related to Xylella fastidiosa in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). http://dx.doi.org/10.4454/JPP.V95I3.035

[3] Olmo *et al.* (2017). First Detection of Xylella fastidiosa Infecting Cherry (Prunus avium) and Polygala myrtifolia Plants, in Mallorca Island, Spain. https://doi.org/10.1094/PDIS-04-17-0590-PDN

[4] Redak *et al.* (2004). The biology of xylem fluid-feeding insect vectors of Xylella fastidiosa and their relation to disease epidemiology. https://doi.org/10.1146/annurev.ento.49.061802.123403

[5] Cornara *et al.* (2017). Transmission of Xylella fastidiosa by naturally infected Philaenus spumarius (Hemiptera, Aphrophoridae) to different host plants. https://doi.org/10.1111/jen.12365

[6] Almeida *et al.* (2005). Vector Transmission of Xylella fastidiosa: Applying Fundamental Knowledge to Generate Disease Management Strategies. https://doi.org/10.1603/0013-8746(2005)098[0775:VTOXFA]2.0.CO;2

[7] Ringenberg *et al.* (2014). Survey of potential sharpshooter and spittlebug vectors of Xylella fastidiosa to grapevines at the São Francisco River Valley, Brazil. https://doi.org/10.1590/S0085-56262014000200013

[8] Bodino *et al.* (2019). Phenology population dynamics and host plants of Philaenus spumarius in Italian olive groves. https://zenodo.org/record/1407670#.Y1p7U7bMKUk

[9] Yaseen *et al.* (2015). On-site detection of Xylella fastidiosa in host plants and in" spy insects" using the real-time loop-mediated isothermal amplification method. https://doi.org/10.14601/Phytopathol_Mediterr-15250

[10] Craud *et al.* (2018). Using insects to detect, monitor and predict the distribution of Xylella fastidiosa: a case study in Corsica. https://www.nature.com/articles/s41598-018-33957-z

using sticky traps, however collection of *Philaenus* is more labour intensive and in this context the development of traps or lures for known vectors should be investigated.
Philaenus spumarius plays an important role in the transmission of *X. fastidiosa* and understanding the biology and behaviour of vectors and potential vectors is vital in preventing the introduction and spread of the disease; therefore, this project aims to improve our knowledge of both vectors and potential vectors of *X. fastidiosa* within differing habitats and climates. As well as sharing vector survey data from across the globe, studies on plant host preferences and vector movement between crops and wild plants were investigated. Different trapping techniques were discussed and reviews on potential biocontrol agents and natural enemies were considered. Transmission experiments on potential vector *Aphrophora salicina* were carried out; and endosymbiotic bacteria studies on vector populations were assessed.

2.2. Project aims

While the vectors of *Xylella* are relatively well studied in South and North Americas, our knowledge needs to be improved in the European countries where the disease has only been detected relatively recently. Therefore, this project aimed to improve our understanding of the biology of the main vectors of *Xylella* in a range of differing habitats and environments in Europe and further afield through surveying for xylem feeding Auchenorrhyncha as potential vectors and determining associations, if any, with plant hosts.
The main objectives of the project were:

- To share knowledge, experiences and data on potential *Xylella* vectors from different countries/regions, including feeding preferences and phenology.
- To study vector abundance and movement between crops and wild plants.
- To investigate and discuss different trapping techniques for collection of potential *Xylella* vectors.
- To undertake transmission studies to determine the efficiency of vectors to transmit *X. fastidiosa*.
- To investigating the composition of secondary endosymbiotic bacteria within *Xylella* vectors.
- To assess potential biocontrol agents for vectors i.e. parasites, fungi.

2.3. Description of the main activities

2.3.1. Vector surveys and collection of voucher specimens:

2.3.1.1. Vector survey summary

Vector survey data collected by partners of the project were shared and collated. Different vector species observed during surveys along with behavioural traits e.g. plant hosts, habitat preferences and nymph/adult timelines from different countries and regions were shared.

2.3.1.2. Vector abundance and movement between crops and wild plants

(i) IVIA (Spain)

The vector survey work carried out by IVIA was concentrated in several plots outside the Demarcated Zone in mainland Spain (the area where *X. fastidiosa* has been detected in the Valencian Community). The population of *X. fastidiosa* vectors was monitored for 4 years (2019-2022), in 6 plots: 2 olive and 1 citrus groves in the town of Segorbe (Castellón); 1 citrus and 1 almond grove in the town of Vila-real (Castellón) and 1 citrus grove in the town of Moncada, at IVIA (Valencia).
For immature sampling: abundance and identification of nymphs present in cover plants was recorded within a 0.25 m^2 (0.25 m x 1 m) rectangle. 10 samples (rectangles) per plot were taken every fortnight. Host plants were identified to establish the relationship between nymphs and plants. For adult sampling: counting and identification of all adults present in cover plants and the crop canopy were carried out using a sweep net. In cover plants, 10 samples/sweeps

(5 m^2/sample) per plot were taken. In canopies, 10 samples/sweeps were taken per plot, with 1 sweep taken from each of the 4 canopy orientations.
The relationship between adults and herbaceous host-plants was identified. Insect behaviour was studied for *P. spumarius* and *N. campestris*.

(ii) BPI (Greece)

Citrus Variegated Chlorosis (CVC) is a serious disease of citrus which is caused by the bacterium *Xylella fastidiosa* subsp. *pauca*. *X. fastidiosa* is exclusively transmitted by sap-feeding insects such as sharpshooter leafhoppers and spittlebugs. Given the tremendous impact that an outbreak of CVC in the Mediterranean basin could lead to, additional data about the bioecology of vectors in citrus groves and in neighbouring areas are urgently needed.
To study the occurrence and the population trends of the insect vectors of *X. fastidiosa*, sampling was conducted during 2022 in two citrus groves located in central Greece and in a natural area that neighbored one of the citrus groves. Spittlebug nymphs were recorded using a quadrat frame (dimension 50x50cm) and by examining the plants within the frame for the presence of spittle. Ten samples for nymphs were taken in each grove. The samplings for nymphs were performed every 10 - 15 days from March to May. Adults were sampled with an entomological sweep net (38cm diameter). Ten samples were taken from the canopy of citrus trees and another ten from the ground vegetation in each grove. Additionally, five samples were taken from the foliage of pine trees (*Pinus halepensis*) and another five samples from the foliage of *Pistacia lentiscus* located in the natural area. Samplings for adults were performed every 10 - 15 days from February to May and every 15 - 20 days from June to December.

(iii) AGES (Austria)

The monitoring for potential *Xylella fastidiosa* vector species in Austrian vineyards was carried out in 2019, 2021 and 2022. Abundance of *Philaenus spumarius*, *Neophilaenus* spp., *Cicadella viridis* and *Aphrophora alni* was recorded from May to the end of September in vineyards in Burgenland (three sites) and Lower Austria (10 sites). In May and June, the number of spittle masses on grapevine leaves was recorded by a visual survey on 400 leaves per site. Adults were monitored using yellow sticky traps (Rebell® giallo) and a beating tray method.

2.3.1.3. Additional vector survey work

(i) Fera (England, GB)

Potential vectors of *X. fastidiosa* were monitored throughout 2021 (every two weeks) and 2022 (every week) within a sown meadow. For nymphs, 40 quadrats of 0.25 m^2 were assessed on the ground cover. Quadrats were distributed within the different plant zones: 10 quadrats were randomly positioned in each of the north and south zones and twenty in the larger middle zone. The vegetation inside each quadrat was examined for the presence of spittle. Identification of nymphs was not reliable below genus level. This protocol therefore produced data only for *P. spumarius* (the only *Philaenus* species present in the United Kingdom), *Neophilaenus*, *Cercopis* and *Aphrophora*.
For adults, sampling started in the meadow from the first appearance of teneral adults in the quadrat samplings and continued until no adult spittlebugs were found for two consecutive sampling dates. The ground vegetation was sampled in 30 randomly distributed locations. Each location consisted of four sweeps of a 0.4 m diameter sweep net, with a step forward taken after each sweep with the result that approximately 2.5 m was covered. In total, 120 sweeps were performed on the ground cover per sampling date, giving 30 discrete random locations consisting of four sweeps each. Potential *Xylella* vectors were identified and counted. Ten randomly selected clusters of shrubs or small trees were also sampled for potential *Xylella* vectors (adults). Shrub/tree species included *Salix babylonica var pekinensis*, *S. cantabrica*, *S. daphnoides*, *S. irrorata*, *S. kinuyanagi*, *S. koriyanagi*, *S. x friesiana*). Ten sets of four sweeps

were used to dislodge individuals from the foliage around each sampling cluster. The number, species and sex of adult spittlebugs were recorded for each sampling location.

2.3.1.4. Traps and lures

Group observations and discussions

Several vector collection techniques e.g. sweep netting and sticky traps were discussed within the group in terms of their success rate, labour intensiveness, and time efficiency. IVIA (Spain) compared yellow sticky traps to sweep netting in olive groves over one year. Clear (transparent) sticky traps were also tested to detect adult movement from cover plants to tree canopies. Fera (England, GB) compared sweep netting and yellow sticky traps for detecting potential insect vectors in the United Kingdom.

2.3.2. Investigate biology of vectors and potential vectors (transmission of X. fastidiosa, endosymbiotic studies and feeding preferences):

2.3.2.1. Transmission of *X. fastidiosa* and secondary endosymbiotic studies:

(i) ILVO (Belgium) - Study on the transmission of Xylella *by* Aphrophora salicina

A reliable and robust protocol is needed to conduct sound transmission tests. The theoretically established protocol was adapted several times during our cage trials. In both years, *Aphrophora salicina* was used as a test case and *Philaenus spumarius* as a positive control, being a proven vector.

During the first year of the project, a protocol adapted from Saponari *et al.* (2014)[11] and Cornara *et al.* (2017)[12] was used. A strain of *X. fastidiosa* subsp. multiplex (*CFBP 8431, St6)* was selected and used to inoculate 15 twigs from two 1.5m-high *Salix alba* trees. Each twig was inoculated five times over a length of 10 cm. This was done using the pin prick method with the *Xylella* cells suspended in phosphate buffer (Hill & Purcell, 1995[13]; Almeida *et al*., 2001[14]; EPPO PM7/24, 2019[15]). After one month, samples were taken to confirm that the inoculation was successful using Harper's qPCR assay (Harper *et al*., 2010)[16]. First, the insects were allowed to feed on the inoculated twig only during the Acquisition Access Period (AAP). With modified breeding dishes, the insects were kept near the inoculation points on the twigs. Each breeding dish contained five insects. After six or seven days of AAP, the breeding dishes and insects were transferred to a healthy twig on a new tree. This is the start of the Inoculation Access Period (IAP), during which the insects might transfer *X. fastidiosa* to the healthy twig. The insects were allowed to feed on the healthy twigs for six days. All (dead and alive) insects were collected and tested separately using Harper's qPCR (2010)[16]. The twigs were tested after one month using the same qPCR method. The study was carried out under field conditions.

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[11] Saponari et al. (2014). Infectivity and Transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. Journal of economic entomology, 107(4), 1316-1319. https://doi.org/10.1603/EC14142.

[12] Cornara et al. (2017). Transmission of *Xylella fastidiosa* by naturally infected *Philaenus spumarius* (Hemiptera, Aphrophoridae) to different host plants. Journal of Applied Entomology, 141(1-2), 80-87. https://doi.org/10.1111/jen.12365.

[13] Hill, B. L., & Purcell, A. H. (1995). Multiplication and movement of Xylella fastidiosa within grapevine and four other plants. Phytopathology, 85(11), 1368-1372.

[14] Almeida et al. (2001). Multiplication and movement of a citrus strain of Xylella fastidiosa within sweet orange. Plant Disease, 85(4), 382-386. https://doi.org/10.1094/PDIS.2001.85.4.382.

[15] PM 7/24 (4) *Xylella fastidiosa*. Bulletin OEPP/EPPO Bulletin (2019) 49 (2), 175–227. https://doi.org/10.1111/epp.12575.

[16] Harper SJ, Ward LI & Clover GRG (2010) Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. Phytopathology 100, 1282–1288. https://doi.org/10.1094/PHYTO-06-10-0168.

During the second year, the protocol was modified with the aim of increasing the number of insects tested, to increase the chances of detecting a positive insect after AAP. Both *X. fastidiosa subsp. Multiplex (*CFBP 8431, St6) and *X. fastidiosa subsp. fastidiosa* (KLN59.3, GFP-labeled) were used. During AAP, breeding cages were used during AAP instead of breeding dishes. Inoculated cuttings were placed in the breeding cages. Depending on the number of insects, a different number of inoculated twigs were added, at least one cutting/2.5 insects (Figure 1). The study was carried out under greenhouse conditions. Insects (dead or alive) were collected after six days of AAP and pooled by five tested via a tetraplex qPCR (Dupas., 2019[17] and Harper *et al*., 2010).

Figure 1. Insect cage with two trays of 10 inoculated *S.alba* cuttings

An extra experiment was set up to explain the high mortality of *A. salicina* during the second year. Cuttings placed in breeding cages were given different treatments instead of inoculation with *Xylella*. The four different treatments were CFBP 8431, KLN59.3, inoculation buffer and not inoculated.
Some preliminary tests were also performed with *Cicadella viridis* as an extra test.

(ii) IVIA (Spain) - Detection of X. fastidiosa *in insect vectors*

Potential insect vectors (adults), collected as described in 3.1.2, were analysed individually for the presence of *X. fastidiosa*. Insect heads were removed and extracted using a CTAB method as described in EPPO (2019)[18]. Real-time PCR methods followed Harper *et al.* (2010; erratum 2013)[19] and EPPO (2019) protocols.
Due to issues obtaining authorisation to work with *X. fastidiosa* outside of the Demarcated Zone, transmission studies on vectors were not carried out.

[17] Dupas, E., Briand, M., Jacques, M. A., & Cesbron, S. (2019). Novel tetraplex quantitative PCR assays for simultaneous detection and identification of *Xylella fastidiosa* subspecies in plant tissues. Frontiers in plant science, 10, 1732. https://doi.org/10.3389/fpls.2019.01732.

[18] PM 7/24 (4) *Xylella fastidiosa*. Bulletin OEPP/EPPO Bulletin (2019) 49 (2), 175–227. https://doi.org/10.1111/epp.12575

[19] Harper SJ, Ward LI & Clover GRG (2010) Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. Phytopathology 100, 1282–1288. https://doi.org/10.1094/PHYTO-06-10-0168.

*(iii) BPI (Greece) - Investigate the composition of the secondary endosymbiotic bacteria (*Wolbachia, Arsenophonus, Rickettsia, Hamiltonella, Cardinium*) in the collected populations of insect vectors*

Adult insects *Philaenus spumarius*, *Aphrophora salicina, A. pectoralis, A. alni, Cicadella viridis, Neophilaenus campestris, N. lineatus, Lepyronia coleoptrata* and *Euscelis lineolatus,* were analyzed for secondary endosymbiotic bacteria. Insects were collected from different regions of Belgium, Spain, Portugal and Scotland between the years 2019-2022. Up to 45 individuals from different populations of the aforementioned species were used, depending on the number individuals available. In total, 108, 50, 6, 26, 65, 60, 15, 40 and 17 individuals of *P. spumarius*, *A. salicina, A. pectoralis, A. alni, C. viridis, N. campestris, N. lineatus, L. coleoptrata,* and *E. lineolatus*, respectively, were screened to extract information about the distribution and infection status of five secondary endosymbionts (*Wolbachia*, *Arsenophonus*, *Hamiltonella*, *Cardinium*, *Rickettsia*) (Table 1).

Table 1. Populations used in the study, including the number of individuals tested for endosymbiont infection.

Insect species	Region (Consortium partner)	Number of individuals
Philaenus spumarius	Belgium (ULB)	45
Aphrophora salicina	Belgium (ULB)	30
Aphrophora pectoralis	Belgium (ULB)	6
Aphrophora alni	Belgium (ULB)	4
Cicadella viridis	Belgium (ULB)	15
Neophilaenus campestris	Spain (IVIA)	40
Philaenus spumarius	Spain (IVIA)	20
Lepyronia coleoptrata	Spain (IVIA)	40
Aphrophora salicina	Belgium (ILVO)	20
Euscelis lineolatus	Portugal (INIAV)	17
Neophilaenus campestris	Portugal (INIAV)	20
Cicadella viridis	Portugal (INIAV)	19
Philaenus spumarius	Portugal (INIAV)	20
Aphrophora alni	Scotland (SASA)	22
Neophilaenus lineatus	Scotland (SASA)	15
Philaenus spumarius	Scotland (SASA)	23
Cicadella viridis	Scotland (SASA)	31

Total genomic DNA was extracted from single individuals using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Extracted DNA from each individual was stored at -20 ºC until use. Each extraction series contained positive and negative controls. Individuals were screened for endosymbiont infection using specific PCR primers targeting the 16S *rRNA* gene for *Hamiltonella*, *Cardinium* and *Rickettsia*, the 23S *rRNA* gene for *Arsenophonus* and the *ftsZ* gene for *Wolbachia*.

2 µl of genomic DNA extract were used as template in 25 µl reactions containing 0.1 mM dNTPs, 0.5 µM of each primer, 0.1 µl Kapa Taq DNA polymerase (Kapa Biosystems) and 1x enzyme buffer (Kapa Biosystems). PCRs were performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) using the following conditions: initial denaturation at 95 °C for 3 minutes,

followed by 35 cycles of 95 °C for 30 seconds, 50-60 °C (depending on the bacterial species) for 45 seconds, and 72 °C for 1 minute; and a final step of extension at 72 °C for 10 minutes. Positive and negative controls (Nuclease-Free water) were included in each PCR reaction to avoid false negative and false positive results. Table 2 shows the different pairs of primers used for the amplification of the different endosymbionts tested and the respective annealing temperatures.

Table 2. Primers used in this study and respective annealing temperatures.

Targeted taxon	Primers	Sequences (5' - 3')	Annealing (ºC)
Arsenophonus	Ars23S.1	CGTTTGATGAATTCATAGTCAAA	58
	Ars23S.2	GGTCCTCCAGTTAGTGTTACCCAAC	
Hamiltonella	Ham_F	TGAGTAAAGTCTGGAATCTGG	60
	Ham_R	AGTTCAAGACCGCAACCTC	
Rickettsia	RB_F	GCTCAGAACGAACGCTATC	60
	RB_R	GAAGGAAAGCATCTCTGC	
Cardinium	CFB_F	GCGGTGTAAAATGAGCGTG	58
	CFB_R	ACCTMTTCTTAACTCAAGCCT	
Wolbachia	ftsZ_F1	ATYATGGARCATATAAARGATAG	54
	ftsZ_R1	TCRAGYAATGGATTRGATAT	

The amplified products were loaded and visualized on a 1.2% agarose gel electrophoresis containing the Midori Green Nucleic Acid gel stain.

2.3.2.2. Feeding preferences

(i) SASA (Scotland, GB) - Feeding survival rates of nymphs

Philaenus spumarius is a polyphagous insect that feeds on a wide range of host plants in the field with a host plant range that exceeds 1000 species (Ossiannilsson, 1981)[20]. Even a generalist will perform differently on different plant hosts and although *P. spumarius* has been recorded feeding on numerous host species they still exhibit preference and avoidance of certain plant hosts (Villa *et al*., 2020, Dongiovanni *et al*., 2018)[21,22].

To better understand what might drive *P. spumarius* host plant choice, nymphal development and survival was recorded over six weeks on several different plant species.

(ii) IVIA (Spain) - Feeding preferences and the behaviours of vectors

The suitability of different host plants for attraction and development of the two main vectors of *X. fastidiosa* (*P. spumarius* and *N. campestris*) were analysed. Three different tests were studied: i) The attraction of plant volatiles to adults, ii) the viability of insect nymphs to reach the adult stage on different plants, and iii) the female preference to egg-laying depending on host-plants.

[20] Ossiannilsson (1981). The Auchenorrhyncha (Homoptera) of Fennoscandia and Denmark. 2. The families Cicadidae, Cercopidae, Membracidae, and Cicadellidae (excl. Deltocephalinae). Fauna Entomologica Scandinavica. https://www.cabi.org/ISC/abstract/19820595593

[21] Villa *et al.* (2020). Populations and Host/Non-Host Plants of Spittlebugs Nymphs in Olive Orchards from Northeastern Portugal. https://doi.org/10.3390%2Finsects11100720

[22] Dongiovanni *et al.* (2018). Plant selection and population trend of spittlebug immatures (Hemiptera: Aphrophoridae) in olive groves of the Apulia region of Italy. https://doi.org/10.1093/jee/toy289

For testing the attraction of plant volatiles to adults, a Y-olfactometer was used, following the protocol of Aure *et al*. (2021)[23]. Nymphal development from the first instar stage to adult was studied in a greenhouse with natural climate and light, between April and June on alfalfa, calendula, grasses and almond. Female egg-laying preferences on alfalfa, calendula and grasses were also analysed in the greenhouse between October and November.

2.3.3. Vector controls

(i) SASA (Scotland, GB) - Summary of Verrallia aucta *survey (In Review)*

Verrallia aucta (Diptera, Pipunculidae) or big-headed fly is a parasite of Aphrophoridae. The parasites' life cycle is synchronous to the life cycle of its host. In addition, female adult *P. spumarius* were found to be sterile when parasitised and there is a possibility that the parasite could be useful as biological control to reduce *P. spumarius* populations. To determine the presence and prevalence of *V. aucta* in Scotland, vectors were collected from several sites across central Scotland in 2021 and screened for presence of *V. aucta* parasitism using a SYBR Green and TaqMan PCR developed by Molinatto *et al.* (2020)[24].

(ii) IVIA (Spain) - Assessment of potential biocontrol agents for vectors

At IVIA, two potential biological control agents of vectors have been tested: an insect predator, *Nesidiocoris tenuis*, and an entomopathogenic fungus, *Beauveria bassiana*. Both agents were analysed against *P. spumarius* nymphs.

The predator *N. tenuis* was tested mainly against the protective effect of the foam/spittle produced by the nymphs rather than the efficacy of *N. tenuis* against *P. spumarius* itself. Both insects were reared at IVIA. Two experimental lines were performed when nymphs were offered to *N. tenuis* predators: i) nymphs in Petri plates and on small pieces of alfalfa plants but without spittle and ii) nymphs on calendula plants with spittle. In both cases males and females of *N. tenuis* were used. The experiments were performed in laboratory conditions.

In the case of the entomopathogenic fungus, a commercial compound, Botanigard®, was sprayed on calendula plants containing *P. spumarius* nymphs with their natural protective foam. The study was performed in laboratory conditions, but spraying was done outside.

(iii) Fera (England, GB) - Literature review on the natural enemies of the UK potential X. fastidiosa *vectors*

Should *X. fastidiosa* arrive in the United Kingdom, control and management of the insect vectors to reduce their populations is likely to play a key role within strategies to manage the spread of the pathogen and limit disease. Little is known regarding effective methods for reducing their populations, especially for methods that are based on some form of biological control. Augmentative biological controls, or nature-based management strategies that seek to enhance natural enemies of the vector species within the habitat under threat from *Xylella*, may be possible options. A comprehensive literature survey was completed by Fera, entitled "Review of the natural enemies of the UK Auchenorrhyncha considered potential vectors for *Xylella fastidiosa*", R. Down, S. Conyers, C. Malumphy, December 2021. This review provides information on the natural enemies of the United Kingdom Auchenorrhyncha species that are considered potential vectors for *X. fastidiosa* in the United Kingdom.

Additionally, Fera set up malaise traps to assess for the presence of *Verrallia aucta* (big-headed fly parasite of Aphrophoridae) in a meadow habitat. Malaise traps were set up for 24

[23] Aure C.M, Herrero-Schell J, Blanes M, Beitia F (2021). First assays on the response of adults of Philaenus spumarius (Hemiptera: Aphrophoridae) to different host plants. https://zenodo.org/search?page=1&size=20&q=4680075

[24] Molianatto *et al.* (2020). Biology and prevalence in Northern Italy of *Verrallia aucta* (Diptera, Pipunculidae), a parasitoid of *Philaenus spumarius* (Hemiptera, Aphrophoridae), the main vector of *Xylella fastidiosa* in Europe. https://doi.org/10.3390/insects11090607

hours at a time in early-mid July 2021 and 2022, during the teneral phase when *P. spumarius* nymphs are emerging as adults.

2.4. Main results

2.4.1. Vector surveys and collection of voucher specimens:

2.4.1.1. Vector survey summary

Appendix 1 details the vector data recorded from surveys carried out by the different institutions of this Euphresco project: Austria, Belgium, Greece, Spain, United Kingdom, Tunisia, Israel and New Zealand. Exchanging this data has helped to observe the similarities and differences in vector diversity and behaviour across different habitats, countries and continents. It is important to note that this is not a comprehensive list of all potential vector species present within each country and not all biological data has been recorded by each institution.

Several other xylem feeding spittlebugs and leafhopper species (i.e. potential vectors of *X. fastidiosa*), have been recorded in both agricultural and natural habitats across various countries e.g. *Aphrophora alni*, *A. salicina*, *Neophilaeus lineatus*, *Cicadella viridis*, *Evacanthus interruptus*. There is vector diversity between countries, however, many have recorded *Philaenus spumarius* and *Neophilaenus campestris* as being present within in agricultural and/or natural habitats.

P. spumarius have frequently been associated with multiple herbaceous plant hosts (particularly Fabaceae and Asteraceae) across all countries where the vector species is present; while *Neophilaenus* spp. are seen to primarily feed from plants from the Poaceae family. Multiple potential vector species (*P. spumarius*, *N. campestris*, *N. lineatus*, *Aphrophora alni*, *A. salicina* and *Cicadella viridis*) have also been recorded in the canopies of various species of deciduous and conifer trees.

Timelines of nymph and adult vector emergences do differ slightly between warmer and colder climates. For more southern European countries, nymph emergence can be observed from as early as mid-February (Spain). In the United Kingdom, nymphs begin to emerge in April-May, with peak nymph abundances recorded in June for Scotland. The emergence of adults within cooler climates are also slightly delayed: late spring/early summer for warmer climates compared to June/July-August for the United Kingdom. In the southern hemisphere i.e. New Zealand, *P. spumarius* nymphs emerge in the spring time (late September to October), with adult populations emerging in October and surviving until winter (August).

2.4.1.2. Vector abundance and movement between crops and wild plants

(i) IVIA (Spain)

Four potential vector species of *X. fastidiosa* were found in the plots: *Philaenus spumarius*, *Neophilaenus campestris*, *Cercopis intermedia* and *Lepyronia coleoptrata*. In addition, individuals of *N. lineatus* were found on herbaceous plants in a woodland area on the IVIA facilities. The more abundant species in all plots were *P. spumarius* and *N. campestris* while *L. coleoptrata* was only detected in one of the citrus plots and *C. intermedia* in one of the olive groves.

Nymphs of all identified species, except for *L. coleoptrata*, were found between mid-February and early-March, while adults were found in early-April. In the case of *L. coleoptrata*, nymphs appeared in mid-April and adults in late-May. In general, the presence of adults was confirmed until early-December.

P. spumarius and *N. campestris* are present as nymphs on herbaceous plants in the plots and nearby areas. Adults appearing in mid-spring remain in the cover crop and do not show evidence of moving to crop canopies (data obtained with transparent sticky traps) as long as the cover crop remains green and temperatures are not very high. In late spring-early summer, the plants in the cover crop start to dry out and adults migrate. In the case of *P. spumarius*, they were detected in pinewoods close to the crops, on shrubs and herbaceous plants. In the case of *N. campestris*, the summer migration area was not detected.

With the arrival of autumn and the reappearance of herbaceous plants in cover crop, the presence of adults is detected again in the cover crop. The females lay their eggs from October-November onwards and the population cycle of the species is completed.
As it has been noted previously, there is a clear difference in the herbaceous plants preferred by the vector species of *X. fastidiosa*. *N. campestris* and *N. lineatus* have an almost exclusive presence on plant species of the family Poaceae. The other three insect species identified have a wider range of host plant species, with noticeable presence on plants from the Asteraceae and Fabaceae families.

(ii) *BPI (Greece)*

In the citrus grove located in Kechries, 39% and 61% of the nymphs collected from the ground vegetation belonged to the species *Philaenus spumarius* and *Neophilaenus campestris*, respectively. In the citrus grove located in Ancient Corinthos, 75%, 20% and 5% of the collected nymphs belonged to the species *P. spumarius*, *N. campestris* and *P. signatus*, respectively.
In both citrus groves the nymphs of *P. spumarius* and *N. campestris* were first recorded in late March until in mid-May. Adults of *P. spumarius* and *N. campestris* appeared in spring and autumn in the two citrus groves. *P. spumarius* adults were recorded in the ground vegetation in late April and they were recorded until mid-May and early June in Kechries and the Ancient Corinthos grove, respectively. *N. campestris* adults were first recorded in late April and they were observed until the end of May in both groves. In the citrus foliage, very few adults were recorded in early and mid-May in the two groves. During the summer months, spittlebug species were absent from the citrus groves. Adults of *P. spumarius* reappeared again in the ground vegetation in late October while *N. campestris* reappeared in early November. *Neophilaenus campestris* adults were recorded in the pine trees of the natural area for first time in mid-May and they were observed constantly until early November. The individuals of this species were captured almost exclusively in pine trees (95%). *Philaenus spumarius* adults were not recorded in the wild plants of the natural area during summer. However, in late October and November a few adults were captured in pine trees.
In summary, spittlebugs occurred mainly in the herbaceous plants in the citrus groves. They were absent from the groves during summer when the adults of *N. campestris* were observed in pine trees. All spittlebug species were rarely found in citrus foliage. Hence, their role in the transmission of CVC in case of an outbreak might be limited.

(iii) AGES (Austria)

Spittle masses were found on grapevine leaves more frequently in vineyards with extensive green cover (32) than in vineyards with no green cover (3). In 2022, numerous adult *P. spumarius* were recorded on grapevines at a very dry monitoring site in Mörbisch in midsummer. In this period, the soil had dry cracks and vegetation was sparse.
The most frequent vector species recorded with yellow sticky traps (Rebell® giallo) and the beating tray method was *P. spumarius* (50); whereas *N. campestris* was found only in small numbers (15). In very few cases, *C. viridis* (3) was detected on yellow sticky traps in August and September. *Aphrophora alni* was not detected in Burgenland or Lower Austria, but in Styrian vineyards, where forests border the vineyards.

2.4.1.3. Additional vector survey work

(i) Fera (England, GB)

For both 2021 and 2022, the number of *P. spumarius* nymphs peaked in early June with adults emerging by mid-June. By late June, no further nymphs or spittle was observed within quadrats. *P. spumarius* nymphs were recorded on 14 different plant species, with varying numbers. The favoured plant species with the highest numbers for *P. spumarius* nymphs in 2022 were *Plantago lanceolata* for the north and middle zones; and *Centaurea nigra* for the

south zone, the zone where the latter plant is dominant. This was similar for *Aphrophora alni* which favoured plants with a rosette growth pattern particularly *Hypochaeris radicata*, despite the fact that it was not particularly common in the meadow. Other plant species with high numbers of *P. spumarius* nymphs were *Rumex acetosa* and *Rhinanthus minor*.
P. spumarius adults peaked in mid-August, with numbers declining until early-October. Other potential *X. fastidiosa* adult vectors recorded in the meadow grassland were: *Aphrophora alni*, *Neophilaenus lineatus*, *Evacanthus interruptus*, *Cicadella viridis* and *Euscelis incises*.
In the shrubs, (non-native *Salix* spp.), numbers of *A. alni* were greater than in the grassland areas. *P. spumarius* and *C. viridis* were collected from areas with a mix of herbaceous stems and low-lying shrub branches. One *Graphocephala fennahi* individual was also collected from the shrubs.

2.4.1.4. Traps and lures

Group observations and discussions

The group were not aware of any lures currently available to attract potential *X. fastidiosa* vectors. There was a general agreement that sweep netting is the most efficient adult vector trapping technique for determining vector population abundances and diversity. Vectors can then be easily collected from the sweep nets using mouth aspirators. As well as collecting noticeably more vectors than other methods, sweep netting is more time efficient and cost effective. For the trapping method comparison studies, both IVIA (Spain) and Fera (England, GB), reported that sweep netting was a much more effective method of trapping and recording vectors.
However, sticky traps can be very useful at monitoring and detecting the movement of spittlebugs from one habitat to another e.g. from crops to natural habitats, or from cover plants to tree canopies. They can therefore be considered as a useful complement to sweep netting for tracking vector movement. Clear or transparent sticky traps were noted to be slightly more successful at trapping spittlebugs compared to yellow sticky traps. Yellow sticky traps can also attract many other unwanted insects. PFR (New Zealand) have successfully trapped and monitored spittlebugs and cicadas moving from natural habitats to crops using clear sticky traps set at heights of 30cm and 150cm, with higher numbers caught at 30cm.
For collecting live nymph and adult vectors for laboratory trials e.g. transmission studies, ILVO (Belgium) noted that it is relatively easy to catch *Aphrophora salicina* on *Salix*. *A. salicina* nymphs (easily spotted by their foam nests, as all spittlebug nymphs), can be collected by cutting small sections of the twigs where the nymphs are positioned and placing them on small, branched *Salix* plants in cages, allowing the nymphs to migrate over. ILVO (Belgium) found it more efficient to visually observe adult *A. salicina* in heavily infested areas and collect specimens directly from the plant using an aspirator, rather than sweep netting. Locations for adult spittlebug vectors are best determined when the nymphal stage is present.

2.4.2. Investigate biology of vectors and potential vectors (transmission of *X. fastidiosa*, endosymbiotic studies and feeding preferences):

2.4.2.1. Transmission of *X. fastidiosa* and secondary endosymbiotic studies

(i) ILVO (Belgium) - Study on the transmission of Xylella *by* Aphrophora salicina

During the first year, the trials were started with 60 *Aphrophora salicina* adults, of which 30 died during AAP, and 16 during IAP. All insects tested negative. Of the three *Philaenus spumarius* individuals we used as a positive control, two died during AAP; the third survived the complete trial, but it too tested negative. Many dead insects were observed, often trapped in condensation droplets. Poor airflow in the modified breeding dishes could explain the high mortality rate. During the second year, however, we encountered similar problems with high mortality rates, but in well-ventilated cages.

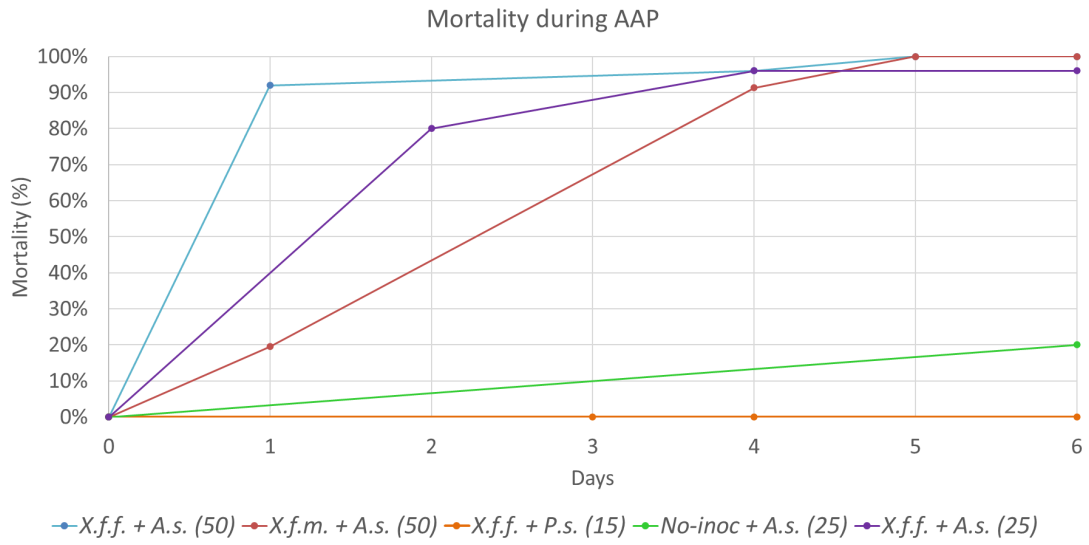

Figure 2. Mortality rate during AAP: X.f.f = *Xylella fastidiosa* subsp. *fastidiosa* (KLN59.3), X.f.m = *Xylella fastidiosa* subsp. *multiplex* (CFBP 8431), A.s = *Aphrophora salicina*, P.s. = *Philaenus spumarius*, the number between brackets = number of insects used.

As shown in Figure 2, there is a large difference in mortality rate of *A. salicina* between the cages inoculated and those not inoculated. For the positive control (*Philaenus spumarius*), the mortality rate after six days was 0%. All 125 *A. salicina* specimens tested negative for *Xylella*. For *P. spumarius,* two pools of five individuals were tested and five insects were placed on one healthy cutting for six days before testing. All *P. spumarius* pools tested positive, with an average Ct of 32.01 (standard deviation = 0.73) for the Xf qPCR and Ct 31.12 (standard deviation= 1.4) for the *Xff* qPCR. After one month, the *S. alba* cutting also tested positive with a Ct of 30.14 for *Xf* and 29.1 for *Xff*. These results were verified by fluorescent microscopy on leaf tissue.
For determining the cause of high mortality, the first experiment was repeated with an additional cage in which the *S. alba* cuttings were inoculated with inoculation buffer (= phosphate buffer) without *Xylella.* Mortality was again very high for all experiments. In *A. salicina* on non-inoculated *S. alba* cuttings, the mortality rate was also high, although this could be due to the insects being older as it was later in the season.

For *Cicadella viridis,* out of 15 adults placed on *S. alba* plants inoculated with *Xylella fastidiosa* subsp. fastidiosa, 13 individuals (86.6%) survived AAP. Two pools of five individuals were placed on healthy twigs. No insect or twig tested positive.
In general, protocols for infecting *Salix* with *X. fastidiosa* via spittlebugs have been optimised and were validated with our positive *P. spumarius* control. The results indicate that *A. salicina* does not transmit *X. fastidiosa* to *S. alba* or at least not as effectively as *P. spumarius.* Further studies with a larger number of insects are needed to obtain tangible and reliable results.

(ii) IVIA (Spain) - Detection of X. fastidiosa *in potential insect vectors*

The presence of the *X. fastidiosa* was analysed in individuals of *Philaenus spumarius*. 38 individuals collected from olive and citrus groves between May and June 2021 were tested. As expected, *X. fastidiosa* was not detected in any of the adults analysed, while amplification was obtained from all positive controls.

*(iii) BPI (Greece) - Investigate the composition of the secondary endosymbiotic bacteria (*Wolbachia, Arsenophonus, Rickettsia, Hamiltonella, Cardinium*) in the collected populations of insect vectors:*

Among the different insect species studied, the infection status and frequency of the endosymbionts did not vary significantly. Very few of the populations collected were associated with the secondary symbionts tested.

Among the five secondary endosymbionts examined, *Wolbachia* was found to infect many of the insect species tested (*P. spumarius, L. coleoptrata, N. campestris, N. lineatus* and *C. viridis*) at low frequencies, with the exception of the population from Scotland where *Wolbachia* was found in half of the individuals of *P. spumarius*. *Cardinium* was found to infect *P. spumarius,* and *N. campestris* from the Scotch and Portuguese populations, respectively. *Rickettsia* infected *P. spumarius* and *A. alni* from the Belgian populations, *N. campestris* from the Spanish population and *C. viridis* from the Portuguese population, all at very low frequencies. Only two individuals from the population of *N. campestris* from Spain was found to be infected by the endosymbiont *Hamiltonella*. There was no evidence for the presence of *Arsenophonus* in any of the examined populations of the insect species tested. *Wolbachia* and *Rickettsia* were the most common among the endosymbiotic bacteria since they were found in samples from three countries (Table 3).

Table 3. Number of individuals (N) within each insect species form each different region infected with secondary endosymbionts.

Region	Insect species	N	*Cardinium*	*Wolbachia*	*Arsenophonus*	*Hamiltonella*	*Rickettsia*
Belgium (ULB)	*P. spumarius*	45	0	0	0	0	1
Belgium (ULB)	*A. salicina*	30	0	0	0	0	0
Belgium (ULB)	*A. pectoralis*	6	0	0	0	0	0
Belgium (ULB)	*A. alni*	4	0	0	0	0	3
Belgium (ULB)	*C. viridis*	15	0	0	0	0	0
Spain (IVIA)	*N. campestris*	40	0	0	0	2	2
Spain (IVIA)	*P.spumarius*	20	0	1	0	0	0
Spain (IVIA)	*L. coleoptrata*	40	0	1	0	0	0
Belgium (ILVO)	*A. salicina*	20	0	0	0	0	0
Portugal (INIAV)	*E.lineolatus*	17	0	0	0	0	0
Portugal (INIAV)	*N. campestris*	20	1	4	0	0	0
Portugal (INIAV)	*C. viridis*	19	0	0	0	0	3
Portugal (INIAV)	*P.spumarius*	20	0	4	0	0	0
Scotland (SASA)	*A. alni*	22	0	0	0	0	0
Scotland (SASA)	*N. lineatus*	15	0	2	0	0	0
Scotland (SASA)	*P. spumarius*	23	3	11	0	0	0
Scotland (SASA)	*C. viridis*	31	0	3	0	0	0

2.4.2.2. Feeding preferences

(i) SASA (Scotland, GB) - Feeding survival rates of nymphs

The percentage of nymphs surviving to adulthood was higher on those plants they generally show preference for in the field, Asteraceae (*Cirsium sp.*) and Fabaceae (*Trifolium sp., Vicia*

sp.) in comparison survival was lower on *Urtica sp.* (Figure 3). Survival on *Lavandula sp*. and *Holcus sp*. was lower than expected.
P. spumarius did feed and survive on both *Pinus* sp. and *Betula* sp. however survival is lower, and this is reflected in that we rarely encounter large numbers of *P. spumarius* adults on broadleaved trees or conifers in the United Kingdom. It is useful to know that they can feed and survive on conifers however this is unusual and not typical feeding behaviour. The same also appears to be true of *Vaccinium sp.* and low abundance of *P. spumarius* adults in polytunnels containing soft fruits has also been observed (authors' observations).

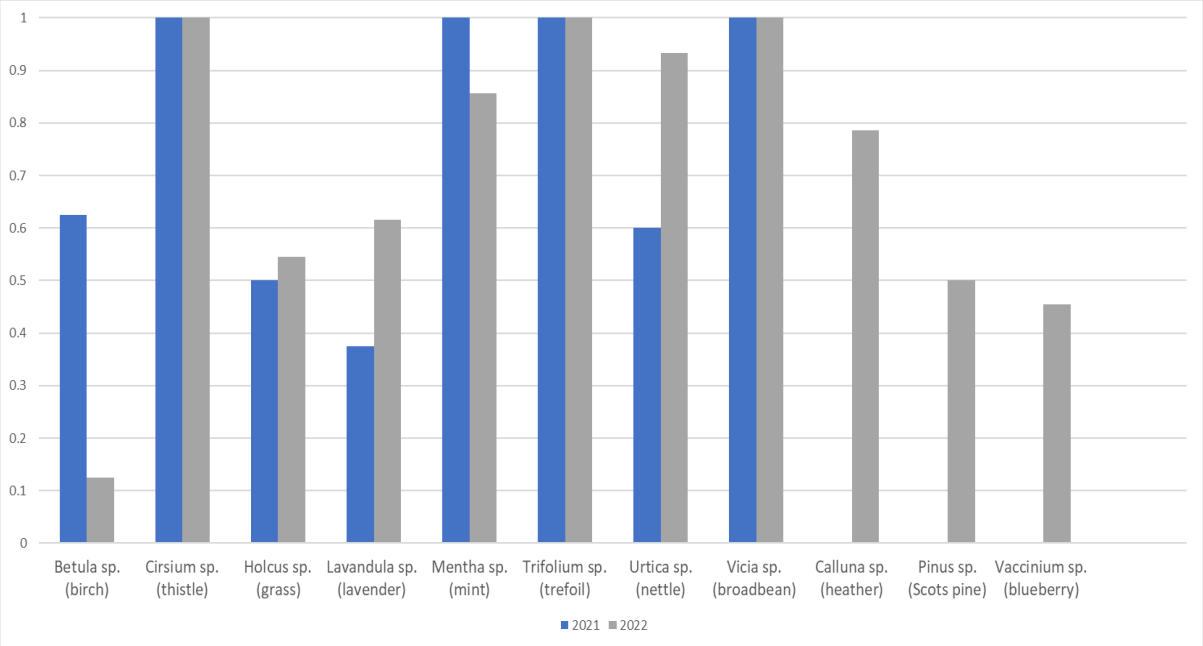

Figure 3. Survival rate of *P. spumarius* nymphs to adults on different plant species in 2021 and 2022. Ratio of total live nymphs recorded on day 1: total adults collected day 38. *Calluna* sp., *Pinus* sp., and *Vaccinium* sp. were not included in 2021.

There was no strong evidence that development occurred more quickly on nitrogen rich plants, and we believe development was more closely linked to environmental conditions (humidity and temperature) than nutrient levels.
In summary this work supports the theory that *P. spumarius* demonstrates a preference for nitrogen-fixing plants such as Fabaceae due to a higher nutritional content (Thompson, 1994, Horsfield, 1977)[25,26].

(ii) IVIA (Spain) - Feeding preferences and the behaviours of vectors

The main results achieved were:
i) Plant volatiles: In the olfactometer tests, adults of *P. spumarius* responded to the plant volatiles while *N. campestris* did not appear to detect these chemicals.

[25] Thompson (1994). Spittlebug indicators of nitrogen-fixing plants. https://doi.org/10.1111/j.1365-2311.1994.tb00257.x
[26] Horsfield (1977). Relationships between feeding of Philaenus spumarius (L.) and the amino acid concentration in the xylem sap. https://doi.org/10.1111/j.1365-2311.1977.tb00889.x

ii) Nymph survival rates: Host plants are essential for the development of nymphs. *P. spumarius* nymphs reached adulthood on alfalfa and calendula (and also on almond); while none did on grasses. *N. campestris* completed nymphal development on grasses but not on the other three host plants.
iii) Ovipositing host plant preference: *P. spumarius* showed no preference for ovipositing on any of the host plants but preferably laid eggs on dry material. In contrast, females of *N. campestris* laid eggs mainly on the grasses.

2.4.3. Vector controls

(i) SASA (Scotland, GB) - Summary of Verrallia aucta *survey (In Review)*

A total of 1,148 *P. spumarius* were screened for *Verrallia aucta*. The overall average percentage of *V. aucta* positive *P. spumarius* was 30% in males and 27% in females. The parasite was also detected in *Neophilaenus lineatus* (n=238, 24% parasitism) and *Aphrophora alni* (n=22, 5% parasitism). This rate of parasitism in *P. spumarius* is much higher than found in the survey carried out by Molinatto *et al.* (2020)[27] in Italy where the percentage of parasitized adults rarely exceeded 15% and more in line with parasitism rates observed by Whittaker (1973)[28] in the UK.
This study provided evidence that the pipunculid parasitoid *Verrallia aucta* is naturally present in Scotland, infects both male and female adult *Philaenus spumarius* and appears to differ in habitat prevalence. Further work is required to better understand the host-parasite relationship in Scotland which will strengthen the concept of using *V. aucta* as a potential biological control agent.

(ii) IVIA (Spain) - Assessment of potential biocontrol agents for vectors

Males and females of *Nesidiocoris tenuis* preyed equally on *P. spumarius* nymphs. However, the predation rate was very low on nymphs protected by foam/spittle, while it was higher on nymphs unprotected by foam. This highlights the possible protective effect the foam has against predators (and perhaps parasitoids).
The use of a commercial compound based on *Beauveria bassiana* induced some mortality on *P. spumarius* nymphs, with evidence of fungal development in some treated individuals. However, considering the optimal conditions in the application of the compound in the assay, it does not seem to be a particularly effective method of population control of the insect.

(iii) Fera (England, GB) - Literature review on the natural enemies of the United Kingdom potential X. fastidiosa *vectors (summary of findings)*

It is clear from the information gathered that relatively little is known about the natural enemies of many species of Auchenorryncha present in the United Kingdom and considered potential vectors of *X. fastidiosa*. Parasitoids are known to attack *P. spumarius*. Of particular note are *Ooctonus vulgatus* (Hymenoptera: Mymaridae), a hymenopteran that attacks *P. spumarius* eggs, and *Verrallia aucta* (Diptera: Pipunculidae), known to attack newly emerged adults of *P. spumarius*. Both these parasitoid species are native to the United Kingdom and some information, albeit dated and from limited locations, is available for rates of parasitism by *V. aucta*. No information has been found on rates of parasitism by *O. vulgatus* in the United Kingdom. As far as is known, *V. aucta* is relatively specific, known only to parasitise *P. spumarius* and *N. lineatus*, and possibly *N. campestris*. Any further work with *V. aucta* should

[27] Molianatto *et al.* (2020). Biology and prevalence in Northern Italy of Verrallia aucta (Diptera, Pipunculidae), a parasitoid of Philaenus spumarius (Hemiptera, Aphrophoridae), the main vector of Xylella fastidiosa in Europe. https://doi.org/10.3390/insects11090607
[28] Whittaker, J.B. (1973). Density regulation in a population of Philaenus spumarius (L.)(Homoptera: Cercopidae). https://www.jstor.org/stable/3410

include these alternative hosts. Very little is known about *O. vulgatus* with the only hosts listed so far being *P. spumarius* and the sciarid fly *Sciara analis*.
As previously mentioned, Molinatto *et al.* (2020)[29] have developed a molecular PCR-based assay that can be used to survey for the presence of *V. aucta* in spittlebug populations. Likewise, Mesmin *et al.* (2020)[30] have used molecular methods to confirm the presence of *O. vulgatus* in *P. spumarius* eggs at locations in Corsica.
It is important to note that *V. aucta* does not immediately kill its host; instead, death of the host typically occurs when the parasitoid larva emerges from the host body to pupate. While the parasitoid develops within the host, the host may still feed and therefore potentially transmit *X. fastidiosa*. However, parasitism by *V. aucta* does render the host sterile. *V. aucta* may therefore be used as an appropriate long-term reduction strategy. Encouraging high numbers of egg parasitoids such as *O. vulgatus* could prevent spittlebug eggs from hatching and impact on subsequent numbers of feeding nymphs and adults in the environment, and thus transmission of the pathogen.
The malaise traps set up by Fera in a meadow habitat collected 4 *V. aucta* individuals in 2021, but none in 2022.

2.5. Conclusions and recommendations to policy makers

2.5.1. Vector surveys

Investigation into the biology of vectors (and potential vectors) of *X. fastidiosa*, including species diversity, feeding preferences and behaviour has been studied by the partners of this Euphresco consortium, covering multiple countries, continents and climates. This research is vital in learning as much as possible about the vectors or potential vectors of *Xylella* in order to prevent the introduction and further spread of the disease. Several other xylem feeding Auchenorrhyncha insects that could act as potential vectors of *X. fastidiosa* have been recorded from surveys within agricultural and natural habitats across multiple countries, many of which feed on similar plant hosts to *P. spumarius* and *N. campestris*. Further work is needed to establish whether these other insects can transmit *Xylella*. Understanding climatic influences on nymph and adult emergence and timelines will further aid in identifying potential plant hosts that may be affected by *Xylella*, particularly with climate change pressures.

2.5.2. Vector movement from crops to wild plants

Studies monitoring vector movement from crops to wild plants have shown that the herbaceous plants below the crops (cover crops) are crucial in the development of spittlebugs. Within olive, citrus and almond groves, spittlebug nymphs (*P. spumarius* and *N. campestris*) develop and feed on the herbaceous plants below the crops (cover crops). The adults will only begin to migrate once the cover crop plants start to dry out as the temperatures increase. In vineyard monitoring in Austria, spittle masses were also found directly on grapevine leaves, particularly within vineyards with extensive green cover. In the United Kingdom, *P. spumarius* are not frequently caught in tree canopies. This could be due to the ground cover plants rarely drying out because of the cooler, wetter climate providing the insects with green plant hosts throughout the whole season.
During the hotter summer months, populations of *P. spumarius* (Spain) and *N. campestris* (Greece) migrated to natural habitats e.g. on pine trees, shrubs or other herbaceous plants nearby where they remained until late autumn/early winter. However, the migration location of

[29] Molianatto *et al.* (2020). Biology and prevalence in Northern Italy of Verrallia aucta (Diptera, Pipunculidae), a parasitoid of Philaenus spumarius (Hemiptera, Aphrophoridae), the main vector of Xylella fastidiosa in Europe. https://doi.org/10.3390/insects11090607
[30] Mesmin *et al.* (2020). Ooctonus vulgatus (Hymenoptera, Mymaridae), a potential biocontrol agent to reduce populations of Philaenus spumarius (Hemiptera, Aphrophoridae) the main vector of Xylella fastidiosa in Europe. https://doi.org/10.7717/peerj.8591

some vector populations was not detected. To establish the network of plants that could be at risk to *Xylella* transmission, continued studies on where the vector populations migrate to is advised.

2.5.3. Traps and lures

With no vector lures currently available, sweep netting is considered to be an efficient and effective method of determining vector diversity and abundances. Sticky traps (clear as opposed to yellow) were seen to complement sweep netting, particularly for monitoring migration between different habitats. Depending on the height on the sticky trap, it is possible for some migrating vectors to be missed i.e. some may migrate by flying at heights greater than the traps. Potential vectors like *Aphrophora salicina* have been observed to fly more frequently and over longer distances than *P. spumarius* who have a preference for jumping rather than flying (Casarin *et al*. 2022)[31].

2.5.4. Transmission and endosymbiotic studies

Transmission studies to determine whether other xylem feeding Auchenorrhyncha insects could act as *X. fastidiosa* vectors in the wild are vital pieces of research to mitigate risk of introduction or further spread of *Xylella* and further research into this topic needs to be explored. Within this project, transmission studies on an important potential vector, *Aphrophora salicina*, were trailed. *A. salicina* adults are known to commonly use Salicaceae as host plants, which are widely distributed within the northern hemisphere. Another recent study by Casarin et al. (2022)[32] has also highlighted *Populus tremula* and *Salix alba* as being potential *X. fastidiosa* hosts. The results from the trial study carried out by ILVO (Belgium) found that *A. salicina* does not transmit *X. fastidiosa* to *S. alba* but that further investigation is needed to optimise experimental testing procedures.
Endosymbiotic diversity studies of potential vectors (BPI, Greece) have provided important data for understanding the biology and population dynamics of these insects from widespread locations. Continued research in this field is vital for establishing possible interactions between vectors, endosymbionts and plant pathogens, which could aid in developing effective *X. fastidiosa* management strategies.

2.5.5. Feeding preferences

Under experimental conditions at SASA (Scotland, GB), *P. spumarius* showed a preference for nitrogen-fixing plants such as Fabaceae, although some did survive to adulthood on grass, blueberry, birch and pine. While *P. spumarius* may not consistently choose to feed on non nitrogen-fixing plants, it is important to be aware that they can if necessary. Contrastingly, in Spain, *P. spumarius* nymphs did not survive on grasses.

2.5.6. Vector controls

The summary of findings from the recent literature review on the natural enemies of *X. fastidiosa* vectors has offered a useful insight into potential biological control methods of vector populations. The pipunculid parasitoid *Verrallia aucta* is naturally present in the United Kingdom and has been found to infect adult *Philaenus spumarius* and *Neohilaenus lineatus*

[31] Casarin, N., Hasbroucq, S., Carestia, G. *et al.* Investigating dispersal abilities of Aphrophoridae in European temperate regions to assess the threat of potential *Xylella fastidiosa*-based pathosystems. *J Pest Sci* 96, 471–488 (2022). https://doi.org/10.1007/s10340-022-01562-9

[32] Casarin, N., Hasbroucq, S., Pesenti, L. et al. Salicaceae as potential host plants of Xylella fastidiosa in European temperate regions. Eur J Plant Pathol 165, 489–507 (2022). https://doi.org/10.1007/s10658-022-02622-7

populations in Scotland. *Nesidiocoris tenuis* predation on *P. spumarius* nymphs was lower on nymphs protected by foam/spittle, highlighting the important protective effect it creates.

2.6. Benefits from trans-national cooperation

The involvement of partners from across Europe, Africa, the Middle East and Australia and New Zealand has been extremely beneficial for the exchange of knowledge and expertise on vectors and potential vectors of *Xylella fastidiosa.* Through regular group meetings, partners were able to discuss and share data on many aspects of vector biology: species diversity, trapping methods, phenology, movement between crops and natural habitats, feeding behaviours, transmission and endosymbiont studies, and potential vector controls. Transnational cooperation also allowed the sharing of material for vector research e.g. vector specimens and DNA was shared from four different countries (five institutions) for secondary endosymbiotic bacteria studies at BPI (Greece). *Philaenus* sp. specimens from Greece were also shared with SASA (UK), which will be kept as useful voucher references, the sequences of which will be added to Genbank. Guest speaker Dr. Stephen Parnell, expert in epidemiological modelling of plant pathogen and insect pest populations from The University of Warwick, was also invited to one of the meetings to present guidelines and advice for developing statistically sound, risk-based surveys of *Xylella fastidiosa* using tools like RiBESS+ for estimating sampling efforts, which the group found very helpful.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

- Casarin, N., Hasbroucq, S., Carestia, G. et al. Investigating dispersal abilities of Aphrophoridae in European temperate regions to assess the threat of potential Xylella fastidiosa-based pathosystems. *J Pest Sci* 96, 471–488 (2022). https://doi.org/10.1007/s10340-022-01562-9.
- Casarin, N., Hasbroucq, S., Pesenti, L. et al. Salicaceae as potential host plants of Xylella fastidiosa in European temperate regions. *Eur J Plant Pathol* 165, 489–507 (2022). https://doi.org/10.1007/s10658-022-02622-7
- Casarin N, Hasbroucq S, López-Mercadal J, Miranda MÁ, Bragard C, Grégoire J-C (2023) Measuring the threat from a distance: insight into the complexity and perspectives for implementing sentinel plantation to test the host range of Xylella fastidiosa. In: Jactel H, Orazio C, Robinet C, Santini A, Battisti A, Branco M, Kenis M (Eds) Conceptual and technical innovations to better manage invasions of alien pests and pathogens in forests. https://doi.org/10.3897/neobiota.84.90024
- Aure C.M., Herrero-Schell J., Montoro M., Beitia F. (2021). Puesta a punto de crías controladas de dos insectos vectores de *Xylella fastidiosa*: *Philaenus spumarius* y *Neophilaenus campestris* (Hemiptera: Aphrophoridae). Agrícola Vergel, 430: 13-18.
- Aure C.M., Herrero-Schell J., Blanes-García M., Beitia F. (2021). First assays on the response of adults of *Philaenus spumarius* (Hemiptera: Aphrophoridae) to different host plants. https://doi.org/10.5281/zenodo.4680075
- Bouvet J.P., Nieves L., Aure C.M., Beitia F. Idoneidad de especies vegetales para el desarrollo poblacional de Neophilaenus campestris (Fallen, 1805) (Hemiptera: Aphrophoridae). Poster. XII National Congress of Applied Entomology. Málaga (Spain), 3-7 Octobre 2022.
- Bernat-Ponce S., García-García R., Aure C.M., Nieves L., Monzó C., Bouvet J.P., Beitia F. Desarrollo, colonización y establecimiento de Philaenus spumarius (Hemiptera: Aphrophoridae) sobre diferentes plantas hospedadoras. Poster. XX Iberian Congress of Entomology. Alicante (Spain), 26-30 June 2023.

4. Open Euphresco data

None.

Appendix 1. Summary of potential *Xylella* vectors data collected during vector surveys.

The table details the *Xylella* vectors or potential vectors present during field surveys carried out by institutions of the 2020-F-341 project. Data that has not been recorded or is incomplete is marked as '-'.

Location of sampling and Institution	Vector (or potential vector) observed during surveys	Agricultural habitats	Natural habitats	Plant species (single or multiple hosts?)	Nymph emergence	Adult emergence	End of season	Additional comments
Austria (AGES)	*Neophilaenus campestris*	Vineyards, Olive groves	-	Multiple plant hosts	April to June	-	-	Low abundance in vineyards
	Philaenus spumarius	Vineyards, Olive groves	-	Multiple plant hosts	April to June	July to September	End of September	Most common species in vineyards. Low abundance in olive groves
	Neophilaenus lineatus	Olive groves	-	Multiple plant hosts	-	-	-	Low abundance in olive groves
	Neophilaenus minor	Olive groves	-	Multiple plant hosts	-	-	-	Low abundance in olive groves
	Cicadella viridis	Vineyards	-	Multiple plant hosts	August to September	-	-	Very low abundance in vineyards
	Aphrophora alni	Vineyards	Forest	Multiple plant hosts	August to September	August to September	-	High abundance on grapevine in Styria
Belgium (ULB)	*Aphrophora salicina*	-	Riparian area	Multiple: Salicaceae (*Salix* sp., *Populus* sp.)	April	June	October	
	Cicadella viridis	Agricultural field margins	Riparian area	Multiple: *Alnus* sp.; Asteraceae; *Carex* sp.; *Corylus avellana*; *Crataegus* sp.; Ericaceae; Fabaceae; *Juncus* sp.; *Pinus* sp.; Poaceae; *Primula* sp.; *Prunus Laurocerasus*; *Quercus* sp.; Renonculaceae; *Rubus* sp.; *Silene* sp.; *Urtica* sp.	First generation: end of April to early May Second generation: June	First generation: end of may to June Second generation: July to August	November	

	Philaenus spumarius	Agricultural field margins, Vineyards	Riparian area, Forest	Multiple: *Alnus* sp.; Asteraceae; *Carex* sp.; *Crataegus* sp.; Fabaceae; *Picea* sp.; Poaceae; *Prunus laurocerasus*; *Quercus* sp.; Renonculaceae; *Rubus* sp.; *Salix* sp.; *Urtica* sp	April	May to June	End of October	
	Aphrophora alni	-	Riparian area, Forest	Multiple: *Alnus glutinosa*; *Alnus* sp.; Asteraceae; *Betula* spp.; *Carex* sp.; *Corylus avellana*; *Crataegus* sp.; Ericaceae; Fabaceae; *Fagus sylvatica*; *Juglans regia*; *Malus domestica*; *Pinus* sp.; Poaceae; *Populus* sp.; *Prunus laurocerasus*; *Quercus* sp.; Renonculaceae; *Salix* sp.; *Sambucus nigra*; *Urtica* sp.	April	Early June	October	
	Neophilaenus lineatus	Agricultural field margins	Riparian area, Forest	Multiple: Herbaceae	-	-	-	
Greece (BPI)	*Neophilaenus campestris*	Citrus groves	Woodland (on conifer)	Multiple: *Avena sterilis* and other plants mainly from Poaceae family (nymphs and adults).	Late March to April	May to early June and November	December	Adults also observed in pine trees (*Pinus halepensis*) during summer and early autumn
	Philaenus spumarius	Citrus groves	-	Multiple: plants from the families Asteraceae, Fabaceae and Poaceae (nymphs and adults)	Late March to April	May to early June and November	December	
	Philaenus signatus	Citrus groves	-	Only in *Asphodelus* sp. (nymphs)	Late March to April	May to early June	-	
	Cercopis sanguinolenta	Citrus groves	-	-	-	May	-	

Israel (MOAG)	*Mesoptyelus impictifrons*	-	-	-	-	-	-	
	Cercopis intermedia	-	-	-	-	-	-	
	Neophilaenus campestris	-	-	-	-	-	-	
New Zealand (PFR)	*Philaenus spumarius*	Apricot orchard, Vineyards, Agricultural field margins	Scrubland	Multiple: Poaceae, Hypericum (adults and nymphs). Cassina, invasive Broom (*Cytisus scoparius),* Lucerne, Yarrow, Fennel (adults).	Late September to October	October	August	Southern hemisphere climate
	Cicada sp.	Agricultural field margins	-	-	-	-	-	
Spain (IVIA)	*Philaenus spumarius*	Olive groves, Citrus groves, Almond groves	-	Multiple herbaceous plant hosts	Mid-February to Early March	Early to Mid-April	Early December	
	Neophilaenus campestris	Olive groves, Citrus groves, Almond groves	Riparian area	Poaceae	Mid-February to Early March	Early to Mid-April	Early December	
	Neophilaenus lineatus	-	Woodland (on conifer)	Poaceae and conifers	Mid-February to Early March	Early to Mid-April	Early December	
	Cercopis intermedia	Olive groves	-	Multiple plant hosts mainly within Fabaceae and Asteraceae	Mid-February to Early March	Early to Mid-April	Early December	
	Lepyronia coleoptrata	Citrus groves	-	Multiple plant hosts mainly within Fabaceae and Asteraceae	April	Late May	Early December	
Tunisia (INRAT)	*Philaenus tesselatus*	Vineyards	Woodland	Multiple plant hosts	March to April	-	-	
	Philaenus maghresignus	-	Woodland	Single plant host	March to May	Early April	December	
	Neophilaenus campestris	Olive groves, Fruit orchards	Woodland, Grassland (dry)	Poaceae	March	-	-	

	Neophilaenus lineatus	Olive groves	Grassland (dry)	Poaceae	April	-	-	
UK, England (Fera)	*Aphrorphora alni*	-	Scrub (adults), Grassland (nymphs)	Nymphs: herbaceous plants; Adults: Salicaceae (*Salix* and *Populus*);	April to June	June to July	September	Nymphal stage develop at base of broadleaved herbs e.g. *P. lanceolata, H. radicata, R. acetosa;* then observed to move up plants in later stages e.g. *H. sphondylium*
	Cicadella viridis	-	Wet grassland	Juncus, Poaceae	-	June to July	September	Adults swept from Salicaceae
	Evacanthus Interruptus	-	Grassland	Poaceae	-	June to July	September	
	Euscelis incisus	-	Grassland	Poaceae	-	July	October	
	Neophilaenus lineatus	-	Grassland	Poaceae	April to June	-	-	
	Philaenus spumarius	-	Grassland	Multiple broadleaved plants	April to June	June to July	October	
UK, Scotland (SASA)	*Aphorphora alni*	-	Woodland, Grassland	Multiple plant hosts	May to June	July to August	October	
	Cicadella viridis	Agricultural field margins	Woodland, Heathland, Grassland	Only found where *Juncus* sp. is present	May to June	July to August	October	Captive insects observed feeding on blueberries
	Evacanthus Interruptus	-	Woodland, Grassland	-	May to June	July to August	October	
	Neophilaenus exclamationis	-	Heathland, Grassland	Poaceae	May to June	July to August	October	
	Neophilaenus lineatus	Agricultural field margins	Heathland, Woodland, Grassland	Poaceae	May to June	July to August	October	
	Philaenus spumarius	Agricultural field margins, Polytunnels, Conifer plantations	Heathland, Woodland, Grassland	Multiple plant hosts	May to June	July to August	October	Low abundance observed within polytunnels and conifers