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ESCUELA DE INGENIERÍA AGRARIA Y FORESTAL

Biological and ecological features of xylem-feeder vectors through establishment of a sustainable strategy for control of *Xylella fastidiosa*-vectors

Doctoral Thesis in Ingeniería De Biosistemas

Isabel Cristina de Sousa Rodrigues

Directors:

Professor Doutor José Alberto Cardoso Pereira

Professora Doutora Paula Cristina dos Santos Baptista

León, 2023

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Características biológicas y ecológicas de los insectos vectores y potenciales vectores de *Xylella fastidiosa* que se alimentan del xilema para desarrollar una estrategia sostenible de control

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As meus pais

Ao meu irmão

"Yesterday is history, tomorrow is a mystery, but today is a gift. That is why it is called the present."

- Master Oogway

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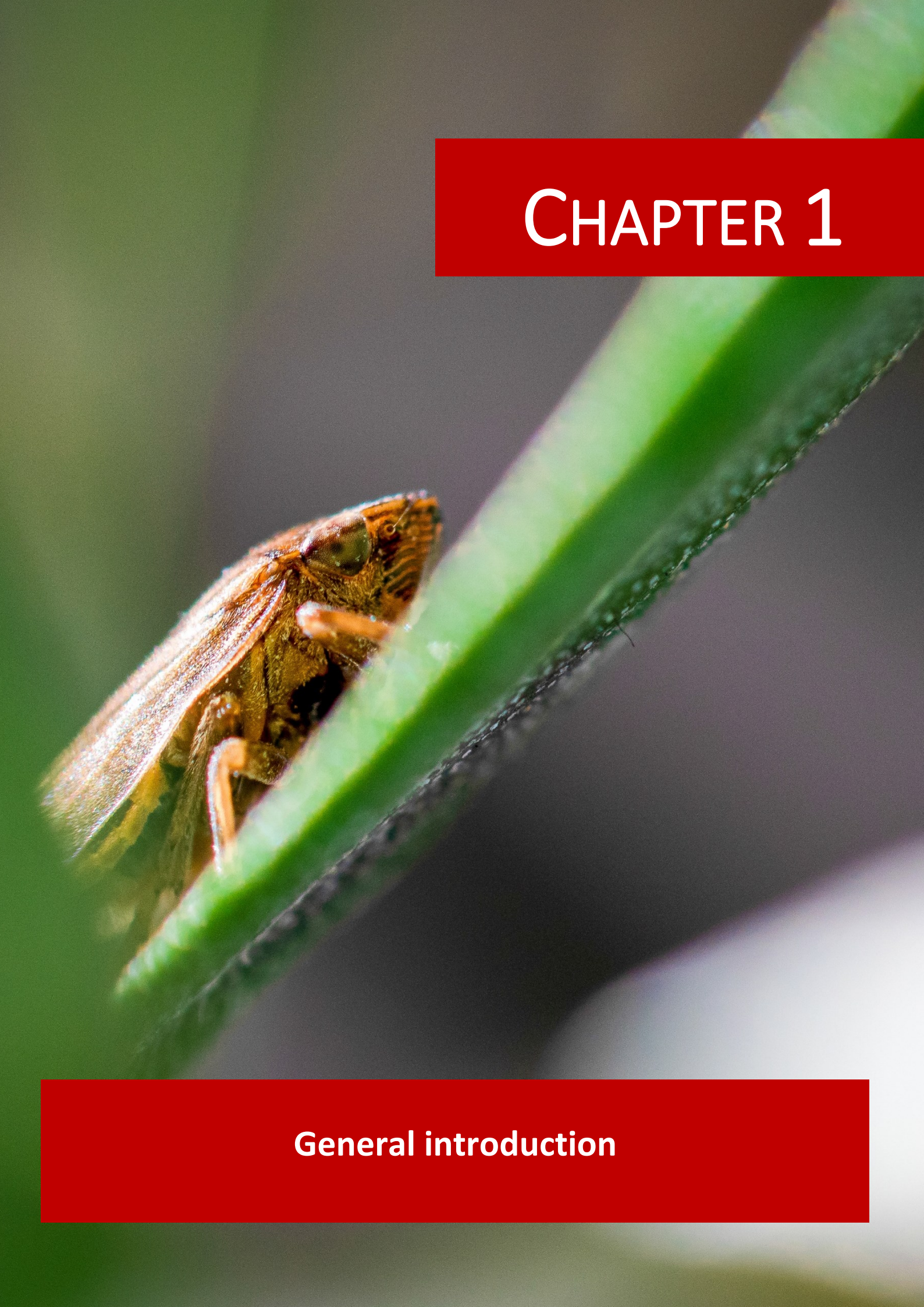
Abstract

Xylella fastidiosa Wells et al., 1987, one of the most dangerous plant pathogenic bacteria worldwide, is disseminated by xylem-sap feeder insects. In Portugal, this pathogen was reported for the first time in January 2019 in Porto. However, in this country, knowledge of this community of insect vectors, potential vectors, and potential predators is still scarce. Thus, this thesis first studied the diversity and abundance of vectors and potential vectors in different Mediterranean agroecosystems. In vineyards, five xylem-sap feeders were identified, being *Cicadella viridis* (Linnaeus, 1758), the most abundant, followed by *Philaenus spumarius* (Linnaeus, 1758), *Neophilaenus campestris* (Fallén, 1805), *Lepyronia coleoptrata* (Linnaeus, 1758), and *N. lineatus* (Linnaeus, 1758). In almond, citrus, olive groves and adjacent scrubland, only two confirmed vectors of *X. fastidiosa* (*P. spumarius* and *N. campestris*) were recorded. The olfactory response of *P. spumarius* and *C. viridis* adults to different concentrations of cis-3-hexenyl acetate and cis-3-hexen-1-ol, volatiles frequently present in almond, olive, and vine leaves, was also studied. While *P. spumarius* females were significantly attracted at the lowest concentration, *C. viridis* did not show a significant preference for the volatiles at any concentration. In comparison to males, females of *P. spumarius* and *C. viridis* walked significantly at longer distances and at higher velocities. The olfactory response of *P. spumarius*, to five Portuguese olive cultivars, showed to be sex- and season-dependent. In Spring, females and males were significantly attracted to "Negrinha de Freixo"; while in autumn, females were significantly attracted to "Cobrançosa". With the aim to identify potential predators of confirmed vectors of *X. fastidiosa*, a polymerase chain reaction (PCR)-based approach was developed to detect DNA of *P. spumarius* in the spider's gut. Among the primers tested, one targeting the *cyt*B gene was able to detect *P. spumarius* in the spider *Xysticus acerbus* Thorell, 1872, with high sensitivity, reaching 50% detection success 82 h after feeding. The feasibility of this primer set to detect predation of *P. spumarius* by spiders was confirmed in the field, where 20% of the collected spiders presented positive amplifications. Overall, the results obtained contributed to enhancing the scarce knowledge of the community of vectors and potential vectors of *X. fastidiosa* in several Portuguese agroecosystems. Moreover, insights into their olfactory behaviour and potential natural predators can help future implementation of approaches to manage the vectors and the spread of *X. fastidiosa*.

Keywords: Emerging plant diseases; *Philaenus spumarius*; *Cicadella viridis;* olfactory behaviour; insect–predator association



Chapter 1

General introduction

Chapter 1

1.1. *Xylella fastidiosa*: taxonomy, host plant, diseases, and symptoms

Xylella fastidiosa is a gram-negative, xylem-limited, slow-growing, fastidious bacterium described for the first time in 1987 by Wells et al. This bacterium belongs to the class of Gammaproteobacteria, order Xathomonadales and family Xanthomonadacea, and it is an obligate endophyte with high genomic variability and plasticity, allowing it to have a wide host range (Schuenzel et al., 2005; Nunney et al., 2013; Vanhove et al., 2019; EFSA et al., 2022). Currently, the bacterium is genetically divided into six subspecies: (1) *X. fastidiosa s*ubsp. *fastidiosa,* (2) *X. fastidiosa* subsp. *multiplex,* (3) *X. fastidiosa* subsp. *pauca,* (4) *X. fastidiosa* subsp. *sandyi,* (5) *X. fastidiosa* subsp. *tashke,* and (6) *X. fastidiosa* subsp. *morus* (Nunney et al., 2013; EFSA et al., 2019). All subspecies are subdivided into sequence types (STs), each with different host ranges (Sicard et al., 2018; Denancé et al., 2017; Nunney et al., 2019; EFSA et al., 2022).

According to the most recent update of the *Xylella* spp. host plant database, the number of hosts of *X. fastidiosa* comprise 655 plant species belonging to 88 different families (EFSA et al., 2022). The plant families with the highest number of host species are Fabaceae (72 plant species), followed by Asteraceae (64 plant species) and Vitaceae (53 plant species) (EFSA et al., 2023). The *X. fastidiosa* subspecies *fastidiosa*, *pauca*, and *multiplex* are considered the most economically damaging, being confirmed in several countries and hosts plants worldwide (Table 1.1) (Denancé et al., 2019; Schneider et al., 2020; EFSA et al., 2022). In nature, the *multiplex* subspecies can infect a large number of plants species, followed by *pauca* and *fastidiosa*, whereas, when artificially inoculated, *fastidiosa* is reported to be the most effective, followed by *pauca* and *multiplex* subspecies (Table 1.1).

Despite the wide *X. fastidiosa* host range, not all plant species express symptoms or have economic importance. Some plant species can sustain long-term infections and remain asymptomatic (EFSA, 2013; Sicard et al., 2018). Specific symptomatic host plants are only susceptible to specific strains of *X. fastidiosa*. Meaning that each subspecies and respective STs of *X. fastidiosa* has a small number of symptomatic host plants (Janse & Obradovic, 2010; Nunney et al., 2013; EFSA et al., 2022). However, each subspecies of *X. fastidiosa* is associated with several insidious diseases in important economic crops worldwide, such as Almond Leaf Scorch (*Prunus dulcis* (Mill.) D.A.Webb), Olive Quick Decline Syndrome (*Olea europaea* L.), Pierce's disease of the vine (*Vitis vinifera* L.*)*, Citrus Variegated Chlorosis (*Citrus* spp.), coffee leaf scorch (*Coffea* spp.), Phony peach disease (*P. persica* (L.) Stokes), Plum leaf scald (*P. domestica* L.), Leaf Scorch (*Nerium oleander* L., *Ulmus* spp., *Platanus* spp., and *Acer* spp.) and alfalfa dwarf

(*Medicago sativa* L.) (Hopkins & Purcell, 2002; Saponari et al., 2013). The subspecies *fastidiosa* is known to cause diseases in vine (*V. vinifera*), almond (*P. dulcis*), coffee (*C. arabica* L. and *C. canephora* Pierre ex A.Froehner), cherry (*P. avium* (L.) L.), oleander (*N. oleander)*, alfalfa (*M. sativa*) and maples (*Acer* spp.) (Schaad et al., 2004; Marcelletti & Scortichini, 2016). The subspecies *pauca* is typically responsible for disease in the citrus (*Citrus* spp.), olive (*O. europaea*), coffee (*C. arabica*), oleander, almond, and cherry (Schaad et al., 2004; Martelli et al., 2016). And the subspecies *multiplex,* as the name implies (numerous), is responsible for several diseases; from wich the most important are in peach (*P. persica*), plum (*P. domestica*), almond, elm (Ulmus spp.), sycamore (*Platanus* spp.), and other forest trees (Schaad et al., 2004; Marcelletti & Scortichini, 2016). Regarding the other subspecies, *sandyi, tashke* and *morus*, they have a limited host spectrum and are associated with plant diseases of less economic interest (EFSA et al., 2022).

Table 1.1. Subspecies of *Xylella fastidiosa*, respective geographic distribution, and the total number of host plants by natural and artificial infection (adapted from EFSA et al., 2022, 2023).

Subspecies	**Geographic distribution**	**Total number of host plants**	
		Natural Infection	**Artificial Infection**
X. fastidiosa subsp. *fastidiosa*	Spain, Costa Rica, Mexico, United States of America	53	74
X. fastidiosa subsp. *multiplex*	France, Portugal, Spain, Brazil, France, Italy, and the United States of America	202	25
X. fastidiosa subsp. *pauca*	Argentina, Brazil, Costa Rica, Ecuador, France, Italy, and Spain	56	27
X. fastidiosa subsp. *sandyi*	Costa Rica, Mexico, France, United States of America, and Honduras	8	7
X. fastidiosa subsp. *tashke*	United States of America	1	1
X. fastidiosa subsp. *morus*	United States of America	4	2

The typical symptoms of *X. fastidiosa* infection are related to the invasion and colonisation of this bacterium in the xylem vessels of the host plants. This pathogen moves upstream and downstream, gradually blocking the xylem due to bacterial growth and plant physiological responses, eventually blocking the flow of water and soluble mineral nutrients through the xylem (Janse & Obradovic, 2010; Sicard et al., 2018). In general, an infected plant can present foliar discolouration, leaf scorch or drying of leaf margins, wilting of apical shoots, dieback of twigs and branches, general decline and even the eventual death of the plant (Figure

1.1) (Janse & Obradovic, 2010; EFSA, 2013). However, the symptoms can vary according to the host plants, the subspecies of *X. fastidiosa* involved, and the climatic conditions (EFSA, 2013).

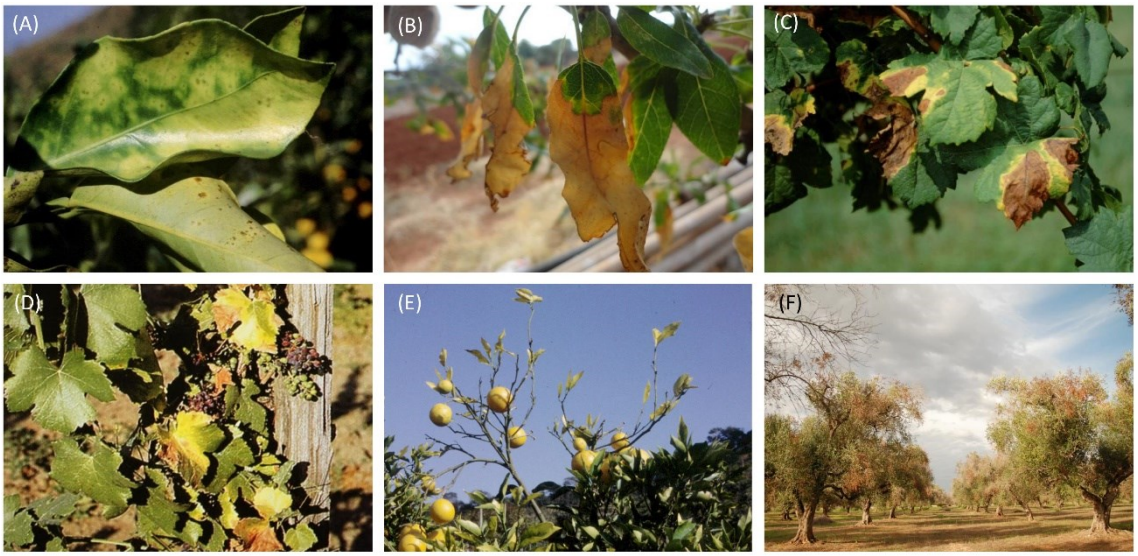


Figure 1.1. Typical symptoms of infection of *Xylella fastidiosa*. (A) foliar discolouration on sweet orange leaves; (B) leaf scorch on almond trees (C) drying of leaf margins and leaf wilting on vine leaves (D) Yellowing and desiccation of line leaves and wilting of bunches; (E) Twig dieback on a sweet orange tree; (F) General decline of the olive trees. (Source: EPPO, 2022a)

1.2. Geographic distribution of *Xylella fastidiosa*

The main dispersal pathways of *X. fastidiosa* over long distances are the commercial trade and the movement of infected plant material (Almeida & Nunney, 2015; Denancé et al., 2017). The insect vectors could also be carried internationally on plants (Janse & Obradovic, 2010), but this situation is less likely. However, when this insect-borne plant pathogen reaches a new location, the spread throughout the landscape could be mediated by native insect vectors (Redak et al., 2004).

1.2.1. Worldwide distribution

Xylella fastidiosa is native to the Americas and is widespread throughout North, Central and South America (Figure 1.2) (Almeida & Nunney, 2015; EPPO, 2022b). In 1892, in southern California, it was reported for the first time a disease caused by *X. fastidiosa*, named California vine disease, later renamed as Pierce's disease (Tumber et al., 2014). Initially, it was thought that the causal agent of the disease was a virus; however, it remained unknown until 1987, when the

fastidious bacterium was described for the first time (Wells et al., 1987). Pierce's disease persists in the United States, from Florida to California, to the present day, representing a severe economic problem in the wine industry (Tumber et al., 2014; Giménez-Romero et al., 2022). In 1987, *X. fastidiosa* was reported in Minas Gerais (Brazil), associated with Citrus Variegated Chlorosis in sweet orange trees (Chang et al., 1993), and in subsequent years, rapidly became established throughout Brazilian citrus industry (Coletta-Filho et al., 2020).

In Asia, *X. fastidiosa* was first identified in 1993 in Taiwan in Asian pear, causing leaf scorch in several regions (Leu & Su, 1993). However, genetic analyses suggested that the bacterial strain isolated from the Asian pear is a new and distinct species tentatively named *X. taiwanensis* sp. nov (Su et al., 2016). In 2002, *X. fastidiosa* was also reported in a commercial vineyard in central Taiwan (Su et al., 2013). In 2014, symptoms resembling those of Pierce's disease and almond leaf scorch were observed in several provinces of Iran (Amanifar et al., 2014), and more recently, the same symptoms were observed in Israel (EPPO, 2022).

In Europe, the first official detection of *X. fastidiosa* was in 2013 in olive groves in Apulia (South Italy) (Saponari et al., 2013). It is estimated that the bacterium was introduced in Italy via infected ornamental coffee plants imported from Costa Rica or Honduras (Martelli et al., 2016). Since then, the pathogen has spread and killed millions of olive trees in Apulia, causing unprecedented socio-economic issues (Saponari et al., 2019). Before 2013, some sporadic reports of *X. fastidiosa* detection in Europe were not confirmed and did not raise concerns (Berisha et al., 1998). After the first official introduction in Italy, this bacterium was reported in other European countries (Figure 1.2) (EPPO, 2022b). In 2015, outbreaks were identified in Corsica and mainland France (Denancé et al., 2017). The outbreaks in mainland France are currently classified as transient, *i.e.,* under eradication (EPPO, 2022b). In Switzerland, the pathogen was also detected in 2015 in imported coffee plants. These plants were eradicated, and potential host plants were banned from the list of imports to prevent further occurrences of this bacterium (EPPO, 2015). In 2016, *X. fastidiosa* was detected in Germany (EPPO, 2016a), and the outbreak was declared eradicated (EPPO, 2018). In the same year, the bacterium was also detected in Spain, in the Balearic Islands (EPPO, 2016b), and in 2017, the Spanish authorities notified the presence *of X. fastidiosa* in almond orchards in Alicante (mainland Spain) (EPPO, 2017); later in 2018, they reported the presence of the bacteria in Madrid (mainland Spain) associated to olive trees (EPPO, 2019a). Currently, the outbreaks in the Balearic Islands are under containment, and the epidemics in mainland Spain are classified as transient (EPPO, 2022a). In 2019, *X. fastidiosa* was reported for the first time in Portugal (EPPO, 2019b).

Monitoring and eradication programs focusing on *X. fastidiosa* are being implemented throughout Europe to prevent the spread and introduction into other countries (Commission Implementing Regulation (EU) 2020/1201 of 14 August 2020). Distribution models of *X. fastidiosa*, indicated that the current geographical range of the bacteria in Europe is small compared to the large extent of climatically suitable areas (Godefroid et al., 2019, 2022).Therefore, according to these models, the future geographical distribution of *X. fastidiosa* can encompass the Mediterranean islands and coastal areas of Spain, Greece, Italy and France, the Atlantic coastal areas of France, Portugal and Spain, and the southwestern regions of Spain, France and lowlands in southern Italy (Giménez-Romero et al., 2022; Godefroid et al., 2019, 2022).

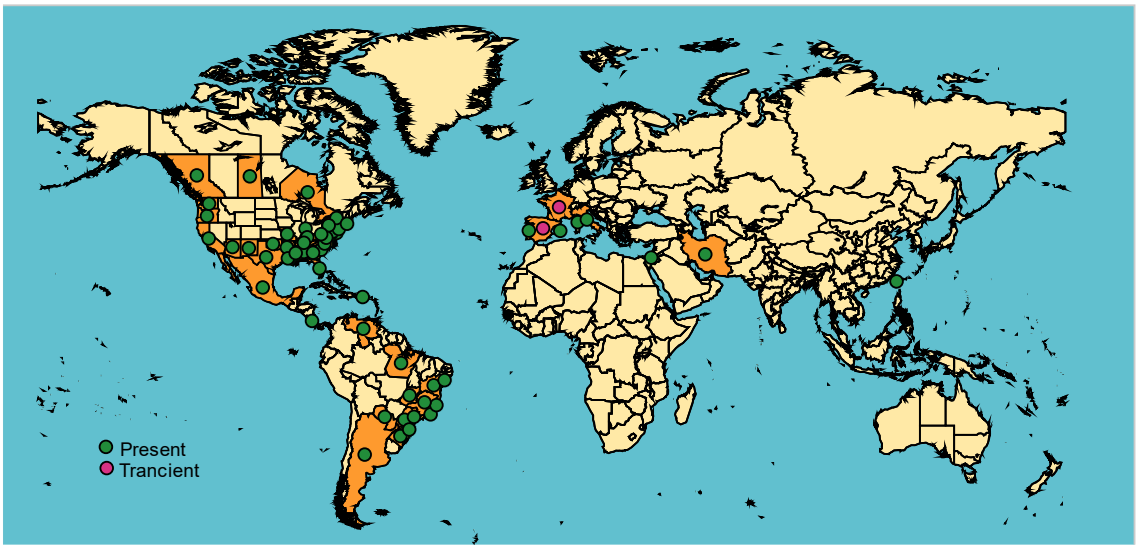


Figure 1.2. Worldwide distribution of *Xylella fastidiosa* (EPPO, 2022b, Last updated: 2022-08-05).

1.2.2. Portugal distribution

In Portugal, *X. fastidiosa* was first described in January 2019 in an ornamental hedge of *Lavandula dentata* L. in Vila Nova de Gaia (Porto) (EPPO, 2019b). Subsequently, intensive prospecting took place in the surrounding area to determine the extent of the outbreak. As a result, several plant species were found to be infected with *X. fastidiosa*, consequently increasing the demarcated area of the Porto metropolitan region over the years (Figure 1.3) (DGAV, 2022a).

In 2021, the presence of the bacterium was laboratory confirmed in samples of *Salvia Rosmarinus* Spenn., collected in Tavira (Algarve) and Sintra (Lisbon). Therefore, two new

demarcated areas were established in the country: the Demarcated area of Tavira (DGAV, 2021a) and the Lisbon metropolitan area (DGAV, 2021b) (Figure 1.3). In the Demarcated area of Tavira, were applied appropriate eradication measures and the absence of infected plants and vectors was confirmed, implying that the initial presence of the bacteria was an isolated case, and the bacteria dispersion did not occur. Therefore, in august 2022, this Demarcated area was considered suppressed (DGAV, 2022b). Regarding the Lisbon metropolitan area, although appropriate eradication measures were also applied, in 2022, the presence of the bacterium was reported in two new locations (Oeiras and Sintra), making a total of 6 outbreaks, thus increasing the Demarcated Zone for *X. fastidiosa* in the Lisbon Metropolitan Region (DGAV, 2022c).

In 2022, ten demarcated areas were established in the country (Figure 1.3) (DGAV, 2022d), showing that the bacteria is progressively spreading throughout the country. In the beginning of 2023 four new outbreaks were reported. Currently, in total, 17 demarcated areas were established (Figure 1.3) (DGAV, 2023). The constant spread may lead to devastating economic and environmental problems, threatening Portugal's agricultural sector and plant diversity. More than 80 host plants have been identified in the country, including plants of economic importance such as olive, citrus, cork oak (*Quercus suber* L.), and almond (DGAV, 2022d; EFSA et al., 2023).

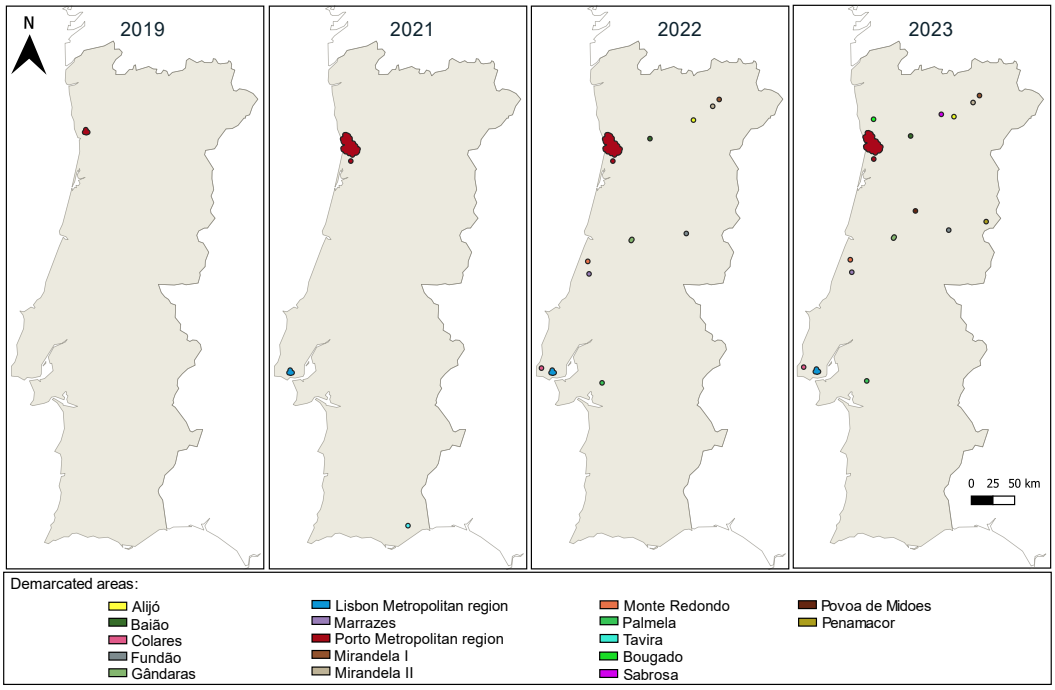


Figure 1.3. Evolution of *Xylella fastidiosa* Demarcated areas in Portugal between 2019-2023. Map is projected in ETRS89/PT-TM06 (DGAV, 2022d and DGAV 2023).

1.3. Vectors and potential vectors of *Xylella fastidiosa*

1.3.1. Xylella fastidiosa - vector relationship

Xylella fastidiosa colonises the host plant's xylem and its insect vectors' mouth parts (Martelli et al., 2016; Sabella et al., 2019). The insect acquires the bacterium when it feeds on the xylem of an infected plant. After the acquisition, *X. fastidiosa* remains restricted to the foregut parts of the insect's alimentary canal (precibarium and cibarium), where it adheres, multiplies, and persists (Almeida et al., 2005; Overall & Rebek, 2017; EFSA et al., 2018). Once acquired, the bacterium does not require a latent period before transmission, meaning that the vector can transmit the pathogen to healthy plants immediately after acquisition (Janse & Obradovic, 2010). Nymphs and adult vectors are able to acquire and transmit the bacteria (EFSA, 2013). However, nymphs lose the transmission capability after moulting, whereas adults, after infection, can transmit the bacteria during their whole lifetime (Almeida et al., 2005; Chatterjee et al., 2008a,b; EFSA et al., 2018). Nevertheless, the bacterium is not transmitted to the progeny (Redak et al., 2004; EFSA et al., 2018; Krugner et al., 2019).

The vectors have different transmission efficiency, which can depend on the vector species, their host plant preference and tissue, pathogen genotype, temperature, and other factors (Sicard et al., 2018).

1.3.2. Vector diversity

As a xylem-limited bacterium, *X. fastidiosa* is transmitted exclusively by xylem sap feeding specialist. All insects that feed solely on xylem fluid are considered potential vectors of the bacterium, until proven otherwise (EFSA et al., 2019). These insects belong to the suborder Auchenorrhyncha (Hemiptera) infraorder Cicadomorpha, Superfamilies Cercopoidea (families: Aphrophoridae and Cercopidae) and Cicadoidea, and the family Cicadellidae (subfamily: Cicadellinae) (Cornara et al., 2019; Redak et al., 2004).

Some studies found that phloem-sap feeders of the subfamily Deltocephaline (Hemiptera: Auchenorrhyncha: Cicadellidae) appear to be infected with the bacteria (Purcell, 1980; Elbeadaino et al., 2014; Ben Moussa et al., 2016; Chuche et al., 2017). Pompon et al. (2011) described that specialised phloem feeders occasionally consume xylem sap to replenish water balance after dehydration and regulate the osmotic potential. Although these individuals have

contact with xylem vessels and can be infected with *X. fastidiosa*, there is no evidence of their ability to transmit the pathogen to plants (Purcell, 1980; Cavalieri et al., 2019).

In the American continent, several insects belonging to the family Cicadellidae, subfamily Cicadellinae, were identified as key vectors of *X. fastidiosa* (Krugner et al., 2019). *Homalodisca vitripennis* (Germar, 1821) is one of the most important vectors in southern and central California (Overall & Rebek, 2017). This Cicadellidae, native to the southeastern United States and northern Mexico, has a very high transmission rate, being able to transmit *X. fastidiosa* to vines, even during winter, when the plants are in vegetative dormancy (Hopkins & Purcell, 2002; Morente & Fereres, 2017). Besides vines, *H. vitripennis*, can also transmit *X. fastidiosa* to other plant species, such as oleander, almond and peach. In the coastal California vineyards, *Graphocephala atropunctata* (Signoret) is the Cicadellidae predominant and is the most efficient vector of *X. fastidiosa* to vines (Daugherty et al., 2010; Overall & Rebek, 2017). Other Cicadellidae, such as *Acrogonia citrina* Marucci & Cavichioli, 2002, *Bucephalogonia xanthophis* (Berg, 1879), *Dilobopterus costalimai* Young, 1977, *Macugonalia leucomelas* (F. Walker, 1851), *Oncometopia facialis* (Signoret, 1854), and *Oncometopia nigricans* (F. Walker, 1851), are associated with Citrus Variegated Chlorosis in Brazil (EFSA et al., 2019).

In Asia, information regarding *X. fastidiosa* vectors is scarce. The Cicadellidae *Kolla paulula* (Walker) was identified as the main vector in the outbreak of Asian pear in Taiwan.

In Europe, more than 90 species are described as xylem sap-feeding specialists (Table 1.2) (EFSA, 2013,2015; Cornara et al., 2019). However, only the Aphrophorids: *Philaenus spumarius* (Linnaeus, 1758), *Philaenus italosignus* Drosopoulos and Remane 2000, and *Neophilaenus campestris* (Fallén, 1805) (Cicadomorpha: Aphrophoridae) are confirmed as vectors of *X. fastidosa* (Cavalieri et al., 2019). *Philaenus spumarius* is considered the main vector of *X. fastidiosa* in Europe since this xylem sap-feeding specialist is the most abundant and widespread in the European territory (Ben Moussa et al., 2016; Cornara et al., 2018; Morente et al., 2018; Tsagkarakis et al., 2018; Villa et al., 2020), and presents a higher transmission rate of this bacteria compared to other vectors (Cavalieri et al., 2019).

Cicadella viridis (Linnaeus, 1758) (Cicadomorpha: Cicadellidae: Cicadellinae), an abundant Cicadellidae in *Europe,* has proved to be a competent vector of *X. fastidiosa*, in laboratory conditions (Bodino et al., 2022). However, the same authors demonstrated that acquisition, and especially inoculation rates, are significantly lower than those registered in *P. spumarius*. Although *C. viridis,* does not feed on olive trees, where it was ruled out as a vector

(Bodino et al., 2022), females oviposit in a variety of host plants, including the vine, where they feed and potentially transmit *X. fastidiosa* (Cornara et al., 2019). Moreover, Markheiser et al. (2022) reported that when individuals of *C. viridis* feed on vines, they display a high frequency of the electrical pattern (higher than *P. spumarius* and *N. campestris*) associated with *X. fastidiosa* inoculation. Therefore, further studies to explore the potential of *C. viridis to transmit X. fastifiosa to the* vineyard or other agroecosystems are needed to understand better this insect's role in the *X. fastidiosa* epidemiology.

Recently, *Draeculacephala robinsoni* Hamilton 1967 (Cicadomorpha: Cicadellidae: Cicadellinae), a Cicadellidae native to eastern North America, was reported in France and Spain. The genus *Draeculacephala* includes species that are vectors of plant pathogens, including *X. fastidiosa* (Rösch et al., 2022).

Regarding Cicadas, Cornara et al. (2020a) showed that cicadas could not transmit *X. fastidiosa* to vine and olive plants, implying that cicadas may not play an important role in spreading *X. fastidiosa*.

Information on the competence of other European xylem sap-feeding specialists transmitting *X. fastidiosa* is non-existent. Although it is expected that xylem sap-feeding can acquire and transmit the bacterium, it is important to demonstrate that species not formally identified as vectors can transmit the bacterium from plant to plant. In this context, performing transmission bioassays and confirming the insect's acquisition and inoculation of the pathogen are needed (Nault, 1997; Chatterjee et al., 2008a,b). Additionally, different vector species may have very different transmission efficiencies depending on host plant species or even by feeding on different tissues of the same host plant (Redak et al., 2004; EFSA, 2013).

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Table 1.2. European potential vectors (most important xylem-sap feeding species) of *Xylella fastidiosa.*

Family	Main species	Role as vector	Portugal	References
Aphrophoridae				
(27 species)	*Aphrophora alni* (Fallén, 1805)	Potential vector	x	(EFSA, 2013, 2015; Cornara el al., 2019)
	Aphrophora salicina (Goeze, 1778)	Potential vector	x	(EFSA, 2013, 2015; Cornara el al., 2019)
	Lepyronia coleoptrata (Linnaeus, 1758)	Potential vector	x	(EFSA, 2013, 2015; Rodrigues et al., 2022ª)
	***Neophilaenus campestris* (Fallén, 1805)**	**Confirmed vector**	x	(EFSA, 2013, 2015; Cavalieri et al., 2019; Rodrigues et al., 2022a)
	Neophilaenus lineatus (Linnaeus, 1758)	Potential vector	x	(EFSA, 2013, 2015; Rodrigues et al., 2022)
	***Philaenus italosignus* Drosopoulos & Remane, 2000**	**Confirmed vector**	-	(EFSA, 2013, 2015; Cavalieri et al., 2019)
	***Philaenus spumarius* (Linnaeus, 1758)**	**Confirmed vector**	x	(EFSA, 2013; Popova, 2020; Cavalieri et al., 2019; Popova, 2020)
	Philaenus tesselatus Melichar, 1899	Potential vector	x	(EFSA, 2013, 2015; Popova, 2020)
Cercopidae				
(7 species)	*Cercopis intermedia* Kirschbaum, 1868	Potential vector	x	(EFSA, 2013, 2015; Popova, 2020)
	Cercopis vulnerata (Rossi, 1807)	Potential vector	-	(EFSA, 2013, 2015; Cornara el al., 2019)
	Cercopis sanguinolenta (Scopoli 1763	Potential vector	x	(EFSA, 2013, 2015; Cornara el al., 2019)
	Haematoloma dorsata (Ahrens 1812)	Potential vector	x	(EFSA, 2013, 2015; Cornara el al., 2019)
Cicadoidea				
(54 species)	*Cicadatra atra* (Olivier, 1790)	Potential vector	-	(EFSA, 2015; Cornara el al., 2019)
	Cicada orni Linnaeus, 1758	Potential vector	x	(EFSA, 2015; Cornara el al., 2019)
	Lyristes plebejus (Scopoli, 1763)	Potential vector	x	(EFSA, 2015; Cornara el al., 2019)
	Cicada barbara Boulard 1982	Potential vector	x	(EFSA, 2015; Cornara el al., 2019)
Cicadellidae				
Cicadellinae	***Cicadella viridis* (Linnaeus, 1758)**	**Competent vector**	x	(EFSA, 2013, 2015; Bodino et al., 2022; Rodrigues et al., 2022a)
(9 species)	*Draeculacephala robinsoni* Hamilton, 1967	Potential vector	-	(Rösch et al., 2022)
	Graphocephala fennahi Young Jr., 1977	Potential vector	-	(EFSA, 2013, 2015)
	Evacanthus acuminatus (Fabricius, 1794)	Potential vector	-	(EFSA, 2013, 2015; Cornara el al., 2019)

(X): species present in mainland Portugal; (-): species absent in mainland Portugal.

1.3.3. Biological and ecological features of vectors of Xylella fastidiosa

Cercopoidea superfamily

The Superfamily Cercopoidea comprises five families (Cercopidae, Aphrophoridae, Epipygidae, Clastopteridae, and Machaerotidae) with approximately 2600 species and 361 genera (Xu et al., 2022). This superfamily, specifically in the Aphrophoridae family, the second largest family of this group, comprises the principal European vectors of *X. fastidiosa*, namely *P. spumarius*, *P. italosignus,* and *N. campestris*. Since only *P. spumarius* and *N. campestris* are present in Portugal, with great dispersion and abundance, the biological and ecological features were focused only on these two species.

Biological cycle

In Cercopoidea, the duration of the biological cycle and the number of generations per year varies with the species and the local climatic conditions (Morente & Fereres, 2017). In Europe, most studies are focused on *P. spumarius* (*e.g.,* Dongiovanni et al., 2019; Bodino et al., 2020, 2021a,b) though some research has also been conducted on *N. campestris* (*e.g.,* Villa et al., 2020; Lago et al., 2021b; Antonatos et al., 2021; Mesmin et al., 2022). These vectors have similar biological cycles; they are univoltine species with a single generation per year (Morente & Fereres, 2017; Cornara et al., 2018; Antonatos et al., 2021; Bodino et al., 2021b). *Philaenus spumarius* and *N. campestris* overwinter in egg form until early spring, when hatching occurs (Bodino et al., 2021b). Once hatched, nymphs crawl to the closest green succulent plant and produce a tenuous foam (Villa et al., 2020; Bodino et al., 2021b; Mesmin et al., 2022). The foam production is a unique feature of the superfamily Cercopoidea, which provides protection against predators and desiccation (Tonelli et al., 2018). Nymphs pass through five instars, and their development takes about 5-6 weeks (Villa et al., 2020; Bodino et al., 2021b; Mesmin et al., 2022). *Philaenus spumarius* nymphs, upon hatching, are orange, and during their development, the colour becomes gradually greenish yellow (Yurtsever, 2000). In contrast, *N. campestris* nymphs are typically pale yellow, with distinctive dark patches on the wing buds (EPPO, 2019c) (Figure 1.4 A and C). The adults start to emerge between May and July (Bodino et al., 2021b; Antonatos et al., 2021) (Figure 1.5). Adult length can vary between 5-7 millimetres, and generally, females are larger than males (EPPO, 2019c). Adults of *P. spumarius* have a high polymorphism (about 20 different colours are known) (Yurtsever, 2000). They are usually yellowish, brownish, or black, with brighter patches on a dark background, but they can also have dark markings on a lighter background (Yurtsever, 2000). *Neophilaenus campestris* adults

are greyish yellow to greyish brown, sometimes with a reddish undertone, generally showing a longitudinal strip extending from the vertex towards the scutellum (EPPO, 2019c) (Figure 1.4 C and D).

Figure 1.4. Nymphs: (A) *Philaenus spumarius* and (B) *Neophilaenus campestris*. Adults: (C) *Philaenus spumarius* and (D) *Neophilaenus campestris*.

Mating occurs after adult appearance and continues throughout the seasons; however, females have ovarian parapause, so egg maturation is delayed until late summer (Witsack, 1973). During late spring, due to mowing or a decrease in the succulence of herbaceous plants, they move to the canopy of the main crop plant or other evergreen or deciduous trees and shrubs (Figure 1.5) (Morente et al., 2018; Cornara et al., 2019; Dongiovanni et al., 2019; Bodino et al., 2021b). In autumn, adults return to the ground cover vegetation where females oviposit in plant debris. Eggs are laid in masses attached and covered by a cement substance (Figure 1.5) (Weaver, 1951; Whittaker, 1973; Yurtsever, 2000). The eggs are ovoid and elongated with about 1 mm long and 0.35 mm wide (Yurtsever, 2000). After oviposition, they are yellowish white and have a dark orange pigmented spot in the shell at one end; with time, the spot gets bigger and

black-coloured, developing in a lid (Yurtsever, 2000). One female can lay up to 350-400 eggs in her lifetime (Yurtsever et al., 2010).

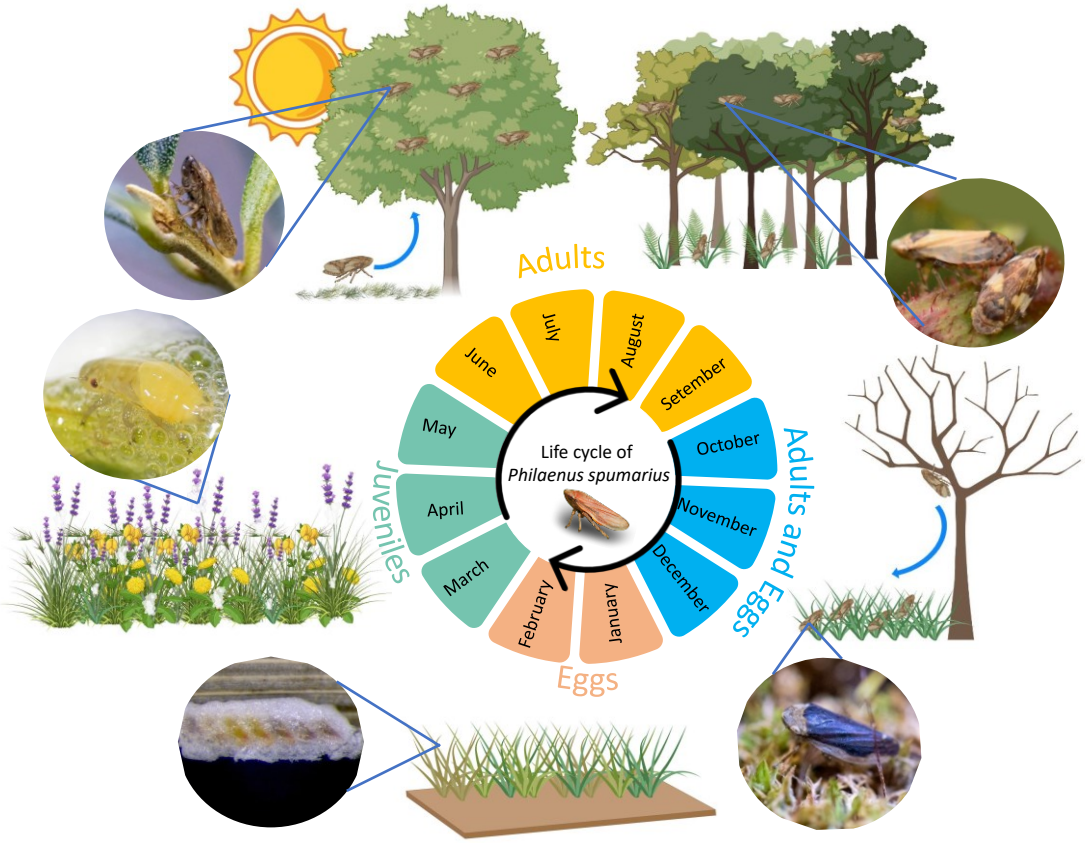


Figure 1.5. A typical life cycle of Aphrophoridae species, the life cycle of *Philaenus spumarius*, is used as a model.

Host plant preference

Philaenus spumarius and *N. campestris* are obligated xylem-feeders during nymphal and adult stages, being able to feed on a wide range of host plants (Weaver & King, 1954; Yurtsever, 2000; Bodino et al., 2020). Despite being polygraphs, they may show preferences for some plant species. The insect's ability to detect and explore the available host plants is related to various stimuli, such as chemicals, visual, and acoustic signals (Finch & Collier, 2008).

Nymphs of *P. spumarius* are mostly found on plant species from families Asteraceae, Fabaceae, Apiaceae, Geraniaceae and Asparagaceae (Morente et al., 2018; Dongiovanni et al., 2019; Villa et al., 2020; Bodino et al., 2021b), whereas nymphs of *N. camprestris* are mostly found in monocotyledonous plants, mainly from the Poaceae family (Weaver & King, 1954; Morente et al., 2018; Dongiovanni et al., 2019; Villa et al., 2020; Bodino et al., 2021b).

Regarding adults, electrophysiological assays demonstrated that *P. spumarius* and *N. campestris* respond to volatile organic compounds (Anastasaki et al., 2021; Germinara et al., 2017). Cascone et al. (2022) reported that females of *P. spumarius* were attracted by the olive cultivars Ogliarola, Rotondella, and Frantoio, and repelled by FS-17, while males did not present any olfactory response. The results of these studies support that, although these vectors have significantly fewer antennal sensilla than other species (Ranieri et al., 2016), they can resort to semiochemical cues to locate and choose host plants. The volatile organic compounds (VOCs) produced by plants can guide the insects towards suitable host plants for feeding or oviposition (Finch & Collier, 2008). Adults of *P. spumarius* can feed on olive and vine trees and other plants of economic importance. Adults of *N. camprestris* can be found on conifers (*Pinus* spp.) during the estivation period, and Cornara et al. (2021a) described that this insect prefers to feed on vines and *Bromus madritensis* L. (Poaceae) than on olive trees.

Geographic distribution, movement, and dispersal behaviour

The geographical distribution of Cercopidae is wide, being found in different latitudes and altitudes of the Holarctic region, from North America to Japan, passing through Europe and Russia (Morente & Fereres, 2017). *Philaenus spumarius* and *N. campestris* are widely distributed *in the* Mediterranean region (Ben Moussa et al., 2016; Tsagkarakis et al., 2018; Dongiovanni et al., 2019; Villa et al., 2020; Bodino et al., 2021b; Antonatos et al., 2021).

Like other animal species, these vectors generally use locomotion to feed, avoid predators, and interact socially (Wilson et al., 2015). Therefore, locomotion is considered an important element of survival (Dickinson et al., 2000) and the main way to spread insect-borne plant pathogens throughout the landscape (Finke, 2012). Nymphs of *P. spumarius* and *N. campestri*s move exclusively by walking; however, their mobility is limited since they only displace short walks when selecting the host plant (Dongiovanni et al., 2019). On the contrary, adults are more mobile, as they can move by flying, jumping, and walking (Burrows, 2003, 2007).

Philaenus spumarius and *N. campestris* adults are poor fliers, opting to walk or jump more often instead of flying (Cornara et al., 2018; Casarin et al., 2023). Indeed, these insects' bodies are designed essentially for jumping, and they have long hind legs to increase leverage for jumping, wedge-shaped heads and rigid front wings that form a continuous smooth structure to reduce drag when jumping (Burrows, 2003, 2007). During horizontal movements, these insects walk with the hind legs, usually dragged (Burrows, 2003, 2006).

Weaver & King (1954) reported that *P. spumarius* can travel more than 30 m in a single flight and move as much as 100 m within 24 hours from the release point, whereas Bodino et al.

(2021a) found that individuals can disperse up to 400 m during the population peak in Italy. Furthermore, Lago et al. (2021a), using a flight mill, concluded that spittlebugs could move up to 500 m in a 30 min flight. Casarin et al. (2023) estimated that the mean daily dispersal of *P. spumarius* was 1.5 m and also reported that this insect could fly 1.54 km in 2.5 h in a flight mill. *Philaenus spumarius* can also jump up to 70 cm above the ground with an acceleration of 400 m/s^2 (Burrows, 2003).

Regarding *N. campestris*, Lago et al. (2021b) reported that these insects can fly long distances, reaching almost 1.4 km in an 82-minute single flight. Additionally, this insect can disperse a maximum distance of 2473 m in 35 days from the release point to areas dominated by pine trees.

Philaenus spumarius and *N. campestris* typically have migratory behaviour; as mentioned before, several authors described that these insects start their migratory journey at the end of spring- early summer, coinciding with the death of the vegetation cover, until September, returning to the vegetation cover to lay eggs (Cruaud et al., 2018; Morente et al., 2018; Antonatos et al., 2021).

Cicadellidae family (subfamily: Cicadellinae)

The subfamily Cicadellinae is a highly diverse group, with more than 2600 species described worldwide (Keil & Lozada, 2021). Some species of Cicadellinae are pests in agriculture (Day & Fletcher, 1994), while many of them also act as a vector of viruses, phytoplasmas (Stiller, 2009), and plant diseases (Nielson, 1968). Most studies on the biology and ecology of these insects have been carried out in the American continent, where these individuals are abundant and diverse (Hopkins & Purcell, 2002; Overall & Rebek, 2017; Krugner et al., 2019). In Europe, information about these individuals' biological and ecological features is still very incipient. *Cicadella viridis* is known as the most abundant and widely dispersed Cicadellinae in Europe and is one of the few Cicadellinae identified as a potential vector of *X. fastidiosa* (Bodino et al., 2022). Therefore, this subfamily's biological and ecological features were focused only on *C. viridis.*

Biological cycle

Cicadella viridis can have one or more generations per year. In the coldest parts of Europe, it has only one generation per year reported (Beok, 1972). Nevertheless, in warmer regions, it can have present two or three generations. According to our personal observations, in north-eastern Portugal *C. viridis* probably has two generations per year (Figure 1.6). The

overwintered eggs hatch in April, starting the first generation (Hasbroucq et al., 2020). The nymphs are yellowish with two brownish stripes that start on the head and run down to the abdominal end (Shah et al., 2019). They pass through five nymphal stages; the nymph's development can take between 49 and 62 days (Beok, 1972). The nymphs are motile and have no protective foam (Hasbroucq et al., 2020) (Figure 1.6).

Between May and June, the first adults start to appear (Figure 1.6); males emerge one week earlier than females, reaching sexual maturity three weeks after emergence (Beok, 1972). Males are smaller than females, ranging from 5-7mm, while adult females range from 7-9mm (Shah et al., 2019), and the adult body is purplish and less commonly green in colour (Beok, 1972), the Scutellum and Pronotum are yellow and green, the front side of the head is pale yellow, and near the compound eyes, two black spots are visible (Shah et al., 2019).

Oviposition occurs seven weeks after the emergence of the females; they deposit their eggs in the grasses (Figure 1.6) (Hasbroucq et al., 2020). Females insert their ovipositor into the stem of plants, where they make long downward slits as eggs are laid (Beok, 1972). The eggs are white and cylindrical, measuring between 1.57 mm and 1.80 m (Hasbroucq et al., 2020). One female can lay an average of 52 eggs in her lifetime (Beok, 1972).

The second generation emerges in August (Figure 1.6) (Hasbroucq et al., 2020). Females of the second generation lay their eggs on grasses and woody trees, frequently causing damage to trees of economic importance (Beok, 1972). Finally, in November, all the adults die due to frost, thus ending the second generation (Beok, 1972).

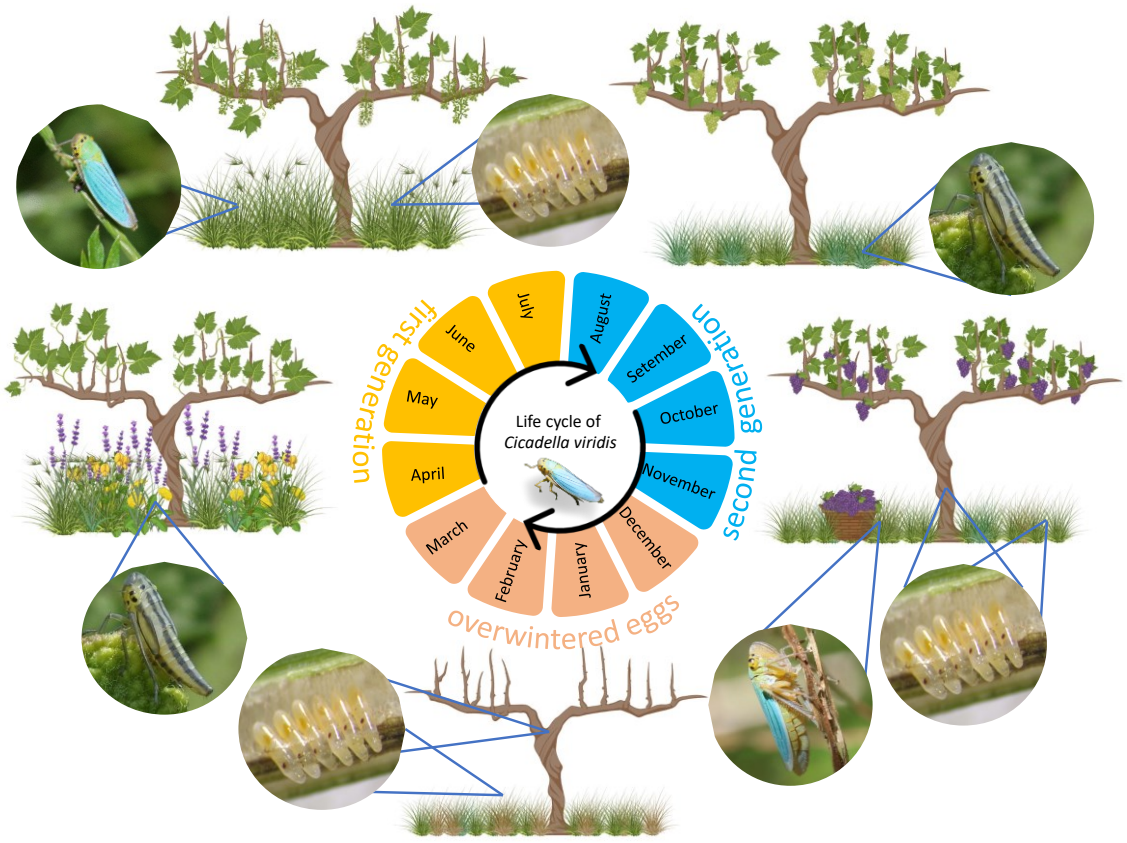


Figure 1.6. Life cycle of *Cicadella viridis*.

Host plant preference

In general, species of Cicadellidae detect suitable host plants based mainly on visual cues or with the combination of olfactory and visual stimuli (Todd et al., 1990; Bullas-Appleton et al., 2004; Cai et al., 2015; Grange et al., 2017). However, information regarding how *C. viridis* chose its host plant is non-existent.

Cicadella viridis is a polyphagous insect that feeds on a large variety of herbaceous plants with a preference for grasses, rushes, and short herbaceous plants (Nickel & Remane, 2002). Nymphs migrate from trees on which they hatch to fields of grasses where they feed and shelter at the bases of plants such as *Polygonum fagopyrum* L., *Phragmites* spp., *Cyperus* spp., and *Juncus* spp. (Cornara et al., 2019). Adults have also been reported to feed on these plants. This species has a different requirement for food and oviposition. Females oviposit on various host plants, including the vine, apple, pear, and peach trees (Nickel & Remane, 2002), whereas Bodino et al. (2022) reported that *C. viridis* is unable to feed on olives.

Geographic distribution, movement, and dispersal behaviour

Cicadella viridis usually lives in humid places like grassy areas, near marshes, and wet meadows and can also be found in dry regions (Shah et al., 2019). This insect is present in East Palaearctic, Europe Near East, the Nearctic region, and the Oriental region, there are no records of this insect on the American continent (Morente & Fereres, 2017).

Like *P. spumarius*, *C. viridis* can also move around by flying, jumping, and walking. *Cicadella viridis*, adults move through the landscape, in response to visual or olfactory stimuli, by jumping or performing short flights (Beok, 1972). On average, *C. viridis* can perform jumps with a take-off velocity of 0.88 m/s and a constant acceleration of 152 m/s^2 (Bonsignori et al., 2013). Compared with *P. spumarius* (Burrows, 2003, 2007), *C. viridis*' jump is slower, which allows the insect to conserve energy (Bonsignori et al., 2013). Regarding flight capacity, Beok (1972) reported that, on average, females of *C. viridis* can fly for four minutes straight, while male flights lasted less than four seconds. However, this author also described that their flight capacity decreases as adults mature. Despite having good locomotion ability, *C. viridis* movements are trivial and confined within the habitat; individuals have been observed to remain in the same spot for days (Beok, 1972).

1.3.4. Vector control strategies

Currently, there is no cure for *X. fastidiosa*. It is expected that if proper prevention and control measures are not implemented, the continuous spread of *X. fastidiosa* over 50 years could cause economic damage higher than 1.9 billion Euros in Europe (Schneider et al., 2020). Therefore, vector control is perceived as the main tool to limit the spread of this bacterium (Schneider et al., 2020).

Previous studies showed that vector control measures based on chemical strategies can significantly reduce the population of *P. spumarius* (*e.g.,* Dongiovanni et al., 2018a,b; Dáder et al., 2019). Furthermore, Lago et al. (2022) reported that Pyrethrin, a potent insecticide targeting the nervous systems of insects, was able to reduce *X. fastidiosa* transmission. However, the extensive use of pesticides in agriculture can pose serious risks to biodiversity, ecosystem services, and human health (Sharma et al., 2019). Therefore, sustainable, and ecological approaches are needed as chemical control alternatives.

Cultural practices could also potentially help reduce vector activity and population density. Sanna et al. (2021) reported that tillage practices can reduce *P. spumarius* density by 60%, while frequent mowing can reduce the density by 20%. López-Mercadal et al. (2022) also reported that tillage and mowing significantly decreased the nymphal density of *X. fastidiosa* vectors in the olive grove and vineyards. The negative effect of tillage on the population of *P. spumarius* was related to the high direct mortality of juveniles combined with the complete removal of the herbaceous vegetation (Sanna et al., 2021). However, cultural practices such as tillage can have stronger negative environmental impacts (Karamahouna et al., 2019). The vegetation cover provides shelter and food to a wide range of beneficial fauna that performs essential ecosystem services in the agroecosystems, such as pollination, decomposition, regulation of the nutrient cycle, and control of pests and diseases (Silva et al., 2010). Moreover, tillage can cause unsustainable rates of soil loss, especially on steep slopes (Sastre et al., 2017).

Trap plants can be useful alternatives to aggressive cultural practices. This practice involves growing plant species that are highly attractive to the pest and different from the main crop to attract the target pest and reduce the pest population. Therefore, identifying plant species that are preferred and less preferred for *P. spumarius* is essential to designing cover crops or field margins to reduce *P. spumarius* populations. In a study by Morente et al. (2022), several plants that are suitable for oviposition and nymphal development and plants that are lethal to nymphs were reported. These authors described that *Anthriscus cerefolium* is a suitable plant for oviposition but can be lethal for nymphs of *P. spumarius.* Moreover, nymphs and adults of *P. spumarius* were attracted to *Taraxacum officinale* and *Lavandula angustifolia*, suggesting that these two species should be avoided in the ground cover vegetation.

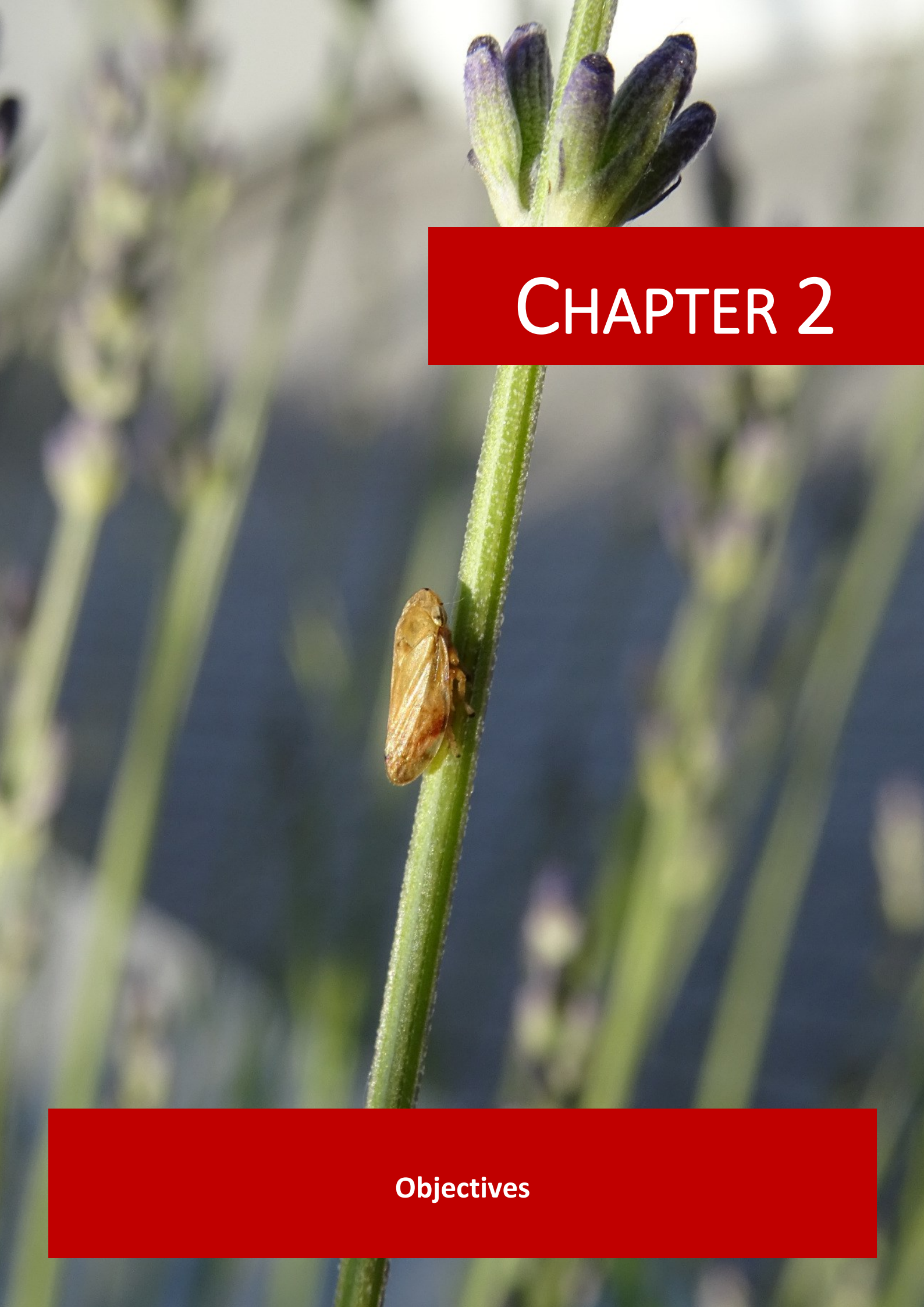
Additionally, identifying the VOCs emitted by plants and disentangling their role in the response of insect vectors can also contribute to the implementation of approaches to manipulate the behaviour of these insects and implement sustainable control strategies such as the push–pull (Cook et al., 2007), lure-and-kill (El-Sayed et al., 2009) or attract-and-kill (Gregg et al., 2018). A study in behavioural responses showed that different aromatic plants and their essential oils could elicit a repellent or attractive behaviour in *P. spumarius* adults (Ganassi et al., 2020).

Philaenus spumarius is known to interact socially via substrate-borne vibrations; therefore, vibrational disturbance can change the behaviour of insects (Avosani et al., 2020). Avosani et al. (2021) showed that the transmission over a suitable host plant of a vibrational

stimulus, designed based on a vibrational signal used by *P.spumarius* for intra-specific communication, significantly affects the probing and feeding behaviour of the insect. However, the authors described that their results could raise numerous further experimental questions, such as: what signal features are responsible for the feeding impairment, how the synthetic interference signal could be transmitted to olive trees and if the synthetic interference signal could reduce *X. fastidiosa* acquisition and/or inoculation. Furthermore, Avosani et al. (2022), demonstrated that frequency-modulated and continuous vibrational noises can interfere with crucial insect behaviours such as mating.

Regarding biological control, some reports show that birds, frogs, arachnids, and other insect species are reported to occasionally feed on *P. spumarius* (Halkka et al., 1967; Phillipson, 1960; Harper & Whittaker, 1976; Benhadi-Marín et al., 2020). In 2020, Liccardo et al. proposed the use of *Zelus renardii* (Kolenati) (Hemiptera: Reduviidae) to control *P. spumarius* populations. However, this insect is considered an alien species and a generalist predator that could put a risk the arthropod biodiversity, including beneficial individuals. Some parasitoids have also been reported to parasite *P. spumarius*. *Ooctonus vulgatus* (Haliday), (Hymenoptera: Mymaridae), and some species of the genus *Centrodora* and *Tumidiscapu* can parasite *P. spumarius* at the egg stage (Weaver & King, 1954; Hayat, 1983; Mesmin et al., 2020). Moreover, *Verralia aucta* (Fallen) *(Diptera: Pipunculidae* can parasite adults of *P. spumarius* (Whittaker, 1973). Various parasitoids have also been reported in *C. viridis* eggs, namely *Polynema woodi*, *Gonatocerus longicornis,* and *Anagrus incarnatus* (Hasbroucq et al., 2020; Arzone, 1972).

Generalist predators, such as spiders, can also play a significant role in the biological control of *P. spumarius*. Spiders are one of the most abundant and diverse arthropod orders and are considered one of the most important groups of natural insect enemies (Nyffeler, 2000; Nyffeler & Birkhofer, 2017). Although there are already reports of spiders preying on *P. spumarius,* knowledge about its antagonist's guild is scarce and outdated (Phillipson, 1960; Harper & Whittaker, 1976). Benhadi-Marín et al. (2020) proposed a guild-based protocol to target spiders as potential natural enemies of *P. spumarius.* In this study, Benhadi-Marín highlights the importance of some spiders as potential predators of *P.* spumarius. However, not always prey choice by predators in field conditions can sometimes be established by using direct observation.



CHAPTER 2

Objectives

2.1. General objectives

The emergence of *X. fastidiosa* in Europe and its presence throughout other European countries, such as France, Spain, and Portugal, has greatly increased awareness of this pathogen's threat to European agriculture. Indeed, this bacterium infects the xylem tissues of a wide range of agriculturally important crops, including the vineyard, citrus, almond and olive, causing high losses. Upon its introduction, the only route for the natural spread of *X. fastidiosa* is by insect vectors, particularly xylem sap-feeding of the infraorder Cicadomorpha, Superfamilies Cercopoidea (families: Aphrophoridae and Cercopidae) and Cicadoidea, and the family Cicadellidae (subfamily: Cicadellinae).

Currently, available control methods against this deadly and highly invasive pathogen are ineffective. Until now, knowledge about the bacterium's vectors and potential vectors in Portugal is scarce. Also, the response of the European vectors or potential vectors to different Organic Volatile Compounds (VOCs) stimulus is also incipient. These VOCs could influence insect behaviours and cultivar preference, which is particularly important for traditional olive cultivars produced under sustainable production systems. Sustainable olive groves are rich agroecosystems in biodiversity; nevertheless, the role of potential predators in suppressing *X. fastidiosa* vectors is unknown.

In this context, the general objective of this thesis was to study aspects of the diversity, abundance of vectors and potential vectors in different agroecosystems (vineyards, citrus, almond, and olive groves) and in the field margins of olive groves (scrublands). As well as to clarify the role of VOCs and traditional olive cultivars on the olfactory behaviour of vectors or potential vectors, and potential of generalist predators on the natural limitation of confirmed vectors of *X. fastidiosa*.

With specific objectives were:

(1) Study the abundance, diversity, and richness of Cicadomorpha individuals in the canopy and inter-row vegetation of Portuguese vineyards;
(2) Study the abundance, diversity, and richness of the Cicadomorpha community in different agroecosystems: almond, olive and citrus groves, vineyards and scrublands;
(3) Assess the olfactory response of *Philaenus spumarius* and *Cicadella viridis* to VOCs emitted by the leaves of olive, almond, and vine plants;

(4) Estimate the movement parameters of *P. spumarius* and *C. viridis* displayed by walking;
(5) Assess the olfactory response of *P. spumarius* to five important traditional Portuguese olive cultivars;
(6) Design and test taxon-specific primers to apply a PCR-based diagnostic method to detect *P. spumarius* within predators.

2.2. Thesis structure

The thesis was structured in eight different chapters, respecting different matters, namely:

Chapter 1. A brief overview of *Xylella fastidiosa*, importance and distribution. A summary of the diversity of potential vectors and vectors of this pathogen, as well as its life cycle, host plant preference, geographic distribution, movement, dispersal behaviour and the respective control strategies.

Chapter 2. The general objectives and structure of the thesis were presented.

Chapter 3. In this chapter, the abundance, diversity, and richness of Cicadomorpha individuals were studied in the canopy and inter-row vegetation of 35 Portuguese vineyards over two consecutive years.

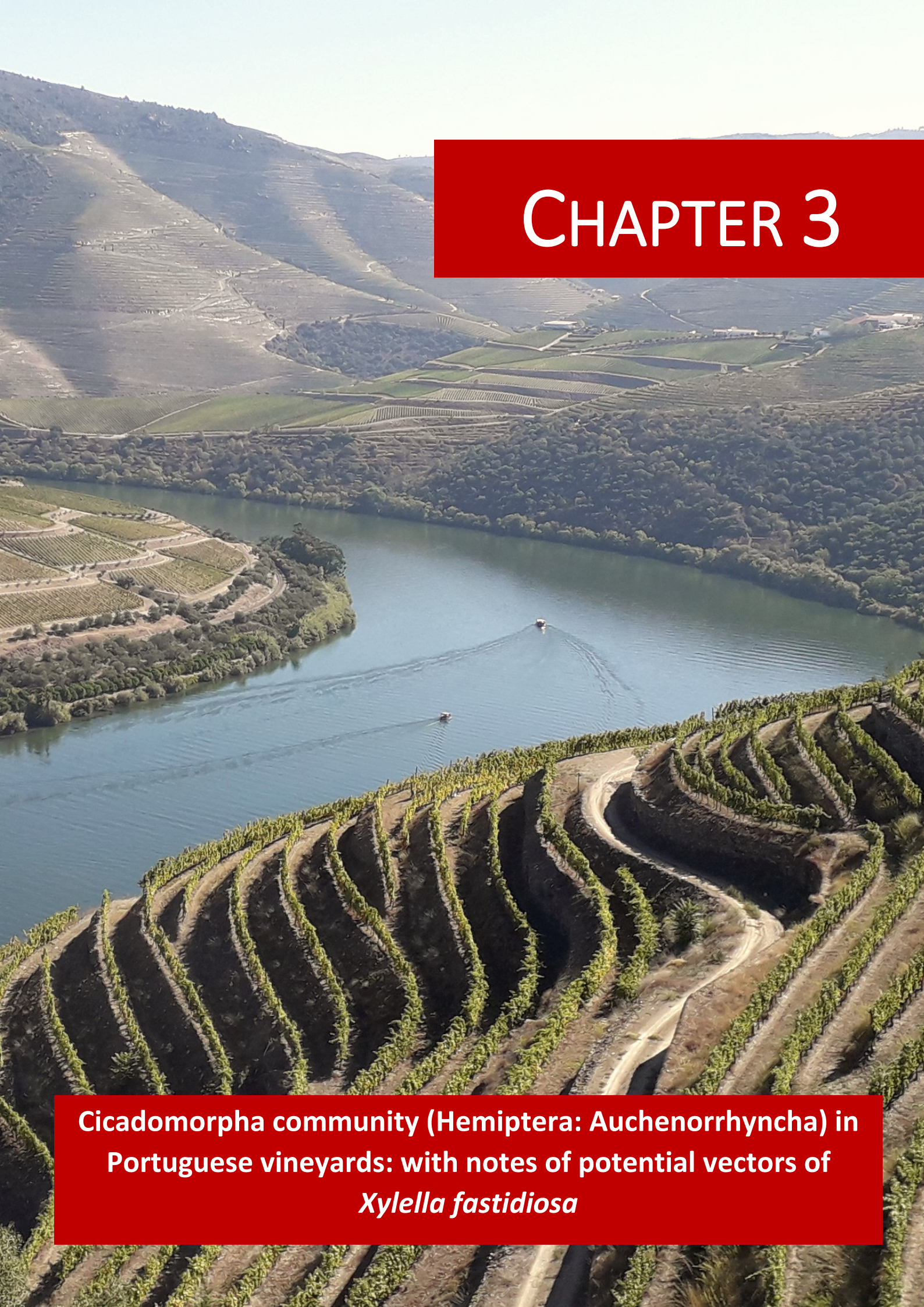
Chapter 4. The characterisation of the abundance, diversity, and richness of the Cicadomorpha community associated with almond, olive and citrus groves, vineyards and natural areas surrounding the olive groves in different sampling periods was presented in this chapter.

Chapter 5. In this chapter, the volatile profile of the olive, almond, and vine leaves, the most important crops in the Mediterranean region, was characterised, and the olfactory response of adults *Philaenus spumarius* and C*icadella viridis* to two VOCs common to the three crops (cis-3-hexenyl acetate and cis-3-hexen-1-ol) was studied. The movement parameters displayed by walking of both species' insects were also assessed.

Chapter 6. The olfactory response of the main vector of *Xylella fastidiosa*, *Philaenus spumarius*, to five traditional Portuguese olive cultivars: "Cobrançosa", "Negrinha de Freixo", "Santulhana", "Madural", and "Verdeal Transmontana" was assessed in Spring (after the emergence of the adults) and Autumn (end of the life cycle of the vector).

Chapter 7. In this chapter, several taxon-specific primers targeting the mitochondrial cytochrome oxidase I (COI) and cytochrome b (cytB) genes were designed and tested to be used for a PCR-based diagnostic method to detect *P. spumarius* within the spider *Xysticus acerbus* Thorell, 1872. Feeding experiments were performed to evaluate the effectiveness of this DNA-based diagnostic tool.

Chapter 8. The general conclusions of the work were presented.



Chapter 3

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in Portuguese vineyards: with notes of potential vectors of *Xylella fastidiosa*

Chapter 3

Adapted from:

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in Portuguese vineyards: with notes of potential vectors of *Xylella fastidiosa*

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Chapter 3

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in Portuguese vineyards: with notes of potential vectors of *Xylella fastidiosa*

Abstract

Cicadomorpha (Hemiptera) insects are currently responsible for a growing negative impact on the agricultural economy due to their ability to directly damage crops or through the capacity to act as vectors for plant pathogens. The phytopathogenic bacterium *Xylella fastidiosa*, the causal agent of the Pierce's disease in vineyards, is exclusively transmitted by insects of this infraorder. Therefore, knowledge of the Cicadomorpha species and understanding their biology and ecology is crucial. In this work, in 2018 and 2019, the canopy and inter-row vegetation of 35 vineyards distributed in mainland Portugal were sampled to investigate species composition, richness, and diversity of the Cicadomorpha community, giving a special focus to vectors and potential vectors of *X. fastidiosa*. A total of 11834 individuals were collected, 3003 in 2018 and 8831 in 2019. Of the 81 species/morphospecies identified, only five are considered vectors or potential vectors of this pathogen, namely, *Cicadella viridis* (Linnaeus, 1758), *Philaenus spumarius* (Linnaeus,1758), *Neophilaenus campestris* (Fallén, 1805), *Lepyronia coleoptrata* (Linnaeus, 1758), and *N. lineatus* (Linnaeus, 1758). *Cicadella viridis* was the most abundant xylem-sap feeder, followed by *P. spumarius*. In addition, Cicadomorpha that cause direct damage to vine and vectors of grapevine yellows' phytoplasmas were also collected and identified in the sampled vineyards. The results suggested that vectors and potential vectors of *X. fastidiosa* and a large proportion of the population of Cicadomorpha have a positive correction with inter-row vegetation.

Keywords: *Cicadella viridis*; Cicadellidae; inter-row vegetation; *Philaenus spumarius*; Pierce's disease

Chapter 3

3.1. Introduction

Viticulture is an important agricultural, environmental, cultural, and economic driving force in the Mediterranean basin. In Portugal, viticulture is an activity of great economic importance, with the vine being cultivated throughout the national territory (Fraga et al., 2017). Unfortunately, this crop, like others, is subject to pests and diseases that threaten production, quality, and wine typicity (Chuche & Thiéry, 2014; Decante & van Helden, 2016; Kyrkou et al., 2018; Sharma et al., 2018).

Cicadomorpha (Hemiptera: Auchenorrhyncha) is one of the richest and phyletically diverse infraorder of Hemiptera, with over 30000 species described worldwide (Dietrich, 2002). This infraorder includes exclusively phytophagous species that feed on mesophyll, xylem, or phloem sap (Raven, 1983). Although most Cicadomorpha do not present a threat to the crops, the infraorder includes several species regarded as economically important pests. These species can damage plants directly through feeding (Decante & van Helden, 2006; Atakan, 2009; Backus, 1988; Scott et al., 2020) and indirectly through the transmission of plant pathogens (Chuche & Thiéry, 2014; Kyrkou et al., 2018; Nielson, 1968; Redak et al., 2004).

When some individuals of the Cicadomorpha feed, their saliva can induce obstructions on plant vascular tissues leading to deformations, a discoloration of leaves, or even premature death of plants (Fornasiero et al., 2016). In vineyards, the green leafhoppers *Jacobiasca lybica* (Bergevim and Zanon, 1922) (Cicadellidae: Typhlocybinae) and *Empoasca vitis* (Göeth, 1875) (Cicadellidae: Typhlocybinae) are considered key pests due to the direct damage caused when feeding (Fornasiero et al., 2016; Román et al., 2021). Cicadomorpha is among the most significant groups of vectors of plant pathogens (Chuche & Thiéry, 2014; Cornara et al., 2019; Ivanauskas et al., 2014; Swisher et al., 2018). Cicadomorpha are considered vectors and potential vectors of two serious plant pathogens in vineyards: i) the causal agent of the Flavescence dorée of the vineyard, the phytoplasma '*Candidatus* Phytoplasma vitis', transmitted exclusively by the Cicadellidae *Scaphoideus titanus* Ball, 1932 (Chuche & Thiéry, 2014), and ii) the xylem-limited bacterium *Xylella fastidiosa* (Wells et al., 1987) (Gammaproteobacteria: Xanthomonadaceae), responsible for Pierce's disease (Hopkins & Purcell, 2002). This bacterium is transmitted exclusively by xylem-sap feeders of the infraorder Cicadomorpha, being the subfamily Cicadellinae (Cicadellidae) and the families Aphrophoridae and Cercopidae, the main groups of potential vectors (Redak et al., 2004; Cornara et al., 2019). In Europe, the spittlebugs, *Philaenus spumarius* (Linnaeus, 1758) (Cicadomorpha: Aphrophoridae), *P. italosigmus*

Drosopoulos and Remane (2000) (Cicadomorpha: Aphrophoridae), and the *Neophilaenus camprestris* (Fallen, 1805) (Cicadomorpha: Aphrophoridae), are confirmed vectors of this pathogen (Cavalieri et al., 2019).

Xylella fastidiosa is a plant endophyte native to the Americas (Almeida & Nunney, 2015; Janse & Obradovic, 2010), which is currently responsible for economic losses in the Californian wine sector of around 92 million euros per year (Tumber et al., 2014). In Europe, despite a sporadic and unconfirmed report of symptoms of *X. fastidiosa* in vineyards in 1997 (Berisha et al., 1998), the bacterium was declared absent on the continent until 2013 (Saponari et al., 2013). The first official widespread detection of *X. fastidiosa* was reported in the Lecce Region of Apulia, Italy, where bacteria have already decimated thousands of olive trees (Saponari et al., 2019). Since this first report, the bacterium has spread to other European countries. Outbreaks have been reported in France, Germany (outbreak eradicated), Spain, and Portugal (EPPO, 2022). In Portugal, the fastidious bacterium was detected in January 2019, in Vila Nova de Gaia, in lavender plants (*Lavandula dentata* Linnaeus) (DGAV, 2021a). More recently, new outbreaks were reported in other regions of the country (DGAV, 2022d, 2023). Since there is no cure for the bacterium, detailed knowledge of the abundance and diversity of potential vectors of *X. fastidiosa* and the remaining adult community of Cicadomorpha in the Portuguese agroecosystems is the first step in preventing diseases or minimising its potential effects.

With this in mind, the present work is dedicated to studying the Cicadomorpha community, focusing on the vectors and potential vectors of *X. fastidiosa* in the canopy and in the inter-row vegetation of Portuguese vineyards.

3.2. Materials and methods

3.2.1. Study area

The study was conducted for two consecutive years, 2018 and 2019, in 35 vineyards (20 vineyards in both years and an additional 15 in the second year) distributed in mainland Portugal (Table S3.1). All vineyards were under sustainable producing systems (integrated or organic), and the inter-rows vegetation was maintained during the sampling periods. Additional information regarding the vineyards' sampling dates and features can be found in

Supplementary Table S3.1. Each vineyard was surveyed in three different periods: late spring, summer, and autumn.

3.2.2. Collection and identification of insects

In each vineyard, Cicadomorpha adults were sampled in the inter-row and the canopy of the vines with a standard entomological sweep net (38 cm). In the inter-row of the vineyards, ten samples of 10 consecutive sweepings randomly distributed over 1 ha were collected. For the canopy, ten samples of 50 successive sweepings were collected. The content of the sweepings was emptied into a plastic bag properly labelled and sealed. Arthropods were sorted under a stereoscopic microscope and conserved in 96% ethanol until further identification. All the adults of the infraorder Cicadomorpha collected were identified. For species identification, the male genitalia was dissected and placed in a heated solution of 10% potassium hydroxide (KOH) at between 20 seconds and 3 minutes, depending on the sclerotisation of each specimen. Subsequently, each genitalia was mounted in glass slides with glycerine and observed under a stereoscopic microscope. The taxonomic classification was based on appropriate keys and illustrations (Nielson, 1968; Biedermann & Niedringhaus, 2009; Dietrich, 2005; Dmitry, 2006; Le Quesne & Payne, 1981). Females were identified to the lowest possible taxonomic level. If all males of a genus in a specific sample belonged to one species, then females of that same genus were considered to be that species. If there were more than one species in a particular genus, females belonging to that genus were identified as morphospecies and designated by genus or subfamily followed by "sp." and a number according to the morphotype (*e.g., Psammotettix* sp.1 or Deltocephalinae sp.1).

3.2.3. Data analysis

The community structure was evaluated in terms of the abundance, richness, and diversity of species/morphospecies. The data for each year of the study, 2018 and 2019, were treated independently to avoid bias from the interannual variability. All the statistical analyses were performed in the R software (R Core Team, 2020). The mean and the total number of individuals captured by stratum (inter-row and canopy of the vines) and sampling year were described. The specific richness and two diversity indices (Shannon-Wiener Diversity Index (H') and Pielou Equitability Index (J')) were determined using the "vegan" package (Oksanen et al., 2019). The specific richness was calculated as the number of species/morphospecies in each

vineyard. The Shannon-Wiener index (H') is the most used index, and it gives greater importance to rare species (Magurran, 1988), while the Pielou Equability Index (J') is derived from the Shannon diversity index and allows the representation of a uniform distribution of individuals among existing species (Pielou, 1966). To analyse the effect of the sampled stratum in the Cicadomorpha community, a permutational multivariate analysis of variance (PERMANOVA) was performed using the function adonis2 from the package "vegan". To assess the sampling effort, species accumulation curves were drawn in function of the number of vineyards sampled per stratum. Species accumulation curves were computed using the specaccum function of the "vegan" package. Additionally, a co-inertia analysis (CIA) was performed to determine the relationship between Cicadomorpha species/morphospecies and the year of sampling and stratum. This analysis was performed using the "ade4" package and the *table.value* function to visualize the results.

3.3. Results

In total, 11834 individuals were collected, of which 3003 in 2018 and 8831 in 2019 (Table 3.1). Over the two years of study, 81 species/morphospecies were identified. *Psammotettix* sp.1 (3314 individuals), *E. vitis* (2866 individuals), and *Zyginidia scutellaris* (Herrich-Schäffer, 1838) (1079 individuals) were the most abundant species/morphospecies.

In the canopy of the vines, a total of 4262 individuals were recovered (987 in 2018 and 3275 in 2019). The population was dominated by individuals of the subfamily Typhlocybinae, which represents 92% of the total recovered in the canopy of the vines.

In the inter-row vegetation, 7572 individuals were recovered (2016 in 2018 and 5556 in 2019). The inter-row vegetation was dominated by individuals of the subfamily Deltocephalinae, representing 68% of the total individuals captured in this stratum.

Concerning vectors and potential vectors of *X. fastidiosa*, five species were captured, namely: *Cicadella viridis* (Linnaeus, 1758) (307 individuals), *P. spumarius* (112 individuals), *N. campestris* (62 individuals), *Lepyronia coleoptrata* (Linnaeus, 1758), and *N. lineatus* (Linnaeus, 1758) (3 individuals) the highest abundance of individuals was observed in the inter-row vegetation in the year 2019.

Table 3.1. Mean and respective standard error (ME±SE) and total number (N) of Cicadomorpha adults collected in 2018 and 2019 in the vine canopy and inter-row vegetation.

Family	Subfamily		Species	2018				2019				Total		
				Canoppy		Inter-row vegetation		Canoppy		Inter-row vegetation				
				ME ± SE	N	ME ± SE	N	ME ± SE	N	ME ± SE	N	ME ± SE	N
Aphrophoridae													
		C1	*Lepyronia coleoptrata* (Linnaeus, 1758)	0.00 ± 0.00	0	0.03 ± 0.03	1	0.00 ± 0.00	0	0.17 ± 0.10	6	0.13 ± 0.07	7
		C2	*Neophilaenus campestris* (Fallén, 1805)	0.10 ± 0.07	2	0.10 ± 0.07	2	0.23 ± 0.11	8	1.43 ± 0.50	50	1.11 ± 0.34	62
		C3	*Neophilaenus lineatus* (Linnaeus, 1758)	0.00 ± 0.00	0	0.05 ± 0.05	1	0.00 ± 0.00	0	0.06 ± 0.04	2	0.05 ± 0.03	3
		C4	*Philaenus spumarius* (Linnaeus, 1758)	0.24 ± 0.10	5	1.29 ± 0.34	27	0.46 ± 0.16	16	1.83 ± 0.67	64	2.00 ± 0.45	112
Cicadellidae													
	Agalliinae	C5	*Agallia consobrina Curtis*, 1833	0.14 ± 0.10	3	0.05 ± 0.05	1	0.34 ± 0.20	12	0.26 ± 0.10	9	0.45 ± 0.15	25
		C6	*Agallia* sp.1	0.05 ± 0.05	1	0.00 ± 0.00	0	0.00 ± 0.00	0	0.09 ± 0.06	3	0.07 ± 0.04	4
		C7	*Anaceratagallia glabra Dmitriev, 2020* (*=A. laevis*)	0.14 ± 0.10	3	1.57 ± 0.80	33	0.46 ± 0.17	16	7.51 ± 1.67	263	5.63 ± 1.21	315
		C8	*Anaceratagallia* sp.1	0.10 ± 0.07	2	1.19 ± 0.31	25	0.14 ± 0.07	5	0.97 ± 0.40	34	1.18 ± 0.28	66
		C9	*Anaceratagallia venosa* (de Fourcroy, 1785)	0.00 ± 0.00	0	0.10 ± 0.10	2	0.23 ± 0.23	8	0.14 ± 0.08	5	0.27 ± 0.15	15
		C10	*Austroagallia sinuata* (Mulsant & Rey, 1855)	0.24 ± 0.15	5	0.86 ± 0.42	18	0.34 ± 0.14	12	2.40 ± 0.72	84	2.13 ± 0.55	119
		C11	*Dryodurgades antoniae* (Melichar, 1907)	0.05 ± 0.05	1	1.05 ± 0.61	22	0.09 ± 0.06	3	0.46 ± 0.22	16	0.75 ± 0.28	42
		C12	*Dryodurgades* sp.1	0.05 ± 0.05	1	0.00 ± 0.00	0	0.00 ± 0.00	0	0.14 ± 0.06	5	0.11 ± 0.04	6
	Aphrodinae												
		C13	*Anoscopus albifrons* (Linnaeus, 1758)	0.00 ± 0.00	0	0.05 ± 0.05	1	0.00 ± 0.00	0	0.00 ± 0.00	0	0.02 ± 0.02	1
		C14	*Aphrodes bicinctus* (Schrank, 1776)	0.00 ± 0.00	0	0.10 ± 0.07	2	0.00 ± 0.00	0	0.00 ± 0.00	0	0.04 ± 0.03	2
		C15	*Aphrodes makarovi Zachvatkin*, 1948	0.00 ± 0.00	0	0.10 ± 0.10	2	0.00 ± 0.00	0	0.03 ± 0.03	1	0.05 ± 0.04	3
		C16	*Aphrodes* sp.1	0.00 ± 0.00	0	0.10 ± 0.07	2	0.00 ± 0.00	0	0.14 ± 0.06	5	0.13 ± 0.04	7
		C17	*Aphrodes* sp.2	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.46 ± 0.25	16	0.29 ± 0.16	16

	C18	*Stroggylocephalus* sp.	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
Cicadellinae																						
	C19	*Cicadella viridis* (Linnaeus, 1758)																				
Deltocephalinae			0.19	±	0.11	4	0.43	±	0.18	9	0.06	±	0.04	2	8.34	±	4.95	292	5.48	±	3.12	307
	C20	*Arocephalus punctum* (Flor, 1861)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
	C21	*Arocephalus* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
	C22	*Artianus manderstjernii* (Kirschbaum, 1868)	0.00	±	0.00	0	0.05	±	0.05	1	0.00	±	0.00	0	0.51	±	0.30	18	0.34	±	0.19	19
	C23	*Artianus* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.11	±	0.09	4	0.07	±	0.06	4
	C24	*Athysanus argentarius Metcalf*, 1955	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.31	±	0.13	11	0.20	±	0.08	11
	C25	*Balclutha frontalis* (Ferrari, 1882)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.11	±	0.07	4	0.07	±	0.04	4
	C26	*Balclutha punctata* (Fabricius, 1775)	0.00	±	0.00	0	0.48	±	0.27	10	0.00	±	0.00	0	1.57	±	0.53	55	1.16	±	0.35	65
	C27	*Balclutha* sp.1	0.00	±	0.00	0	0.05	±	0.05	1	0.09	±	0.05	3	1.11	±	0.55	39	0.77	±	0.37	43
	C28	*Cicadula* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
	C29	*Circulifer* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.04	2	0.04	±	0.03	2
	C30	*Circulifer tenellus* (Baker, 1896)	0.00	±	0.00	0	0.48	±	0.31	10	0.00	±	0.00	0	0.00	±	0.00	0	0.18	±	0.12	10
	C31	*Cosmotettix panzeri* (Flor, 1861)	0.05	±	0.05	1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.02	1
	C32	*Doliotettix lunulatus* (Zetterstedt, 1838)	0.00	±	0.00	0	0.00	±	0.00	0	0.11	±	0.07	4	4.94	±	2.89	173	3.16	±	1.84	177
	C33	*Doratura homophyla* (Flor, 1861)	0.00	±	0.00	0	0.05	±	0.05	1	0.00	±	0.00	0	0.49	±	0.38	17	0.32	±	0.24	18
	C34	*Doratura stylata* (Boheman, 1847)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.06	2	0.04	±	0.04	2
	C35	*Enantiocephalus* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
	C36	*Euscelidius schenckii* (Kirschbaum, 1868)	0.00	±	0.00	0	0.05	±	0.05	1	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.38	1
	C37	*Euscelidius variegatus* (Kirschbaum, 1858)	0.00	±	0.00	0	0.38	±	0.10	8	0.06	±	0.04	2	1.14	±	0.57	40	0.89	±	0.02	50
	C38	*Euscelidius* sp.1	0.00	±	0.00	0	0.10	±	0.07	2	0.06	±	0.06	2	0.06	±	0.06	2	0.11	±	0.91	6
	C39	*Euscelis incisus* (Kirschbaum, 1858)	0.05	±	0.05	1	3.29	±	1.76	69	0.03	±	0.03	1	3.57	±	0.99	125	3.50	±	0.04	196
	C40	*Euscelis lineolatus Brullé*, 1832	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.06	2	0.04	±	0.08	2
	C41	*Euscelis ohausi W*.Wagner, 1939	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.23	±	0.11	8	0.16	±	0.61	9
	C42	*Euscelis* sp.1	0.05	±	0.05	1	1.57	±	0.82	33	0.20	±	0.09	7	2.09	±	0.80	73	2.04	±	4.42	114

	C43	*Exitianus capicola* (Stål, 1855)	0.86	±	0.67	18	17.48	±	9.46	367	0.06	±	0.06	2	9.46	±	3.70	331	12.82	±	0.41	718
	C44	*Exitianus* sp.1	0.00	±	0.00	0	1.62	±	0.99	34	0.00	±	0.00	0	0.40	±	0.26	14	0.86	±	0.21	48
	C45	*Goniagnathus brevis* (Herrich-Schäffer, 1835)	0.00	±	0.00	0	0.29	±	0.17	6	0.00	±	0.00	0	0.63	±	0.32	22	0.50	±	0.45	28
	C46	*Goniagnathus guttulinervis* (Kirschbaum, 1868)	0.00	±	0.00	0	0.57	±	0.31	12	0.03	±	0.03	1	2.17	±	0.68	76	1.59	±	0.10	89
	C47	*Goniagnathus* sp.1	0.05	±	0.05	1	0.05	±	0.05	1	0.00	±	0.00	0	0.29	±	0.16	10	0.21	±	0.02	12
	C48	*Hardya* sp.1	0.00	±	0.00	0	0.05	±	0.05	1	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.03	1
	C49	*Hardya tenuis* (Germar, 1821)	0.00	±	0.00	0	0.05	±	0.05	1	0.03	±	0.03	1	0.00	±	0.00	0	0.04	±	0.07	2
	C50	*Macrosteles alpinus* (Zetterstedt, 1828)	0.00	±	0.00	0	0.10	±	0.10	2	0.03	±	0.03	1	0.11	±	0.09	4	0.13	±	0.08	7
	C51	*Macrosteles sexnotatus* (Fallén, 1806)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.17	±	0.12	6	0.11	±	0.05	6
	C52	*Macrosteles* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.09	±	0.06	3	0.06	±	0.06	2	0.09	±	0.06	5
	C53	Deltocephalinae sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.77	±	0.77	27	0.48	±	0.48	27
	C54	*Neoaliturus fenestratus* (Herrich-Schäffer, 1834)	0.24	±	0.10	5	0.62	±	0.23	13	0.37	±	0.14	13	3.34	±	0.70	117	2.64	±	0.52	148
	C55	*Phlepsius ornatus* (Perris, 1857)	0.05	±	0.05	1	0.10	±	0.07	2	0.00	±	0.00	0	0.71	±	0.20	25	0.50	±	0.13	28
	C56	*Phlepsius* sp.1	0.00	±	0.00	0	0.24	±	0.14	5	0.03	±	0.03	1	0.06	±	0.06	2	0.14	±	0.06	8
	C57	*Platymetopius major* (Kirschbaum, 1868)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.06	2	0.04	±	0.04	2
	C58	*Psammotettix* sp.1	1.86	±	0.46	39	37.81	±	11.44	794	3.09	±	0.76	108	67.80	±	15.43	2373	59.18	±	10.79	3314
	C59	*Rhopalopyx vitripennis* (Flor, 1861)	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.63	±	0.23	22	0.39	±	0.15	22
	C60	*Sardius argus* (Marshall, 1866)	0.00	±	0.00	0	0.38	±	0.13	8	0.09	±	0.05	3	1.26	±	0.39	44	0.98	±	0.25	55
	C61	*Scaphoideus titanus Ball*, 1932	0.00	±	0.00	0	0.00	±	0.00	0	0.09	±	0.06	3	2.34	±	2.03	82	1.52	±	1.27	85
	C62	*Selenocephalus sacarroi Rodrigues*, 1968	0.00	±	0.00	0	0.05	±	0.05	1	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.02	1
	C63	*Sonronius binotatus* (Sahlberg, 1871)	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.03	±	0.03	1	0.04	±	0.04	2
	C64	*Stegelytra putoni Mulsant* & Rey, 1875	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.11	±	0.06	4	0.09	±	0.05	5
	C65	*Eupelix cuspidata* (Fabricius, 1775)	0.00	±	0.00	0	0.19	±	0.09	4	0.09	±	0.05	3	0.57	±	0.21	20	0.48	±	0.06	27
Idiocerinae																						
	C66	*Idiocerus lituratus* (Fallén, 1806)	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.00	±	0.00	0	0.02	±	0.02	1
	C67	*Idiocerus* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.14	±	0.12	5	0.03	±	0.03	1	0.11	±	0.05	6
Megophthalminae																						

	C68	*Megophthalmus scabripennis Edwards*, 1915	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
Typhlocybinae																						
	C69	*Alebra coryli Le* Quesne, 1977	0.00	±	0.00	0	0.00	±	0.00	0	0.63	±	0.63	22	0.06	±	0.06	2	0.43	±	0.39	24
	C70	*Arboridia* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.04	2	0.04	±	0.03	2
	C71	*Empoasca* sp.1	8.19	±	3.90	172	2.05	±	1.25	43	7.83	±	1.68	274	0.86	±	0.33	30	9.27	±	1.82	519
	C72	*Empoasca vitis* (Göthe, 1875)	18.38	±	6.07	386	6.29	±	2.50	132	59.97	±	17.18	2099	7.11	±	1.94	249		±	11.87	2866
	C73	*Eupteryx* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.14	±	0.07	5	0.14	±	0.12	5	0.18	±	0.10	10
	C74	*Fruticidia bisignata* (Mulsant & Rey, 1855)	0.00	±	0.00	0	0.00	±	0.00	0	0.11	±	0.05	4	0.00	±	0.00	0	0.07	±	0.03	4
	C75	*Jacobiasca lybica* (de Bergevin & Zanon, 1922)	13.76	±	7.37	289	0.00	±	0.00	0	13.29	±	12.12	465	0.26	±	0.19	9	13.63	±	8.06	763
	C76	*Ribautiana tenerrima* (Herrich-Schäffer, 1834)	0.00	±	0.00	0	0.00	±	0.00	0	0.46	±	0.21	16	0.06	±	0.06	2	0.32	±	0.15	18
	C77	*Zygina lunaris* (Mulsant & Rey, 1855)	0.05	±	0.05	1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.02	1
	C78	*Zygina ordinaria* (Ribaut, 1936)	0.00	±	0.00	0	0.00	±	0.00	0	0.14	±	0.06	5	0.03	±	0.03	1	0.11	±	0.05	6
	C79	*Zygina* sp.1	0.00	±	0.00	0	0.10	±	0.10	2	1.43	±	1.40	50	0.00	±	0.00	0	0.93	±	0.87	52
	C80	*Zyginidia scutellaris* (Herrich-Schäffer, 1838)	2.62	±	0.64	55	14.52	±	5.13	305	2.43	±	0.47	85	18.11	±	3.75	634	19.27	±	3.11	1079
Ulopinae																						
	C81	*Uteca* sp.1	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.03	1	0.02	±	0.02	1
Total			12.01	±	6.12	987	24.30	±	11.11	2016	39.46	±	25.97	2099	66.94	±	29.92	5556	146.10	±	65.13	11834

The specific richness and the Shannon Wiener Diversity Index (H') were significantly higher in the inter-row vegetation (Table 3.2). However, the Pielou Equitability Index (J') showed no significant differences between the canopy and inter-row vegetation, indicating a uniform species distribution (Table 3.2).

Table 3.2. Cicadomorpha richness and diversity indices for each stratum sampled per year. Mean Richness, Shannon Wiener Diversity Index (H'), and Pielou Equitability Index (J').

		2018	2019
Richness	Canopy	4.95 ± 0.5	6.43 ± 0.68
	Inter-row vegetation	9.62 ± 0.9	13.86 ± 0.10
	p-value	<0.001	<0.001
H'	Canopy	1.00 ± 0.10	1.01 ± 0.10
	Inter-row vegetation	1.44 ± 0.09	1.75 ± 0.11
	p-value	0.002	<0.001
J'	Canopy	1.48 ± 0.60	1.42 ± 0.41
	Inter-row vegetation	1.55 ± 0.40	1.60 ± 0.43
	p-value	0.64	0.14

According to the NMDS analysis based on the Bray-Curtis index (Figure 3.1) and the PERMANOVA analysis (df = 1; F=2; p = 0.001 for 2018, and df = 1; F=5; p = 0.001 for 2019), the sampling stratum significantly influences the Cicadomorpha community.

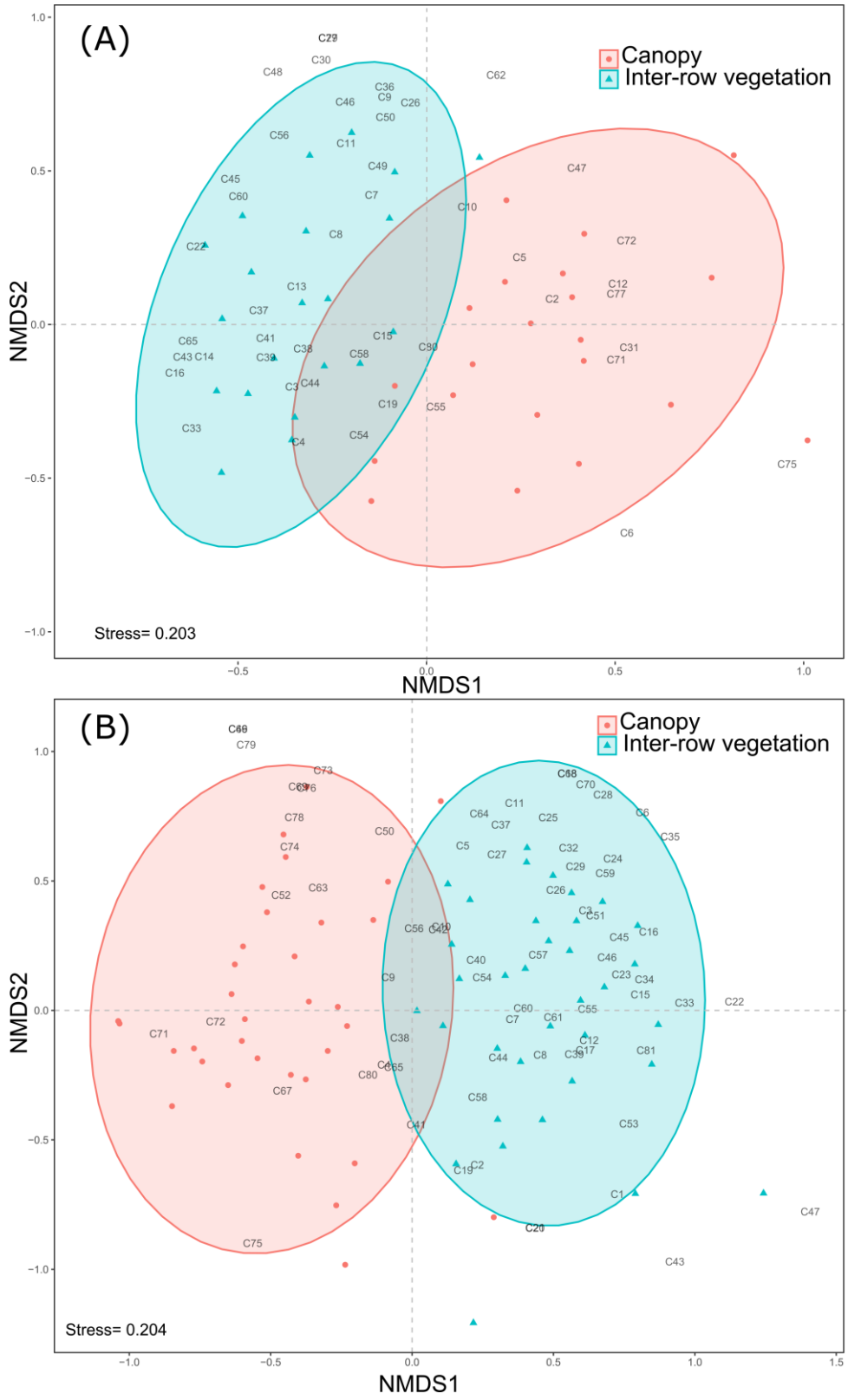

Figure 3.1. Non-metric multidimensional scaling (NMDS) analysis for Cicadomorpha abundance in the canopy and inter-row vegetation on (A) 2018 and (B) 2019. The numbers within the panels correspond to the numbers of the species/morphospecies in Table 3.1.

In both years and stratum, the species accumulation curves showed a tendency toward stabilisation (Figure 3.2), which indicates that the sampling effort was sufficient to detect most of the species of the Cicadomorpha community present in the vineyards.

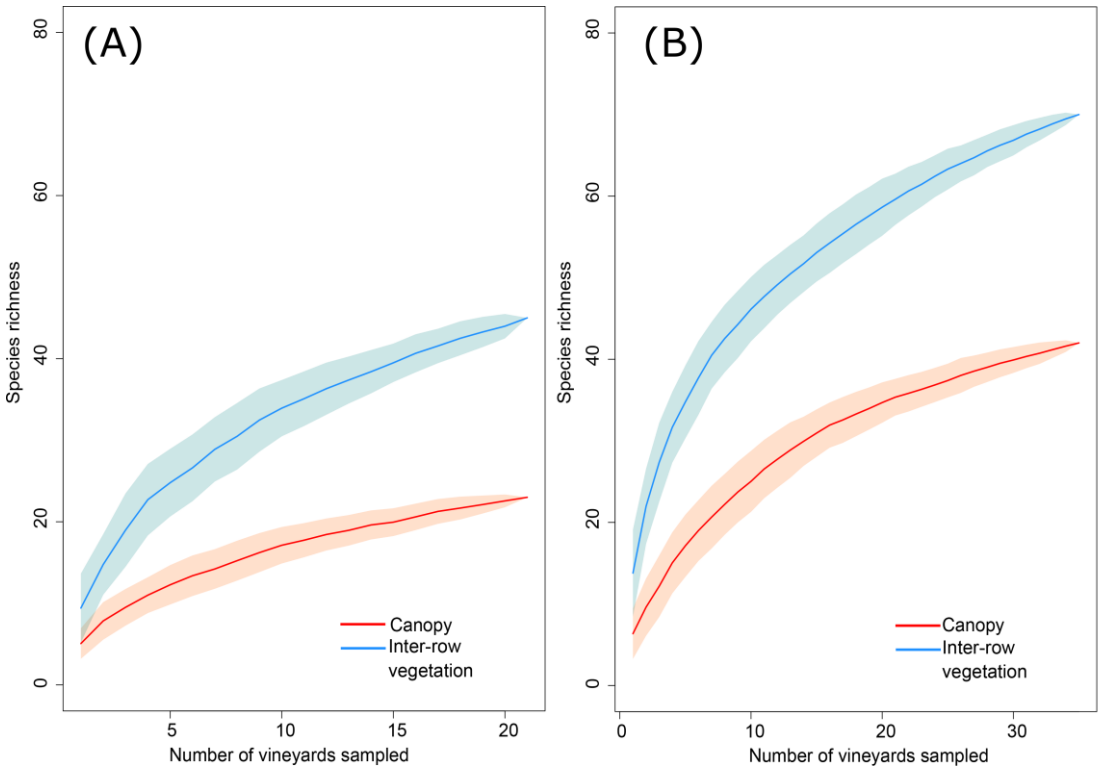

Figure 3.2. Species accumulation curves based on the number of vines sampled for (A) 2018 and (B) 2019 in the canopy and inter-row vegetation. The envelopes correspond to the 95% confidence interval.

The majority of the Cicadomorpha species, including vectors and potential vectors of *X. fastidiosa*, showed a positive correlation with inter-row vegetation (Figure 3.3).

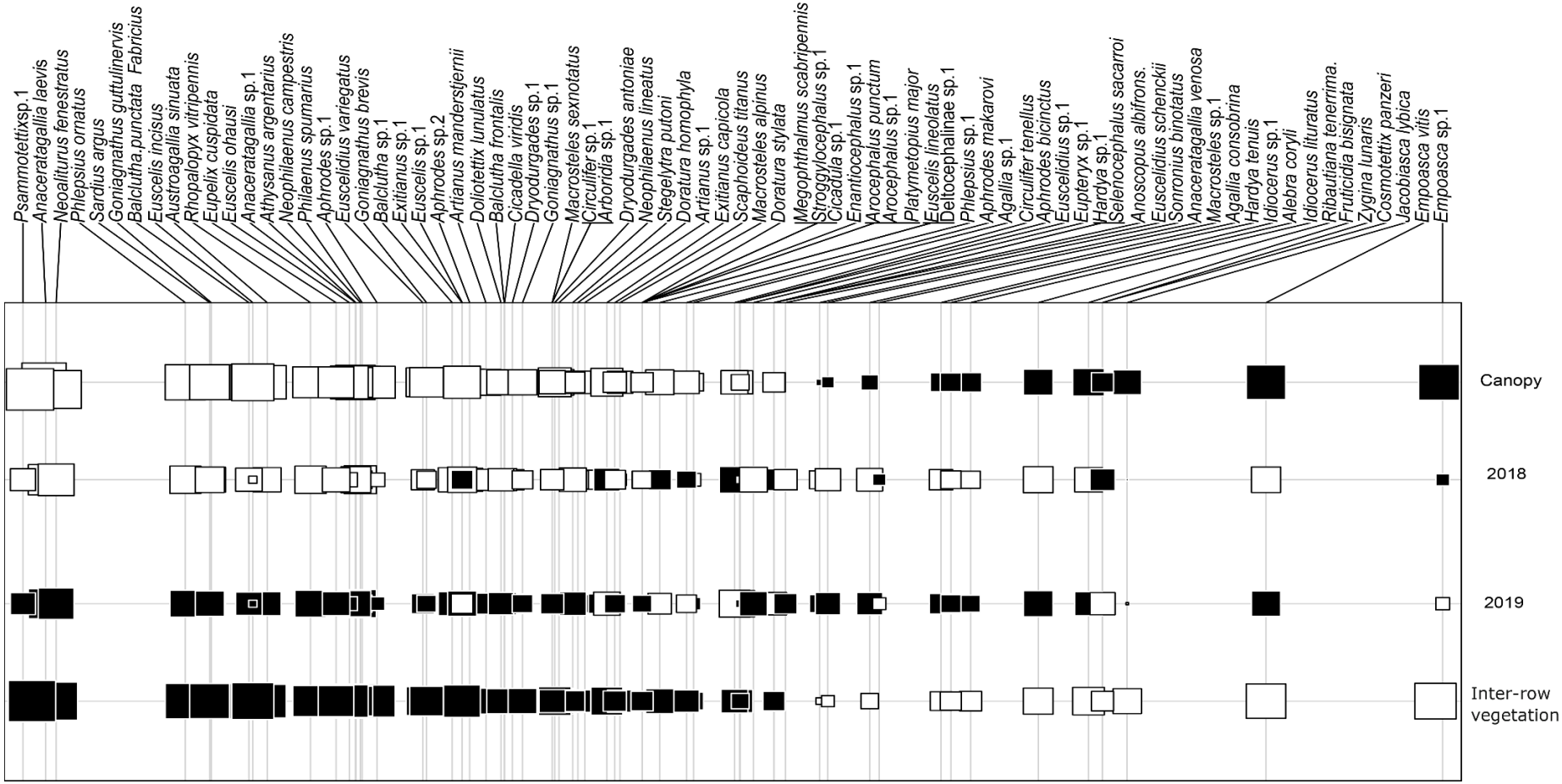

Figure 3.3. Co-inertia factorial map. Black squares represent positive relationships, and white squares negative relationships. Square sizes are pro-portional to the magnitude of the correlation.

3.4. Discussion

Sustainable agriculture requires knowledge of the abundance and diversity of pests and vector insects to protect crops and implement long-term and safe control measures. This principle is the basis of the present work that allowed the identification of the Cicadomorpha community in Portuguese vineyards together with its preference for the different strata.

All insects that feed exclusively on xylem are considered potential vectors of *X. fastidiosa* (Cornara et al., 2019). In the European continent, 96 species specialised in xylem have been recorded (EFSA, 2015). Among them, only five were captured in the sampled vineyards, namely, *P. spumarius*, *N. campestris*, *N. lineastus, L. coleoptera*, and *C. viridis*. Species such as *Aphrophora* sp., *Cercopis intermedia* Kirschbaum 1868, and *Philaenus tesselatus* Melichar, 1899, reported in other Portuguese agroecosystems (Guerreiro, 2020; Nascimento, 2020; Neto, 2017; Popova, 2020) can be considered potential vectors of this pathogen (Cornara et al., 2019), but were not collected in the sampled vineyards. Until now, only *P. spumarius* and *N. campestris*, were shown to be competent vectors of *X. fastidiosa* (Cavalieri et al., 2019). Several studies have demonstrated that *P. spumarius* can efficiently transmit *X. fastidiosa* to vineyards (Beal et al., 2021; Cornara et al., 2016; Severin, 1950). Little is known about the other three species' ability to transmit the bacteria. Nonetheless, according to Bodino et al. (2019) when acquiring the pathogen through an artificial diet, *C. viridis* is an inefficient vector of *X. fastidiosa*, since it can transmit the pathogen to periwinkle with very low efficiency but with no successful transmission from plant to plant. Since this insect was the most abundant xylem feeder captured in the sampled Portuguese vineyards, a particular effort should be made to clarify and understand the potential role of the sharpshooter in spreading the fastidious bacterium within this agroecosystem.

Philaenus spumarius was the most abundant spittlebug in the sampled vineyards, consistent with other studies carried out in European and Californian vineyards (Beal et al., 2021; Bodino et al., 2021; López-Mercadal et al., 2021). However, in the present work, the registered abundance was much lower than those reported in the bibliography.

All the vectors and potential vectors of *X. fastidiosa* collected showed a higher abundance in the inter-row vegetation. In fact, the co-inertia analysis (Figure 3.3) indicated that all the xylem sap feeders present a positive correlation with the inter-row vegetation, which is in line with the literature (Antonatos et al., 2021; Carpio et al., 2020; Elbeadaino et al., 2014; Morente et al., 2018; Tsagkarakis et al., 2018; Villa et al., 2020). Spittlebugs and *C. viridis* spend

a large part of their life cycle in the vegetation cover, mainly in grasses, where they feed, mate, and lay eggs (Cornara et al., 2019; Morente et al., 2018; Bodino et al., 2020, 2021). Nevertheless, with the exception of *N. lineatus* and *L. coleoptrata*, the remaining xylem feeders recovered were also present in the canopy of the vines. Previous studies have also reported the presence of these insects in the canopy of vines (Cornara et al., 2019; Beal et al., 2021; Bodino et al., 2021b; López-Mercadal et al., 2021). One factor influencing their distribution between the vine canopy and inter-row vegetation is the hour of the day. It is expected that the movement of the insects from the different strata during the day would occur, but we don't have observations that corroborate this.

The remaining species of Cicadomorpha captured in the vineyards are phloem or mesophyll feeders. Some studies have reported that some phloem-sap feeders of the subfamily Deltocephalinae, the most abundant subfamily in the sampled vineyards, presented to be infected with the bacteria (Elbeadaino et al., 2014; Ben Moussa et al., 2016; Chuche et al., 2017). However, there is no evidence that they can transmit the pathogen (Cavalieri et al., 2019; Purcell, 1980). As a result, all the remaining species of Cicadomorpha captured in the sampled vineyards most likely do not threaten the vineyards regarding the transmission of *X. fastidiosa*. Further studies on the ability of these individuals to transmit the bacteria are required.

Nonetheless, it should be noted that in addition to vectors and potential vectors of *X. fastidiosa*, some of the species collected in the sampled vineyards are also considered vectors or potential vectors of yellow disease phytoplasmas responsible for destructive damage in the vineyard. Among these, *S. titanus,* the main vector of the Flavescence dorée phytoplasma (Chuche & Thiéry, 2014), should be highlighted. *Euscelidius variegatus* (Kirschbaum, 1858) is another species with potential importance; it demonstrated the ability to acquire and transmit the Flavescence dorée phytoplasma, under laboratory conditions (Picciau et al., 2020) and also tested positive for '*Candidatus* Phytoplasma solani' (Laviña et al., 2006; Quaglino et al., 2019). *Neoaliturus fenestratus* (Herrich-Shaffer, 1834) has been reported to carry the '*Candidatus* Phytoplasma solani' (Batlle et al., 2000; Riolo et al., 2007; Minuz et al., 2013). *Anaceratagallia glabra* Dmitriev, 2020 (=*A. laevis*), *Austroagallia sinuata* (Mulsant & Rey, 1855), and *Z. scutellaris* have also been established as potential vectors of the phytoplasmas of yellow grapevine diseases (Batlle et al., 2000). It is also noted that the main vector of *X. fastidiosa*, *P. spumarius*, tested positive for the phytoplasma '*Candidatus* Phytoplasma solani', but there was no evidence of transmission to grapevine (Quaglino et al., 2019).

Within the Cicadomorpha community, some species recovered in the sampled vineyards can also cause physical damage to the plants, consequently leading to economic losses. *Empoasca vitis* and *J. lybica*, commonly known as green leafhoppers, are key pests in several European wine-producing regions (Decante & van Helden, 2006; Fornasiero et al., 2016). These green leafhoppers feed by puncturing the phloem vessels of the leaves. This induces an obstruction of the vessels, a reddening, and necrosis of leaves, thus reducing photosynthesis and resulting in delayed maturity (Fornasiero et al., 2016).

A great abundance, richness, and diversity of Cicadomorpha individuals were observed in the inter-row vegetation over the two years of study. In fact, the co-inertia analysis showed that most of the captured individuals exhibited a positive correlation with the inter-row vegetation, with only 14 species, mostly belonging to the family Typhlocybinae, showing a positive correlation with the vine canopy (Figure 3.3). Data in agreement with the analysis of PERMANOVA and NMDS showed differences between the communities of the sampled strata. A study by Carpio et al. (2020), whose objective was to understand the role of herbaceous vegetation in structuring the Cicadomorpha community, showed that olive groves with herbaceous vegetation showed higher diversity and abundance of Cicadomorpha compared to olive groves without herbaceous vegetation. Other studies also highlight the importance of vegetation cover in structuring the Cicadomorpha community (Altieri et al., 1985; Geppert et al., 2021; Kőrösi et al., 2012; Masters et al., 1998; McClure, 1982). Herbaceous vegetation can provide a wide range of food sources, shelter, mating places, and substrates for laying eggs (Abad et al., 2021). Tillage or mowing of the vegetation cover can be the one solution to reduce Cicadomorpha population levels in agroecosystems; however, these techniques might have significant side effects. The vegetation cover also provides shelter and food to a wide range of beneficial fauna that performs essential ecosystem services in the vineyard, such as pollination, decomposition, regulation of the nutrient cycle, and control of pests and diseases.

3.5. Conclusions

In conclusion, this study focused on the species composition, richness, and diversity of the Cicadomorpha community in vineyards distributed in mainland Portugal, with special emphasis on vectors and potential vectors of *X. fastidiosa*. The results demonstrate that vectors and potential vectors of this pathogen are present in Portuguese vineyards. *Cicadella viridis* was the more abundant potential vector in the sampled vineyards. Further studies on transmission

rates are necessary to better understand this insect's role in *X. fastidiosa* epidemiology. *Philaenus spumarius*, the main European vector, was the most abundant spittlebug.

Additionally, vectors of phytoplasmas of yellow grapevine diseases and species that can physically damage vines were also collected and identified in the sampled vineyards. Vectors and potential vectors of *X. fastidiosa* and a large part of the population of Cicadomorpha showed a positive correction with inter-row vegetation.

Further research on how the landscape, agricultural practices, application of phytosanitary treatments, the variety present at the sampling site, and environmental conditions shape the Cicadomorpha community is essential to design new techniques to prevent the spread of this pathogen in Portuguese vineyards.

Acknowledgments

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Supplementary Table S3.1. Vineyards' information: sampling dates (2018 and 2019), metric characteristics, and management data.

Vineyards	2018 Sampling dates			2019 Sampling dates			Y	X	Elevation (m)	Spacing (m)	Variety	Training System	Insecticide	Herbicide	Fungicide	Production mode	Soil management
	LS	S	A	LS	S	A											
V1	27/jun	30/aug	19/oct	16/jul	-	21/oct	40.213508	-8.455542	24,219	2.10 x 0.90	Marselan	Cordon de Royat (unilateral)	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al. + Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August	Integrated Production	Tillage between vines
V2	27/jun	30/aug	19/oct	16/jul	-	21/oct	40.207294	-8.451814	43,258	2.30 x 1	Castelão; Baga, Bical, Arinto	Cordon de Royat (bilateral)	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al. + Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August	Integrated Production	Tillage between vines

V3	27/jun	30/aug	19/oct	16/jul	24/sep	21/oct	40.472186	-8.55042	46,770	2.5 x 1	Typical varieties of the region	Unilateral Cordon	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al. + Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August	Integrated Production	No
V4	27/jun	30/aug	19/oct	16/jul	24/sep	21/oct	40.472386	-8.555479	59,408	2.5 x 2	Typical varieties of the region	Unilateral Cordon	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al. + Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August	Integrated Production	No
V5	27/jun	30/aug	19/oct	16/jul	24/sep	21/oct	40.461199	-8.531627	54,375	2.30 x 1	Typical varieties of the region	Unilateral Cordon	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al.	Integrated Production	No

														+ Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August		
V6	27/jun	30/aug	19/oct	16/jul	-	21/oct	40.212725	-8.454453	27,714	2.30 x 0.90	Touriga Nacional; Tinta Roriz; Alfrocheiro	Cordon de Royat (unilateral)	Lambda-Cyhalothrin - July/ August	Glyphosate + Oxifluorfen - February	Mancozeb + Metalaxyl M - March/ April; Mancozeb - April; Wettable sulfur - April/ May; Cymoxanil + Folpet- April; Folpet + Fosetil al. + Iprovalicarb - May; Metirame + Piraclostrobin - May/ June; Kresoxim-methyl + diphenoconazole - June; Copper Oxychloride - June; Penconazole - July/ August; Pirimetanil - July/ August	Integrated Production	Between vines
V7	-	-	-	19/jun	23/sep	21/oct	40.140458	-7.512300	473,104	3 x 1.50	Touriga Nacional; Trincadeira, Syrah	Bilateral Cordon	-	Glyphosate - March	Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July	Integrated Production	Vegetation cover mowing - June and August
V8	-	-	-	19/jun	23/sep	21/oct	40.164472	-7.520331	439,722	3 x 1	Touriga Nacional, Jaen	Bilateral Cordon	-	-	Wettable sulfur - April	Integrated Production	Vegetation cover mowing - March and June
V9	-	-	-	19/jun	23/sep	21/oct	40.168936	-7.510456	409,972	3 x 1	Jaen, Rufette, Syrah	Bilateral Cordon	-	-	Wettable sulfur - April	Integrated Production	Vegetation cover mowing - April and June

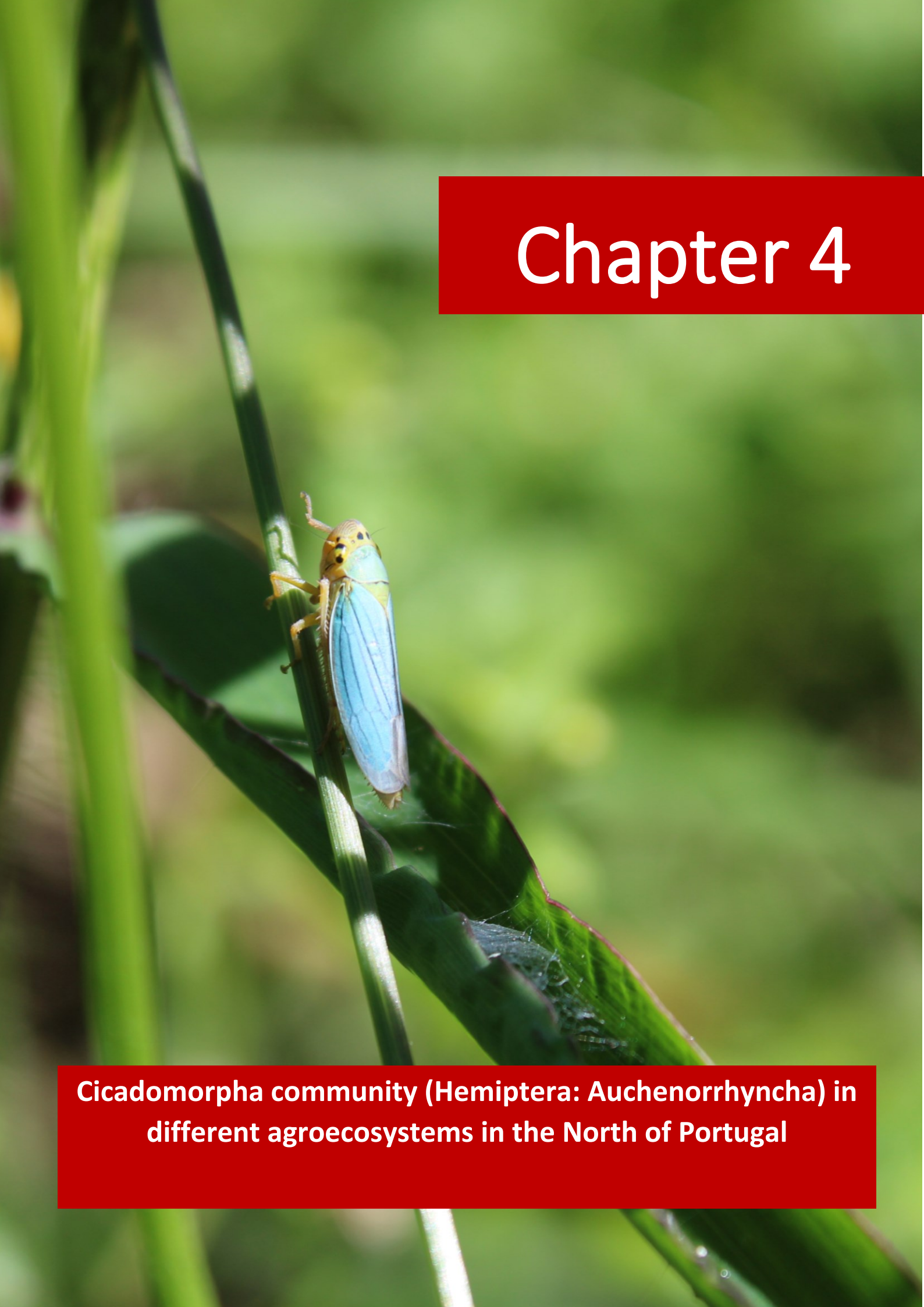
V10	-	-	-	19/jun	23/sep	21/oct	40.326719	-7.418683	459,561	3 x 1	Jaen, Moscatel, Maroco	Bilateral Cordon	-	Glyphosate - April;	Azoxystrobin + Folpet - May; Mancozeb + metalaxyl-M - June; Tetraconazole - June; Fenebuconazole - July	Integrated Production	Vegetation cover mowing - April and June
V11	-	-	-	19/jun	23/sep	21/oct	40.317892	-7.302781	484,359	3 x 2	Touriga Nacional	Unilateral Cordon	-	Glyphosate - April;	Wettable sulfur - April; Dimethomorph + Dithianon - April/June; Penconazole - June	Integrated Production	Vegetation cover mowing - April and June
V12	-	-	-	27/jul	26/sep	25/oct	41.1485948	-7.1271711	151,303	2.20 x 1	Sousão	Cordon de Royat (bilateral)	-	-	Wettable sulfur + Fosetyl aluminium - March; Cymoxanil + Folpet + Fosetyl aluminium + Spiroxamine - April; Kresoxim-methyl and Penconazole -June; Boscalid + Kresoxim-methyl - July	Integrated Production	Vegetation cover mowing - March, April and May
V13	4/jul	29/aug	16/oct	27/jul	26/sep	25/oct	41.184728	-7.109831	133,797	2 x 10	Tinta Cão	Unilateral Cordon	-	-	Wettable sulfur + Fosetyl aluminium - March; Mancozeb -May/ June	Integrated Production	No
V14	11/jul	29/aug	16/oct	27/jul	26/sep	22/oct	41.224727	-7.091073	125,710	2.20 x 0.95	Tourina Nacional	Cordon	-	-	Wettable sulfur + Fosetyl aluminium - March; Mancozeb -May	Integrated Production	No
V15	-	-	-	27/jul	26/sep	24/oct	41.1169499	-7.9869087	211,473	2 x 1.2	Viognier	Cordon de Royat (unilateral)	-	-	Sulfur - April/ May/ June; Copper - April/ May/ June	Organic	Vegetation cover mowing - in March and April
V16	-	-	-	27/jul	26/sep	24/oct	41.1550118	-7.7978446	77,685	2 x 1	Touriga Nacional, Tinta Roriz, Touriga Franca, Tinta Barroca	Cordon de Royat (unilateral)	-	Glyphosate - March;	Sulfur - May; Mandipropamid + zoxamid - May	Integrated Production	No

V17	-	-	-	27/jul	26/sep	24/oct	41.154265	-7.687574	176,179	4.5 x 0.90	Touriga Franca	Cordon de Royat (unilateral)	-	-	Sulfur - April/ May/ June; Copper - April/ May/ June	Organic	Vegetation cover mowing - February and June
V18	-	-	-	27/jul	26/sep	24/oct	41.171111	-7.556944	289,758	2.30 x 0.80	Touriga Nacional	Cordon de Royat (unilateral)	-	Glyphosate - March;	Sulfur - May; Mandipropamid + zoxamid - May	Integrated Production	No
V19	-	-	-	27/jul	26/sep	24/oct	41.175709	-7.530771	267,565	2.5 x 1	Tinta Francista, Touriga Nacional, Vinhas Velhas	Cordon de Royat (unilateral)	-	Glyphosate - March;	Folpet + Metalaxyl - April/ May; Sulfur and Penconazole - April; Fluopyram + tebuconazole - June; Copper sulfate - July; Boscalid + Kresoxim-methyl - July	Integrated Production	No
V20	-	-	-	27/jul	26/sep	24/oct	41.180833	-7.476667	285,160	2.20 x 1	Touriga Nacional, Touriga Franca, Tinta Roriz, Tinto Cão	Cordon de Royat (unilateral)	-	Glyphosate - March;	Folpet + Metalaxyl - April/ May; Sulfur and Penconazole - April; Fluopyram + tebuconazole - June; Copper sulfate - July; Boscalid + Kresoxim-methyl - July	Integrated Production	No
V21	-	-	-	27/jul	26/sep	24/oct	41.108056	-7.241389	300,447	2 x 1	Touriga Franca	Cordon de Royat (bilateral)	-	-	Folpet + Metalaxyl - April/ May; Sulfur and Penconazole - April; Fluopyram + tebuconazole - June; Copper sulfate - July; Boscalid + Kresoxim-methyl - July	Integrated Production	No
V22	26/jun	11/sep	19/oct	15/jul	23/sep	21/oct	38.522541	-8.953211	50,382	3 x 1	Tinta Roriz, Touriga Nacional, Tinta Amarela	Unilateral Cordon	-	-	Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July	Integrated Production	No

V23	26/jun	11/sep	19/oct	15/jul	23/sep	21/oct	38.567994	-8.928173	99,902	3 x 1	Moscatel Roxo, Touriga Nacional	Unilateral Cordon	-	-	Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July	Integrated Production	No
V24	26/jun	11/sep	19/oct	15/jul	-	-	38.490498	-9.022675	108,713	3 x 1	Fernão Pires, Moscatel de Setúbal	Unilateral Cordon	-	-	Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July	Integrated Production	No
V25	26/jun	11/sep	19/oct	15/jul	23/sep	21/oct	38.540465	-8.985373	96,520	3 x 1	Castelão, Trincadeira e Touriga Nacional	Unilateral Cordon	-	-	Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July	Integrated Production	No
V26	11/jul	29/aug	16/oct	15/jul	17/sep	22/oct	41.5162149	-7.092967	344,346	2 x 1	Touriga-Franca; Sousão	Unilateral Cordon	-	-	Sulfur and Copper - June/ July	Organic	Vegetation cover mowing - June
V27	-	-	-	25/jun	25/sep	22/oct	41.550195	-7.259052	260,920	2 x 1	Touriga Franca, Touriga Nacional, Bastardo,	Unilateral Cordon	-	-	Wettable sulfur - May/ June/ July	Integrated Production	Vegetation cover mowing - March and June
V28	-	-	-	25/jun	25/sep	22/oct	41.59775	-7.363637	510,649	2 x 2	Viosinho, Códega do Larinho, Malvasia Fina,	Unilateral Cordon	-	-	Wettable sulfur - May/ June/ July	Integrated Production	Vegetation cover mowing - March and June
V29	11/jul	29/aug	19/oct	26/jun	25/sep	22/oct	41.6473833	-7.5843222	385,721	2 x 1	Alvarinho	Unilateral Cordon	-	-	Malcozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	Vegetation cover mowing - June
V30	11/jul	29/aug	16/oct	26/jun	1/oct	22/oct	41.292179	-7.112580	393,805	2 x 0.90	Touriga-Franca, Touriga Nacional, Rabigato,	Cordon	-	-	Wettable sulfur + Fosetyl aluminium - March; Mancozeb -May;	Integrated Production	No

V31	22/jun	28/aug	26/oct	8/jul	25/sep	21/oct	41.680022	-8.53092	168,628	3 x 1	Espadeiro, Borraçal, Alvarinho	Unilateral Cordon	Deltamethrin - June/July	-	Folpet + Metalaxyl - April/ May; Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	No
V32	22/jun	28/aug	26/oct	8/jul	24/sep	21/oct	41.678658	-8.531356	165,049	3 x 1	Alvarinho	Unilateral Cordon	Deltamethrin - June/July	-	Folpet + Metalaxyl - April/ May; Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	No
V33	22/jun	28/aug	26/oct	8/jul	25/sep	21/oct	41.785855	-8.494984	68,918	3 x 1	Alvarelhão, Borraçal, Pedral	Unilateral Cordon	Deltamethrin - June/July	-	Folpet + Metalaxyl - April/ May; Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	No
V34	22/jun	28/aug	26/oct	8/jul	25/sep	21/oct	41.815375	-8.410264	57,641	3 x 1	Amaral, Rabo de Anho, Vinhão	Unilateral Cordon	Deltamethrin - June/July	-	Folpet + Metalaxyl - April/ May; Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	No
V35	22/jun	28/aug	26/oct	8/jul	25/sep	21/oct	41.792375	-8.538943	39,478	3 x 1	Vinhão, Espadeiro	Unilateral Cordon	Deltamethrin - June/July	-	Folpet + Metalaxyl - April/ May; Mancozeb + cymoxanil - May; Wettable sulfur- June; Fluopyram + tebuconazole - July;	Integrated Production	No

LS – Late spring; S – Summer; A – Autumn; Y – Latitude; X – Longitude.



Chapter 4

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in different agroecosystems in the North of Portugal

Chapter 4

Adapted from:

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in different agroecosystems in the North of Portugal

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Submitted

Chapter 4

Cicadomorpha community (Hemiptera: Auchenorrhyncha) in different agroecosystems in the North of Portugal

Abstract

The infraorder Cicadomorpha is a very diverse group that comprises several species considered important pests of economic crops and species that may act as vectors of plant pathogens. In Europe, the gram-negative bacterium *Xylella fastidiosa* is one of the most important and severe insect-borne plant pathogens associated with the infraorder Cicadomorpha. Therefore, the knowledge of the abundance and diversity of native Cicadomorpha insect vectors related to the different agroecosystems is essential to design and implementing specific measures to control insect-borne plant pathogens. In this work, in two consecutive years (2018 and 2019), five almond orchards, five vineyards, five olive orchards, and five scrublands distributed in the North of Portugal were sampled in three different periods (early summer, summer and autumn) to investigate species composition, richness, and diversity of the Cicadomorpha community. Also, in 2019, five lemon orchards were sampled. A total of 6056 individuals were collected (2322 in 2018 and 3734 in 2019), belonging to 71 species of three families. Within these species, observing several considered pests or vectors of vicious pathogens was possible. The confirmed vectors of *X. fastidiosa* (*Philaenus spumarius* (Linnaeus, 1758) and *Neophilaenus campestris* (Fallen, 1805)) were captured in all agroecosystems and, in general, with more abundance in autumn. The highest abundance, richness, and diversity of Cicadomorpha were observed in vineyards. However, these parameters (abundance, richness, and diversity) significantly differed between the agroecosystem and sampling period. Further research on how the composition of the vegetation cover shape the Cicadomorpha community is essential to implement strategies to reduce the spread of insect borne pathogens if they are introduced into agroecosystems.

***Keywords**:* Cicadellidae; Insect-borne pathogens; *Philaenus spumarius*; *Xylella fastidiosa*.

Chapter 4

4.1. Introduction

The majority of microbial plant pathogens, including bacteria, fungi, and some viruses, are transmitted and disseminated through the landscapes by insect vectors (Eigenbrode et al., 2018). Consequently, insect-borne plant pathogens are an increasing concern in worldwide agriculture since they are causal agents of devastating diseases that threaten the economy, diversity, and public health (Anderson et al., 2004; Huang et al., 2020; Schneider et al., 2020; Tumber et al., 2014). It is estimated that diseases derived from pathogens and phytophagous arthropods are responsible for more than 20% of the world's crop yield losses, which could feed one billion people annually (Ye et al., 2021).

The infraorder Cicadomorpha is a highly diverse group whit more than 30000 species described worldwide (Dietrich, 2002). This infraorder comprises several species that are considered pests in important economic crops; that, through feeding and oviposition cause damage to plants (*e.g.,* Alaserhat, 2021; Atakan, 2009; Backus, 1988; Decante & van Helden, 2006) while many of them act as vectors of plant pathogens (*e.g.,* Chuche & Thiéry, 2014; Kyrkou et al., 2018; Nielson, 1968; Redak et al., 2004). For example, species of the infraorder Cicadomorpha are reported to transmit several phytoplasmas responsible for important economic diseases, such the '*Candidatus* Phytoplasma vitis', '*Candidatus* Phytoplasma phoenicium' and *Spiroplasma citri* (Abu Alloush et al., 2023; Bertaccini et al., 1995; EFSA PLH Panel, 2014). The phytoplasma '*Candidatus* Phytoplasma vitis' is responsible for the Flavescence dorée (Bertaccini et al., 1995), one of the most important diseases in the European vineyards, and it is transmitted by the insect *Scaphoideus titanus* Ball (Chuche & Thiéry, 2014). '*Candidatus* Phytoplasma Phoenicium' is the causal agent of the almond witches-broom, a quarantine pest currently reported only in Iran and Lebanon (Abu Alloush et al., 2023). The phytoplasma *Spiroplasma citri* is transmitted by *Circulifer tenellus* (Baker, 1896) and is currently distributed in the United States, Northern Africa, the Mediterranean countries, and Southeast Asia, where it is responsible for the citrus disease known as "Stubborn disease" (EFSA PLH Plane, 2014). Furthermore, Cicadomorpha individuals can also transmit viruses and bacteria to monocots and dicot plants (Eigenbrode et al., 2018; Ye et al., 2021). However, in Europe, one of the most important and severe insect-borne plant pathogens associated with the infraorder Cicadomorpha is the bacterium *Xylella fastidiosa* Wells (Xanthomonadales: Xanthomonadaceae) (Huang et al., 2020).

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Xylella fastidiosa is a xylem limit bacterium native to the Americas, responsible for several diseases in crops of economic importance, such as the Almond Leaf Scorch, Olive Quick Decline Syndrome, Pierce's disease in the vine and Citrus Variegated Chlorosis (Hopkins & Purcell, 2002; Saponari et al., 2013). Currently, there is no cure for this insect-born pathogen, and it is expected that in the absence of proper control measures, the total economic loss in Europe can reach up to 1.9 billion Euros over the next 50 years (Schneider et al., 2020). Therefore, implementing sustainable measures to manage insect populations is perceived as the main tool to limit the spread of this bacterium (Schneider et al., 2020). This bacterium is transmitted exclusively by xylem sap-feeding specialists (Almeida et al., 2005). They have sucking mouthparts (mandibular and maxillary stylets) that allow them to reach the xylem of plants, from which they ingest sap (EFSA, 2013). In Europe, more than 90 species are described as xylem sap-feeding specialists, and these insects belong to the infraorder Cicadomorpha superfamilies Cercopoidea and Cicadoidea and the family Cicadellidae (subfamily Cicadellinae) (EFSA, 2013; Cornara et al., 2019).

In Portugal, the knowledge of potential vectors of *X. fastidiosa* is still very incipient; however, there is an urgent requirement to know the diversity and abundance of potential vectors in the agroecosystems since this pathogen was reported for the first time in 2019 in the north of the country, and several new outbreaks, distributed mainly in the north and centre have been reported (DGAV, 2022d). The bacterium was confirmed in 80 host plants in Portugal, including plants of economic importance, such as almonds, olives, vines, and citrus (EFSA et al., 2022a; DGAV, 2022d). These crops are characteristic of the landscape of the northern region, where some have a long tradition and represent the main means of survival for farmers in the region. Therefore, establishing and spreading this bacterium through Portugal can have devastating effects on the cultural and agricultural sectors. The dissemination of this pathogen into a new area depends on host plant availability, climatic conditions, and the presence of its vectors (Almeida & Nunney, 2015).

Therefore, the objectives of the present study were to characterise the abundance and diversity of the Cicadomorpha community associated with almonds, olives, and citrus orchards, vineyards and natural areas surrounding the olive groves.

4.2. Material and methods

4.2.1. Study area

Field surveys were conducted in five almond orchards, five vineyards, five olive orchards, five scrublands (surrounding the olive groves) in two consecutive years (2018 and 2019) and five lemon orchards (hereinafter referred to as citrus orchards), only in 2019, distributed in the North of Portugal (Figure 4.1). All the agroecosystems were under sustainable producing systems, and the herbaceous vegetation cover was maintained in the inter-rows during the sampling periods.

The herbaceous vegetation cover of the almond orchards was dominated by plant species such *Chrysanthemum segetum* L., *Anthemis cotula* L., *Calendula arvensis* L., *Coleostephus myconis* (L.) Rchb.f., *Lolium rigidum* subsp. rigidum Gaudin, and *Bromus madritensis* L..

The herbaceous vegetation of the vineyards was characterised by *Papaver* sp., *Avena barbata* Link in Schrad, *Cynodon dactylon* (L.) Pers., *Bromus tectorum* L., *Lathyrus angulatus* L., *Hypericum perfoliatum* L., *C. myconis,* and *Trifolium* sp..

The herbaceous vegetation of the olive orchards was dominated by plant species such *Sonchus tenerrimus* L., *C. myconis, Bromus diandrus* Roth, *C. dactylon*, *Medicago* sp., *Ornithopus compressus* L., and *Rumex bucephalophorus* L. The shrublands sampled were characterised by the herbaceous stratum dominated by plants belonging to Asteraceae and Poaceae families, the shrub stratum dominated by plant species such as *Cistus ladanifer* L., *Lavandula pedunculata* (Mill.) Cav., *Rubus ulmifolius* Schott, and *Crataegus monogyna* Jacq, and the tree stratum dominated by *Quercus rotundifolia* Lam., *Quercus pyrenaica* Willd., and *Arbutus unedo* L..

And finally, the herbaceous stratum of the citrus orchards was dominated by plant species such *C. dactylon, B. diandrus*, *Echium plantagineum* L., *Crepis capillaris* (L.) Wallr., *Dactylis glomerata* L., *Hedera hibernica* (G. Kirchn.) Bean, and *R. ulmifolius*. More details of the study sites are provided in Table S4.1.

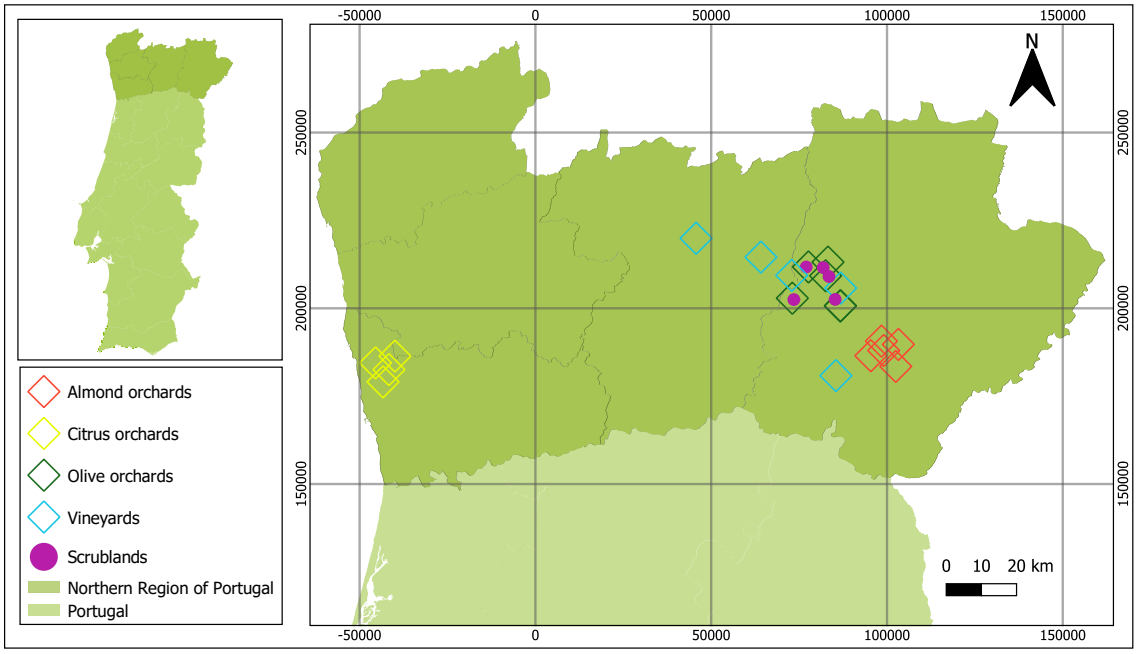

Figure 4.1. Location of the sampled agroecosystems. Map projected in ETRS89/PT-TM06.

4.2.2. Sampling of insects

In each year and agroecosystem, adults of the Cicadomorpha infraorder were sampled in three different periods: early summer, summer, and autumn. The sampling of adults was performed in the natural ground vegetation and the canopy of the plants with an entomological sweep net (38 cm diameter). In each sampling date in all the sampling sites, randomly distributed over 1 ha, ten samples of ten consecutive sweeps were collected in the ground vegetation. Each sweep was performed by moving the entomological sweep 180 degrees. In the canopy of the plants of all sampling orchards, ten samples of two sweeping in six trees, randomly selected, were performed. Furthermore, ten samples of 50 sweepings were collected in the canopy of the vineyards. Each sample was performed in a 40-meter transect. The content of the sweepings was emptied into a plastic bag properly labelled and sealed. All samples were frozen at −20°C. Arthropods were separated under a stereoscopic microscope and were conserved in ethanol 96% until further identification. The adults of the infraorder Cicadomorpha were identified according to described in Chapter 3, section "3.2.2. Collection and Identification of Insects".

4.2.3. Data analysis

The data for each year of the study, 2018 and 2019, were treated independently to avoid bias from the interannual variability. All statistical analyses and modelling were performed in R (R Core Team, 2020). The R package' bipartite' was used to visualise the presence of the species in the different agroecosystems.

The total Cicadomorpha abundance (N), the species richness (S), and the Shannon index (H') per sampling period were calculated. The species richness was calculated as the number of species/morphospecies in each agroecosystem per sampling period, and the Shannon-Wiener index (H') was calculated according to the following formula: H' =SUM [Pi log (Pi)], where Pi = ni/N, ni = the number of individuals of a species, N = a total number of individuals.

The effect of the agroecosystem and sampling period on the abundance and diversity of Cicadomorpha was investigated using general linear mixed models (GLMMs), followed by a post hoc multiple comparisons analysis ($\alpha = 0.05$). Agroecosystems (almond orchards, vineyards, olive orchards, scrublands, and citrus orchards), the sampling period (early summer, summer and autumn) and the interaction between the two terms were used as explanatory variables and the abundance, richness and the Shannon index as response variables; the sampling plots were used as a random factor. Negative binomial distribution: quadratic parameterisation (nbinom2) was used to account for the overdispersion. The function glmmTMB from the "glmmTMB" package was used for fitting the models (Brooks et al., 2017). Models were validated using the simulateResiduals function from the "DHARMa" package (Hartig, 2023).

A non-metric multidimensional scaling (NMDS) was carried out using the Bray-Curtis's index (999 permutations) in order to assess the variability in the Cicadomorpha community along the Agroecosystems and sampling periods. In addition, a permutational multivariate analysis of variance (PERMANOVA) was performed, using the function adonis from the package "vegan" to corroborate the results of the NMDS.

Finally, a co-inertia analysis ("cross-table" multivariate analysis) was performed in order to determine the relationship between Cicadomorpha species/morphospecies and the agroecosystem and sampling period. This analysis was performed using the "ade4" package and the table.value function to visualise the results.

4.3. Results

In total, 6056 individuals of the infraorder Cicadomorpha were collected, of which 2322 in 2018 and 3734 in 2019 (Table S4.2 and Table S4.3). Over the two years of study, 71 species/morphospecies were identified (54 in 2018 and 63 in 2019).

In almond orchards, 1691 individuals (701 in 2018 and 990 in 2019) belonging to 42 species (28 species in 2018 and 36 in 2019) were recovered. In 2018*, Fruticidia sanguinosa* (Rey, 1891) (204 individuals) was the most abundant specie, followed by *Zygina schneideri* (Günthart, 1974) (143 individuals) and *Psammotettix* sp. (125 individuals). Whereas in 2019, the species: *Psammotettix* sp. (516 individuals), *Fruticidia bisignata* (Mulsant & Rey, 1855) (105 individuals), *F. sanguinosa* (87 individuals) were the most abundant. The species *F. bisignata*, *F. sanguinosa*, and *Z. schneideri* were only present in the almond agrosystem (Figure 4.2).

In vineyards, 2908 individuals (1244 in 2018 and 1664 in 2019) belonging to 44 species (36 species in 2018 and 32 in 2019) were recovered. *Psammotettix* sp. (460 in 2018 and 321 in 2019), *Empoasca vitis* (Göthe, 1875) (311 in 2018 and 806 in 2019), and *Emposasca* sp. (Herrich-Schäffer, 1838) (149 in 2018 and 131 in 2019) were the most abundant species/morphospecies. Moreover, the species *E. vitis*, *J. lybica,* and *S. titanus* (only in 2019) were only present in this agroecosystem (Figure 4.2).

In olive orchards, 568 individuals (208 in 2018 and 360 in 2019) belonging to 41 species (26 species in 2018 and 34 in 2019) were recovered. *Psammotettix* sp. was the most abundant morphospecies in both years of study (217 in 2018 and 204 in 2019). *Anoplotettix* sp. was the only captured just in the olive grove in both sampling years (Figure 4.2).

In the scrublands, 336 individuals (169 in 2018 and 167 in 2019) belonging to 31 species were recovered (20 species in 2018 and 27 in 2019). *Circulifer tenellus*, *Selenocephalus sacarroi* Rodrigues, 1968, and *Centrotus cornuta* Linnaeus, 1758 were the most abundant species; that were only present in this agroecosystem.

In the citrus orchard, 553 individuals belonging to 23 species were recovered. *Zyginidia scutellaris* (Herrich-Schäffer, 1838) (226 individuals) was the most abundant specie, followed by *Psammotettix* sp (92 individuals) and *P. spumarius* (55 individuals). Nevertheless, species such *Cicadella viridis* (Linnaeus, 1758) were only captured in the citrus agroecosystem (Figure 4.2).

Regarding the vectors and potential vectors of *X. fastidiosa*, these were present in different agroecosystems (Figure 4.2).

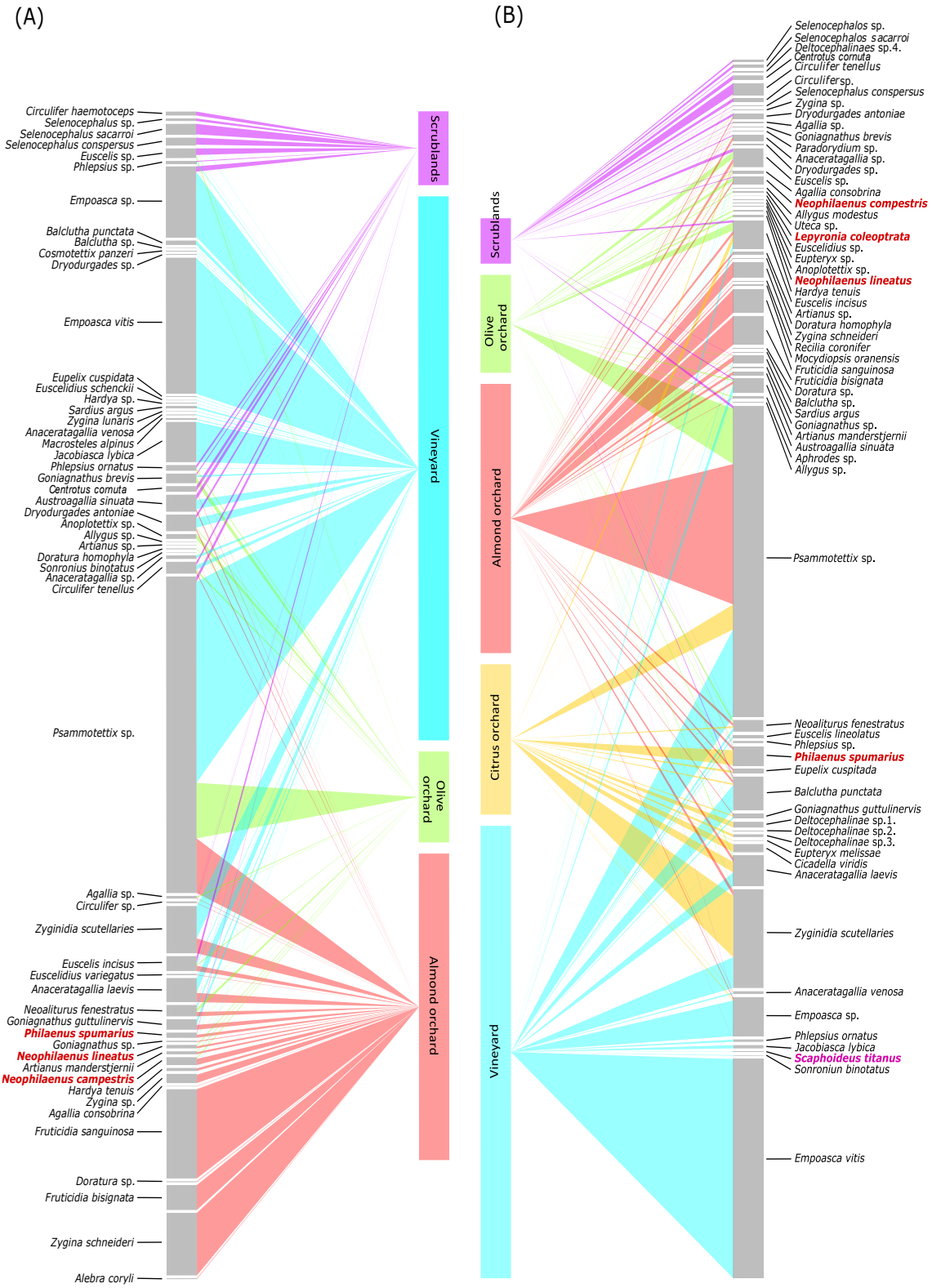

Figure 4.2. Cicadomorpha species/morphospecies -agroecosystem type interaction networks for (A) 2018 and (B) 2019. Red represents the potential vectors of *Xylella fastidiosa*, and pink is the vector of the Flavescence dorée phytoplasma.

In 2018 and 2019, in the different sampling periods, the abundance and richness of Cicadomorpha significantly differed between agroecosystems (Table 4.1). In 2018, the agroecosystem vineyard showed the highest abundance and richness in the early summer and autumn (Figure 4.2). However, in summer, the almond orchard showed more abundance of Cicadomorpha than the olive orchard. Furthermore, the olive orchard showed a lower species richness in this sampling period (Figure 4.2). Concerning Shannon's index, the sampling period did not affect the Cicadomorpha diversity since only significant differences were observed between agrosystems. The olive orchard and the scrublands presented the lowest diversity ($P<0.01$).

In 2019, the agroecosystem vineyard had a higher abundance of Cicadomorpha than the Citrus orchards and olive orchards in the early summer and autumn and a higher abundance than the scrublands in the summer. Additionally, in general, citrus orchards, olive orchards and scrublands showed a statistically significantly lower richness and diversity (H') than the agroecosystem vineyard, except for scrublands in early summer (Table 4.1 and Figure 4.3).

Table 4.1. Results of the GMMLs developed for the effect of the agroecosystem and sampling period as their interaction on the Cicadomorpha abundance, species richness and diversity (Shannon index (H')) in 2018 and 2019.

Explanatory variables	**Response variable**	***2018***			***2019***		
		df	**χ^2**	**P**	**df**	**χ^2**	**P**
Agroecosystems	Abundance	3	32.59	<0.01	4	14.16	<0.01
Period		2	20.03	<0.01	2	33.77	<0.01
Agroecosystems: period		6	66.07	<0.01	8	44.5	<0.01
Agroecosystems	Richness	3	40.38	<0.01	4	16.69	<0.01
Period		2	15.67	<0.01	2	1.23	0.53
Agroecosystems: period		6	37.37	<0.01	8	38.37	<0.01
Agroecosystems	H'	3	23.75	<0.01	4	8.96	0.06
Period		2	5.14	0.07	2	0.12	0.93
Agroecosystems: period		6	8.35	0.21	8	19.86	0.01

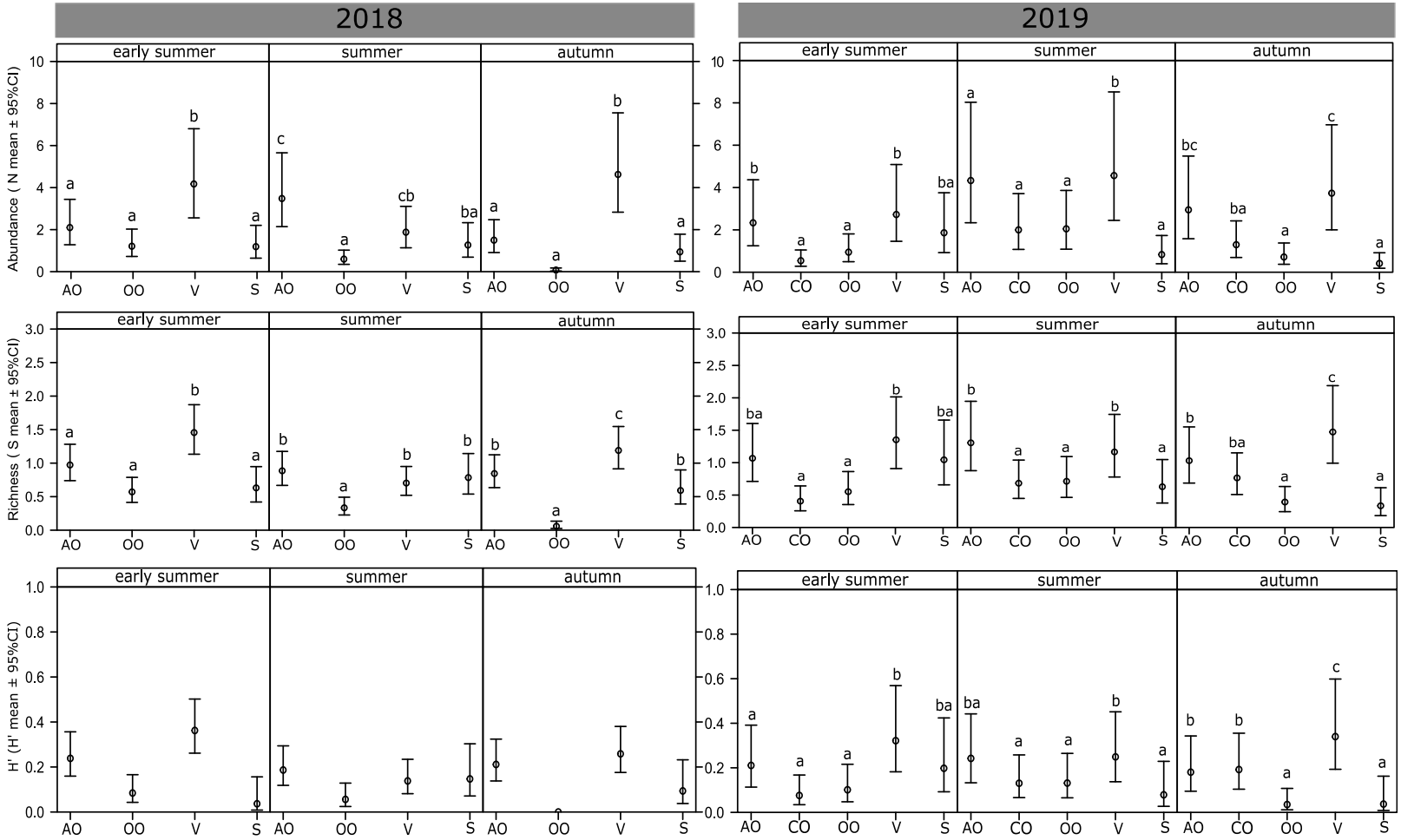

Figure 4.3. Cicadomorpha abundance, species richness and diversity (Shannon index (H')) (mean ± 95% CI) in the different agroecosystems ((AO) almond orchard, (OO) olive orchard, (V) vineyard, (S) scrublands and (CO) citrus orchard) per sampling period in 2018 and 2019.

The NMDS suggested a change in the Cicadomorpha community between agroecosystems per sampling period in both years under study (Figure 4.4). Indeed, the PERMANOVA analysis showed significant differences between agroecosystems per sampling period (df = 6; F= 2.15; P<0.01 for 2018, and df = 8; F= 1.46; P<0.01 for 2019).

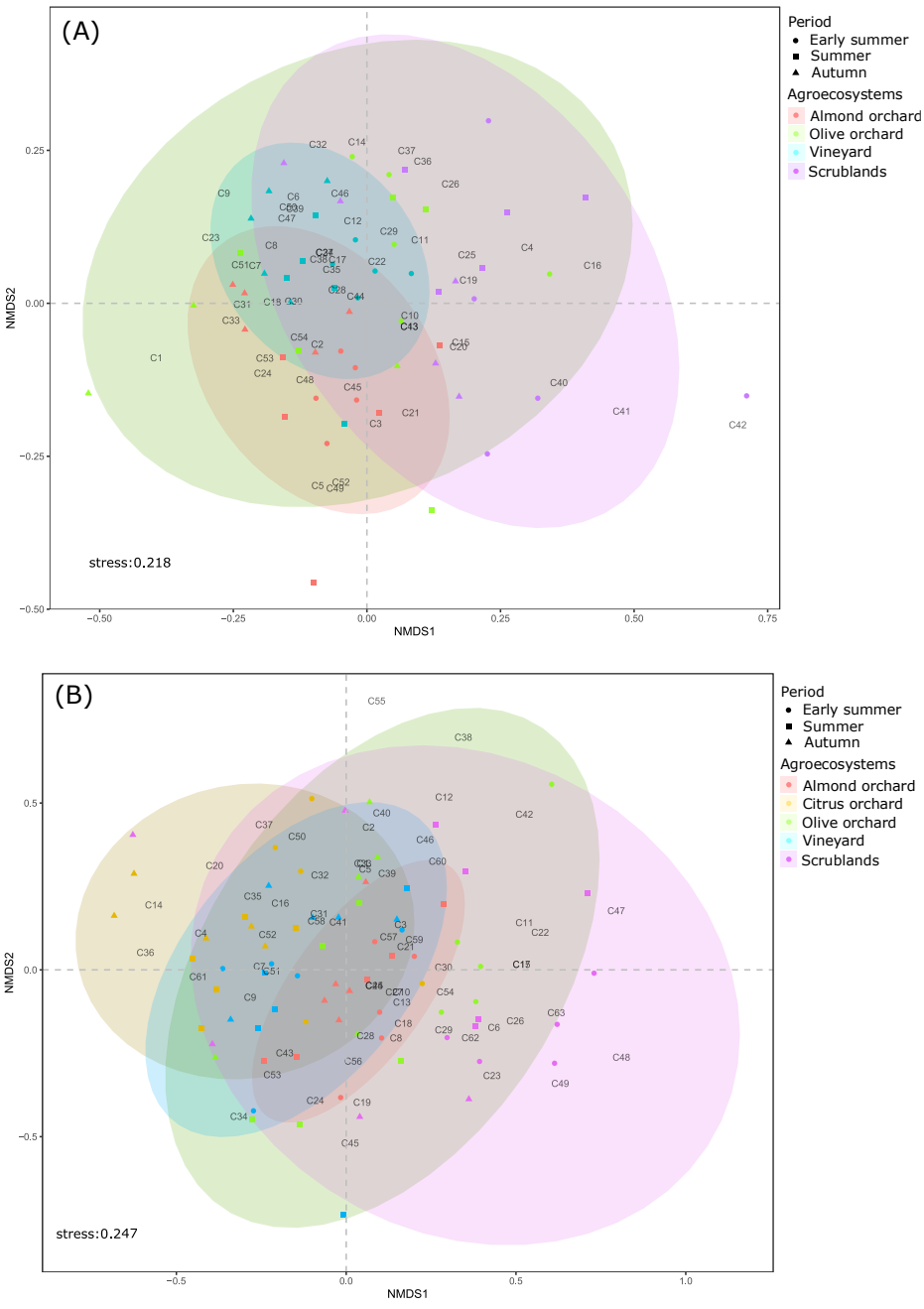

Figure 4.4. Non-metric multidimensional scaling (NMDS) analysis for Cicadomorpha abundance in the different agroecosystems and the sampling period in (A) 2018 and (B) 2019. The numbers within the panels correspond to the numbers of the species/morphospecies in Table S4.2 and S4.3.

In order to further explore the Cicadomorpha species associated with each agroecosystem and sampling period and to evaluate the contribution of these aspects to the structure of the Cicadomorpha community, co-inertia analyses were performed (Figure 4.5 and 4.6). The results obtained in the co-inertia analysis corroborate with the previously observed in the NMDS and PERMANOVA analyses. In 2018, the Cicadomorpha community composition observed in the almond orchard, olive grove, and scrublands were distinctly different from that observed in the vineyard (Figure 4.5A). The species *Psammotettix* sp., *E. vitis,* and *J. lybica* were positively correlated with the agroecosystem vineyards, *F. sanguinosa*, *P. spumarius, N. camprestris,* and *N. lineatus* were positively correlated with the almond orchard. In addition, *C. comotus* was positively correlated with the scrublands and *Goniagnathus brevis* (Herrich-Schäffer, 1835) with the olive orchard (Figure 4.5A). In 2019, vineyards and almond orchards showed a distinctly different community. In contrast, no differences were observed in olive groves and scrub communities. The species *J. lybica*, *E. vitis,* and S. *titanus* had a positive correlation with vineyards, the species *F. bisignata* and *Anaceratagalia* sp. had a positive correlation with almond orchards, *C. cornuta* once again is positively correlated with scrublands. The potential vectors of *X. fastidiosa, N. campestris*, *N. lineatus,* and *L. coleoptera* were positively correlated with olive orchards, and *C. viridis* and *P. spumarius* were positively correlated with citrus orchards (Figure 4.5B).

The sampling period was also found to impact the structure of the Cicadomorpha community. In both sampling years, it was found that the community of Cicadomorpha present at the beginning of summer distinctly differs from that in summer and autumn (Figure 4.6). However, the Cicadomorpha community is not very different in the summer and autumn. In 2018, 30 species were positively correlated with the early summer; the most positively correlated species are *N. fenestratus, Artianus manderstjernii* (Kirschbaum, 1868) and species of the genus *Selenocephalus*. *Philaenus spumarius* also showed a positive correlation with this sampling period (Figure 4.6A).

In 2019, 28 species showed a positive correlation with the early summer; species of the genus *Euscelis* and *Selenocephalus* were among the species that showed the highest positive correlation. Moreover, species such *N. campestris* and *P. spumarius* showed a positive correlation with autumn, while *C. viridis* presented a positive correlation with summer and autumn, whereas *N. lineatus* and *S. titanus* showed a positive correlation with summer (Figure 4.6B).

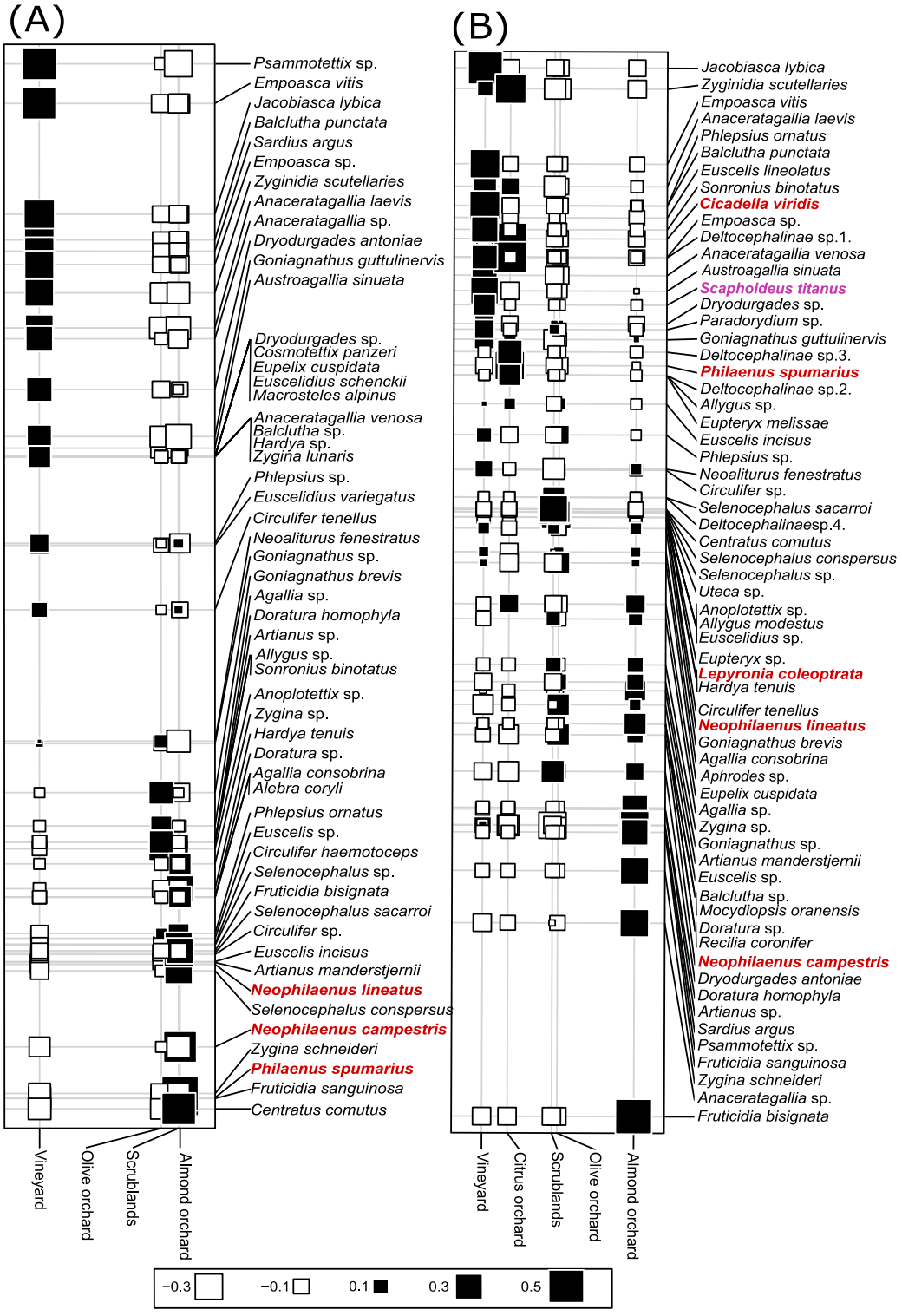

Figure 4.5. Co-inertia factorial map for the agroecosystems in (A) 2018 and (B) 2019.Black squares represent positive relationships, and white squares negative relationships. Square sizes are pro-portional to the magnitude of the correlation. Red represents the potential vectors of *Xylella fastidiosa*, and pink is the vector of the Flavescence dorée phytoplasma.

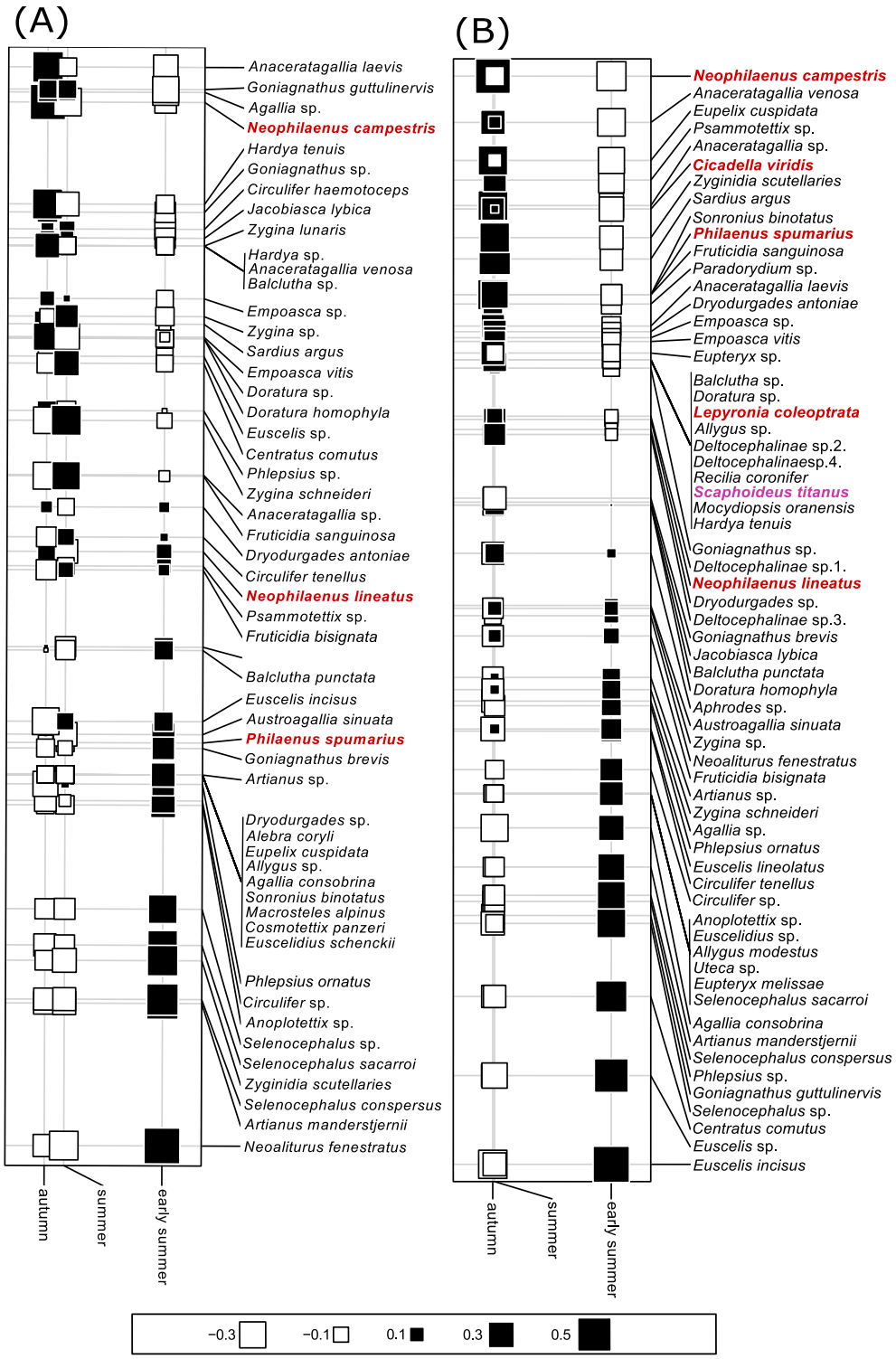

Figure 4.6. Co-inertia factorial map for the sampling period (A) 2018 and (B) 2019.Black squares represent positive relationships, and white squares negative relationships. Square sizes are proportional to the magnitude of the correlation. Red represents the potential vectors of *Xylella fastidiosa*, and pink is the vector of the Flavescence dorée phytoplasma.

4.4. Discussion

When insect-borne plant pathogens reach a new location, the spread throughout the landscape could be mediated by native insect vectors (Redak et al., 2004). Therefore, knowing the abundance and diversity of native insect vectors associated with agroecosystems is essential to design and implementing specific measures to control insect-borne plant pathogens. Cicadomorpha species are well-known as plant pathogen vectors. In this study, the abundance and diversity of Cicadomorpha in several agroecosystems were studied in different sampling periods. In addition, the species and structure community associated with each agroecosystem were also studied.

Our results indicate that the agroecosystems of the north of Portugal can harbour abundant and diverse assemblages of Cicadomorpha insects. The integration agroecosystem and period of samplings plays an essential role in shaping the structure and composition of the Cicadomorpha community. This might be associated with their herbaceous host plants, which depend on the agroecosystems management and season (Aguyoh et al., 2004; Carpio et al., 2020; Villa et al., 2020). Also, Cicadomorpha species may have different biological cycles and phenology, which may differ with environmental conditions like relative humidity and temperature (Weaver & King, 1954; Whittaker, 1965). Some species only have one generation per year; this is the case of the aphrophorids (*e.g.,* Bodino et al., 2020; Morente et al., 2018), while others, like Cicadellidae, may develop more than one generation per year (*e.g.,* Beok, 1972; Decante & van Helden, 2006; Khfif et al., 2022). Furthermore, some species of Cicadomorpha exhibit a migratory behaviour, i.e., they leave the main crop when the host plants of the herbaceous cover die or are cut, returning in autumn with the regrout of the herbaceous cover after the first rains (Antonatos et al., 2021; Cruaud et al., 2018; Morente et al., 2018). These characteristics allow these insects to be present in the agroecosystems at different times throughout the year.

In vineyards, the agroecosystem with more abundance and diversity of Cicadomorpha individuals was dominated by the species *Psammotettix* sp., *E. vitis*, *Empoasca* sp. and *J. lybica*. In fact, these species showed a positive correlation with this agroecosystem. Previous studies demonstrated that species of the genus *Psammotettix* tested positive for '*Candidatus* Phytoplasma solani', the causal agent of Bois noir and Phytoplasmas of the aster yellows in vines (Quaglino et al., 2019). However, these herbivorous insects in Europe only cause great concern

in the wheat, where species of *Psammotettix* can act as vectors for the persistent wheat dwarf virus (Lindblad & Sigvald, 2004).

Empoasca vitis and *J. lybica* are key pests in several European wine regions (Decante & van Helden, 2006; Fornasiero et al., 2016). These green leafhoppers, feed on phloem tissues (Fornasiero et al., 2016), which reduces the possibility of acting as potential vectors of xylem-associated bacteria. However, when these species achieve heavy infestations in vineyards, they can result in severe yield losses (Decante & van Helden, 2006; Fornasiero et al., 2016).

Scaphoideus titanus is a univoltine species that feed specifically on vines. Native to the Nearctic Region, it is widely disseminated in Europe (Lessio & Alma, 2004), this species is known to be the vector of the phytoplasma agent of flavescence dorée, an economically important persistent grapevine disease (Chuche & Thiéry, 2014). However, in the present study, this species was captured in low abundance and only in 2019.

Furthermore, some of the genera recovered in the vineyard, such as *Austroagallia, Anaceratagallia, Euscelidius, Euscelis*, *Circulifer*, *Neoaliturus,* and *Zyginidia* were previously reported as potential vectors of phytoplasmas of yellow grapevine disease (Batlle et al., 2000; Laviña et al., 2006; Minuz et al., 2013; Picciau et al., 2020; Quaglino et al., 201; Riolo et al., 2007). Also, *P. spumarius* and *N. campestris*, efficient and competent vectors of *X. fastidiosa* (Cavalieri et al., 2019), were recovered in this agroecosystem, similar to those observed in other regions (*e.g.,* Beal et al., 2021; Bodino et al., 2021b; López-Mercadal et al., 2021). Nevertheless, its abundance was much lower than that reported in the literature. Indeed, Beal et al. (2021), Cornara et al. (2016), and Severin (1950) reported that *P. spumarius* could effectively transmit *X. fastidiosa* to vines.

Species of the subfamily Typhlocybinae and the genera *Psammotettix* were dominant in the almond orchard. This subfamily comprises serious agricultural pests due to the direct feeding injury they inflict on plants (Backus, 1988); when they reach high population levels, they feed on almond leaves, causing them to turn yellowish and curl up at the edges, leading to eventual drying and leaf fall (González-Zamora et al., 2021). *Asymmetrasca decedens* a highly polyphagous species capable of acting as a vector for '*Candidatus* Phytoplasma Phoenicium', a pathogenic plant bacterium responsible for almond witches-broom disease (Dakhil et al., 2011). Although this insect was already reported in Portugal (Coutinho et al., 2015), it was not captured in the sampled almond orchards.

In 2018 the highest abundance of *X. fastidiosa* vectors, namely: *N. campestris, N. lineatus,* and *P. spumarius* was observed in almond orchards. Still, in 2019, these insects were reduced, probably associated with the interannual variation.

To our best knowledge, except for *X. fastidiosa* (Saponari et al., 2013), the olive tree is not affected by other insect-borne pathogens. In this sense, the presence in this agroecosystem of species that have been reported to transmit pathogens, such as species of the genera *Neolituros*, *Euscelis,* and *Anoplotettix* (Jakovljević et al., 2015; Laviña et al., 2006), is not alarming. However, in olive groves, four species of aphrophorids were captured, two confirmed vectors of *X. fastidiosa* (*P. spumarius* and *N. campestris*) (Cavalieri et al., 2019) and two whose transmission efficiency and competence are still unknown (*L. coleoptera* and *N. lineatus*). Although it is low in abundance compared to that observed in olive orchards from other regions (Antonatos et al., 2021; Bodino et al., 2020; Cornara et al., 2017a; Morente et al., 2018; Tsagkarakis et al., 2018), their presence in this agroecosystem is alarming. *Xylella fastidiosa* does not need a latency period to be transmitted (Janse & Obradovic, 2010), and once acquired by the insect vector, it persists in the insect during its entire adult life (Almeida et al., 2005).

Our results suggest that olive orchards and scrublands have a similar Cicadomorpha community, probably because the sampled scrublands surround the olive groves. Scrublands may act as reservoirs of arthropods providing food and optional shelter from the main crop (Kubiak et al., 2022). Some works carried out in olive groves in Spain, Corsica, and Greece, demonstrate that in summer *P. spumarius* and *N. campestris* migrate from olive groves to adjacent areas. This is probably related to the fact that on olive groves, the vegetation cover dies or is removed, and the insect migrates for a more favourable environment. As autumn approaches, after the first rains, they tend to return to the olive grove when there is regrowth of the herbaceous vegetation where these individuals lay the eggs (Antonatos et al., 2021; Bodino et al., 2020; Cruaud et al., 2018; Morente et al., 2018; Tsagkarakis et al., 2018). Furthermore, Cornara et al. (2021b) also described that natural and semi-natural areas surrounding cultivated orchards could act as *X. fastidiosa* vector reservoirs, sustaining their populations during the summer and possibly acting as both recipient and source areas for *X. fastidiosa.* This corroborates our data since *P. spumarius*, and *N. campestris* were recovered from the Scrublands in the early summer and summer, and the olive orchards were mainly present in the autumn. The same pattern is verified in the vineyards, almonds, and citrus orchards, where these two species were captured with greater abundance in autumn. This

indicates that these vectors have a behavioural pattern in these agroecosystems similar to that observed in olive orchards.

The citrus tree is affected by several insect-borne pathogens (*e.g.,* Coletta-Filho et al., 2020; EFSA PLH Plane, 2014) that can be transmitted by individuals of Cicadomorpha, such as *S. citri* and *X. fastidiosa*. *Spiroplasma citri* is the causal agent of Citrus Stubborn Disease, the main vector of this pathogen, *C. tenellus* (EFSA PLH Plane, 2014); although this specie was present in the other sampled agroecosystems, it was not captured in the sampled citrus orchards.

Xylella fastidiosa is responsible for Citrus Variegated Chlorosis, a very important disease in Brazil, where this bacterium is transmitted mainly by individuals of the Cicadellidae subfamily; however (Coletta-Filho et al., 2020), these vector species are absent in Europe (EFSA, 2013). *Cicadella viridis* is the most abundant Cicadellinae in Europe; although it cannot transmit *X. fastidiosa* to the olive tree (Bodino et al., 2022), there is still no evidence that it can transmit the bacteria in other agroecosystems. *Cicadella viridis* and *P. spumarius* were abundantly captured in the sampled citrus. It should be noted that in Portugal, *X. fastidiosa* has been reported in commercial citrus orchards (DGAV, 2022d). Therefore, it is urgent to know the transmission capacity and efficiency of *C. viridis* and *P. spumarius* in citrus to implement proper control measures against these potential vectors.

4.5. Conclusions

Understanding and knowing the diversity and abundance of insects that may constitute important pests or play an important role in disseminating pathogens is the first step for implementing appropriate measures to combat the negative effects of these insects. With the present study, it was possible to verify that the agroecosystems under study can harbour a great diversity and abundance of Cicadomorpha. Moreover, this diversity and abundance may vary with the agrosystem and the time of year. The confirmed vectors of *X. fastidiosa* (*P. spumarius*, and *N. campestris*) were recovered in all sampled agroecosystems in different abundances.

Further research on how the landscape, agricultural practices, and composition of the vegetation cover shape the Cicadomorpha community is essential to better understand what contributes to variations in the composition of the Cicadomorpha community, to implement strategies to reduce the spread of insect-borne pathogens if they are introduced into agroecosystems.

Acknowledgments

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Chapter 4
Supplementary material

Table S4.1. Agroecosystems' information: sampling dates (2018 and 2019) and metric characteristics.

Plot	Agroecosystems	2018 Sampling dates			2019 Sampling dates			Spacing (ha)	Elevation (m)	coordinates
		es	s	a	es	s	a			
Almond orchard1	Almond orchard	2/jul	28/ago	16/out	26/jun	2/set	25/out	2	616	41°22'01.9"N 6°57'27.2"W
Almond orchard2	Almond orchard	2/jul	28/ago	16/out	26/jun	2/set	25/out	3	595	41°21'57.9"N 6°57'16.8"W
Almond orchard3	Almond orchard	2/jul	28/ago	16/out	26/jun	2/set	25/out	3	565	41°21'17.9"N 6°56'57.3"W
Almond orchard4	Almond orchard	2/jul	28/ago	16/out	26/jun	2/set	25/out	6	573	41°21'17.9"N 6°57'10.1"W
Almond orchard5	Almond orchard	2/jul	28/ago	16/out	26/jun	2/set	25/out	9	610	41°21'33.4"N 6°57'38.5"W
Citrus orchard1	Citrus orchard	-	-	-	27/jun	27/ago	23/out	1	170	41°18'45.3"N 8°38'17.3"W
Citrus orchard2	Citrus orchard	-	-	-	27/jun	27/ago	24/out	2	92	41°18'23.7"N 8°38'37.9"W
Citrus orchard3	Citrus orchard	-	-	-	27/jun	27/ago	25/out	1	101	41°18'28.8"N 8°38'32.0"W
Citrus orchard4	Citrus orchard	-	-	-	27/jun	27/ago	26/out	3	82	41°19'41.0"N 8°40'35.1"W
Citrus orchard5	Citrus orchard	-	-	-	27/jun	27/ago	27/out	2	90	41°19'41.0"N 8°40'34.1"W
Olive orchard1	Olive orchard	3/jul	20/ago	23/out	26/jun	2/set	25/out	3	354	41°29'19.2"N 7°07'34.9"W
Olive orchard2	Olive orchard	3/jul	20/ago	23/out	26/jun	2/set	25/out	3	344	41°29'15.8"N 7°07'49.1"W
Olive orchard3	Olive orchard	3/jul	20/ago	23/out	26/jun	2/set	25/out	5	356	41°32'53.8"N 7°08'37.0"W
Olive orchard4	Olive orchard	3/jul	20/ago	23/out	26/jun	2/set	25/out	3	237	41°34'12.4"N 7°09'56.8"W
Olive orchard5	Olive orchard	3/jul	20/ago	23/out	26/jun	2/set	25/out	6	348	41°29'30.3"N 7°15'28.7"W
Scrublands1	Scrublands	3/jul	20/ago	23/out	26/jun	2/set	25/out	1.7	361	41°29'35.8"N 7°15'01.2"W
Scrublands2	Scrublands	3/jul	20/ago	23/out	26/jun	2/set	25/out	1.9	361	41°29'22.2"N 7°07'24.8"W
Scrublands3	Scrublands	3/jul	20/ago	23/out	26/jun	2/set	25/out	3.0	361	41°29'19.8"N 7°08'00.9"W
Scrublands4	Scrublands	3/jul	20/ago	23/out	26/jun	2/set	25/out	3	259	41°34'17.5"N 7°09'47.0"W
Scrublands5	Scrublands	3/jul	20/ago	23/out	26/jun	2/set	25/out	3	258	41°32'52.8"N 7°08'27.2"W
Vineyard1	Vineyard	11/jul	21/ago	19/out	26/jun	2/set	25/out	3	342	41°30'58.4"N 7°05'34.7"W
Vineyard2	Vineyard	11/jul	21/ago	19/out	26/jun	2/set	25/out	6	259	41°33'00.7"N 7°15'32.6"W
Vineyard3	Vineyard	11/jul	21/ago	19/out	26/jun	2/set	25/out	3	507	41°35'51.9"N 7°21'49.1"W
Vineyard4	Vineyard	11/jul	21/ago	19/out	26/jun	2/set	25/out	6	390	41°38'50.6"N 7°35'03.6"W
Vineyard5	Vineyard	11/jul	21/ago	19/out	26/jun	2/set	25/out	1	396	41°17'31.8"N 7°06'45.3"W

Table S4.2. Total number (N) of Cicadomorpha adults collected in the almond orchard, olive orchard, vineyard and scrublands in early summer, summer, and autumn in 2018.

Family	Subfamily		Species	Almond orchard			Olive orchard			Vineyard			Scrublands			Total
				es	s	a	es	s	a	es	s	a	es	s	a	
Aphrophoridae																
		C1	*Neophilaenus campestris (*Fallén, 1805)	3	0	11	0	0	3	0	0	1	0	0	0	18
		C2	*Neophilaenus lineatus* (Linnaeus, 1758)	1	0	4	3	0	0	0	0	0	0	0	0	8
		C3	*Philaenus spumarius* (Linnaeus, 1758)	5	1	1	0	0	1	0	1	0	1	2	0	12
Cicadellidae																
	Agalliinae															
		C4	*Agallia consobrina* Curtis, 1833	1	0	0	0	0	0	0	0	0	0	0	0	1
		C5	*Agallia* sp.	0	0	1	0	3	0	0	0	1	0	0	1	6
		C6	*Anaceratagallia glabra* Dmitriev, 2020 (=*A. laevis*)	1	1	19	0	0	1	7	12	13	0	0	1	55
		C7	*Anaceratagallia* sp.	0	1	0	0	1	0	3	0	3	0	0	0	8
		C8	*Anaceratagallia venosa* (de Fourcroy, 1785)	0	0	0	0	0	0	0	0	2	0	0	0	2
		C9	*Austroagallia sinuata* (Mulsant & Rey, 1855)	2	1	2	1	0	0	19	2	0	0	2	10	39
		C10	*Dryodurgades antoniae* (Melichar, 1907)	1	0	4	3	0	0	11	1	10	0	8	0	38
		C11	*Dryodurgades* sp.	0	0	0	0	0	0	1	0	0	0	0	0	1
	Deltocephalinae															
		C15	*Allygus* sp.	0	0	0	1	0	0	0	0	0	0	0	0	1
		C16	*Anoplotettix* sp.	0	0	0	11	0	0	0	0	0	0	0	0	11

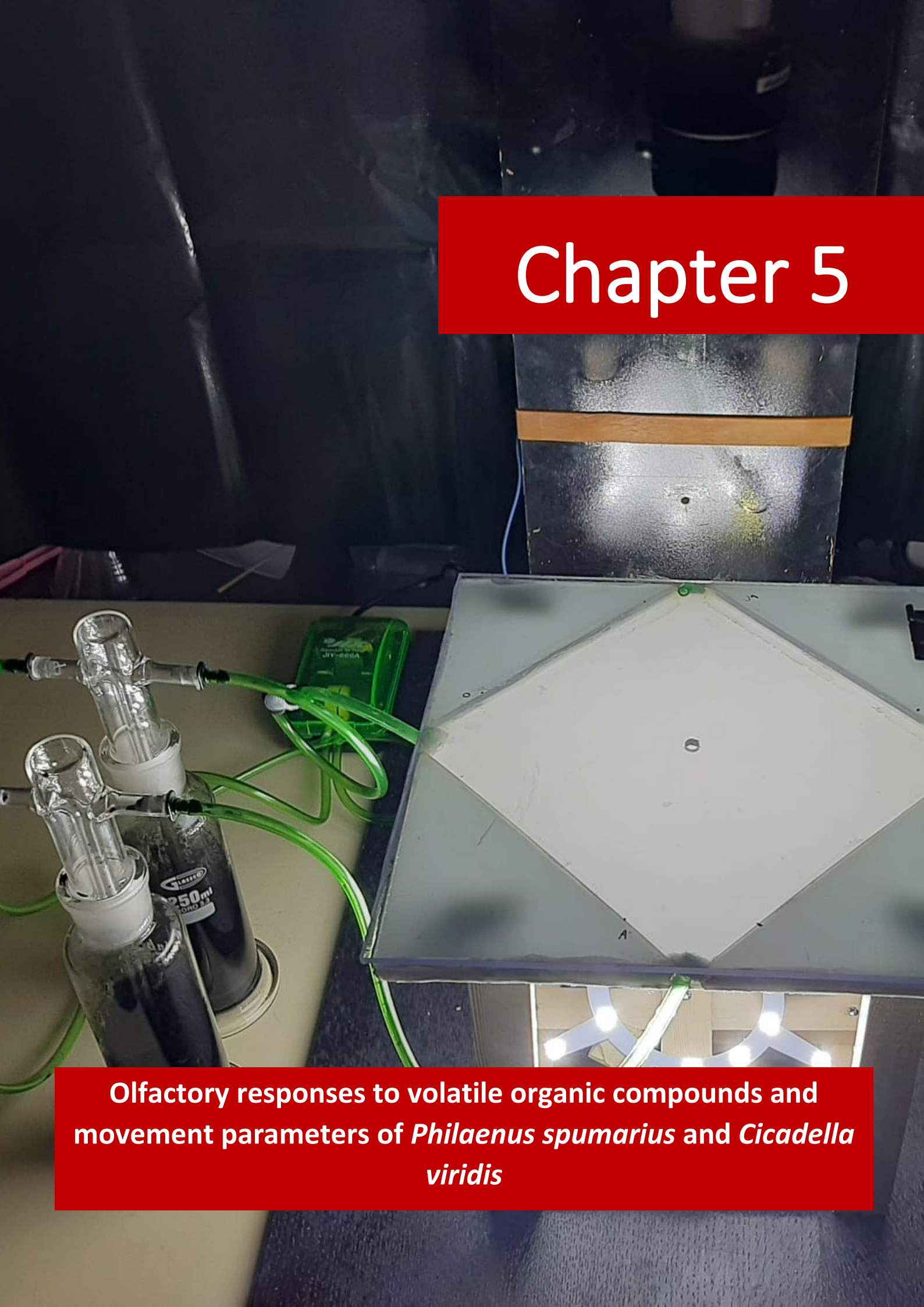
	C17	*Artianus manderstjernii* (Kirschbaum, 1868)	5	0	0	2	0	0	0	0	0	0	0	0	7
	C18	*Artianus* sp.	0	0	0	1	0	0	0	0	0	0	0	0	1
	C19	*Balclutha punctata* (Fabricius, 1775)	0	0	0	0	0	0	6	1	3	0	0	0	10
	C20	*Balclutha* sp.	0	0	0	0	0	0	0	0	1	0	0	0	1
	C21	*Circulifer haematoceps* (Mulsant & Rey, 1855)	0	0	0	0	0	0	0	0	0	0	5	4	9
	C22	*Circulifer* sp.	1	0	0	0	1	0	0	0	0	2	0	0	4
	C23	*Circulifer tenellus* (Baker, 1896)	1	1	2	2	1	2	6	3	1	1	7	0	27
	C24	*Cosmotettix panzeri* (Flor, 1861)	0	0	0	0	0	0	1	0	0	0	0	0	1
	C25	*Doratura homophyla* (Flor, 1861)	0	0	0	0	2	0	0	0	0	0	0	0	2
	C26	*Doratura* sp.	0	2	0	0	0	0	0	0	0	0	0	0	2
	C27	*Euscelis incisus* (Kirschbaum, 1858)	12	0	0	2	1	0	3	1	0	0	14	1	34
	C28	*Euscelis* sp.	0	0	0	3	3	0	1	0	0	0	12	3	22
	C29	*Euscelidius schenckii* (Kirschbaum, 1868)	0	0	0	0	0	0	1	0	0	0	0	0	1
	C30	*Euscelidius variegatus* (Kirschbaum, 1858)	0	0	1	0	0	0	2	0	0	0	0	0	3
	C31	*Goniagnathus brevis* (Herrich-Schäffer, 1835)	0	0	0	12	2	0	1	0	3	1	3	0	22
	C32	*Goniagnathus guttulinervis* (Kirschbaum, 1868)	3	2	6	0	1	0	0	8	4	0	0	0	24
	C33	*Goniagnathus* sp.	0	0	2	0	0	0	0	1	0	0	0	0	3
	C34	*Hardya* sp.	0	0	0	0	0	0	0	0	1	0	0	0	1
	C35	*Hardya tenuis* (Germar, 1821)	1	0	5	0	0	0	0	0	1	0	0	0	7
	C36	*Macrosteles alpinus* (Zetterstedt, 1828)	0	0	0	0	0	0	2	0	0	0	0	0	2
	C37	*Neoaliturus fenestratus* (Herrich-Schäffer, 1834)	9	0	1	5	1	2	6	1	0	0	0	1	26
	C38	*Phlepsius ornatus* (Perris, 1857)	0	1	0	4	0	0	0	1	0	4	3	0	13
	C39	*Phlepsius* sp.	0	0	0	0	1	0	1	0	2	1	0	1	6
	C40	*Psammotettix* sp.	14	26	85	78	48	1	199	121	140	1	3	8	724
	C41	*Sardius argus* (Marshall, 1866)	0	0	0	0	0	0	1	0	3	0	0	0	4
	C42	*Selenocephalus conspersus* (Herrich-Schäffer, 1834)	0	0	0	2	0	0	0	0	0	16	0	0	18
	C43	*Selenocephalus sacarroi* Rodrigues, 1968	0	0	0	0	0	0	1	0	0	22	2	0	25
	C44	*Selenocephalus* sp.	0	0	0	0	0	0	0	0	0	6	0	0	6
	C45	*Sonronius binotatus* (Sahlberg, 1871)	0	0	0	1	0	0	0	0	0	0	0	0	1
Dorycephalinae															
	C46	*Eupelix cuspidata* (Fabricius, 1775)	0	0	0	0	0	0	1	0	0	0	0	0	1
Typhlocybinae															
	C47	*Alebra coryli* Le Quesne, 1977	2	0	0	0	0	0	0	0	0	0	0	0	2
	C48	Empoasca sp.	0	0	0	0	0	0	28	60	63	0	2	10	163
	C49	*Empoasca vitis* (Göthe, 1875)	0	0	0	0	0	0	92	0	219	0	0	0	311
	C50	*Fruticidia bisignata* (Mulsant & Rey, 1855)	26	31	0	0	0	0	0	0	0	0	0	0	57
	C51	*Fruticidia sanguinosa* (Rey, 1891)	60	143	1	0	0	0	0	0	0	0	0	0	204
	C52	*Jacobiasca lybica* (de Bergevin & Zanon, 1922)	0	0	0	0	0	0	8	43	41	0	0	0	92
	C53	*Zygina lunaris* (Mulsant & Rey, 1855)	0	0	0	0	0	0	0	0	1	0	0	0	1
	C54	*Zygina schneideri* (Günthart, 1974)	35	107	1	0	0	0	0	0	0	0	0	0	143
	C55	Zygina sp.	0	19	0	0	0	0	0	0	2	0	0	0	21
	C56	*Zyginidia scutellaris* (Herrich-Schäffer, 1838)	25	6	3	0	0	0	54	6	14	0	0	0	108
Membracidae															
	C57	*Centrotus cornuta* Linnaeus, 1758	0	2	0	0	0	0	0	0	0	3	6	2	13
Total			208		149	133	67	8	453	262	529	58		44	2322

Table S4.3. Total number (N) of Cicadomorpha adults collected in the almond orchard, citrus orchard, olive orchard, vineyard and scrublands in early summer, summer and autumn in 2019

Family	Subfamily		Species	Almond orchard			Citrus orchard			Olive orchard			Vineyard			Scrublands			Total
				es	s	a	es	s	a	es	s	a	es	s	a	es	s	a	
Aphrophoridae																			
		C1	*Lepyronia coleoptrata* (Linnaeus, 1758)	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
		C2	*Neophilaenus campestris (*Fallén, 1805)	0	0	9	1	0	0	1	4	7	0	1	2	0	3	0	28
		C3	*Neophilaenus lineatus* (Linnaeus, 1758)	0	0	0	0	0	0	1	0	5	0	0	0	0	0	0	6
		C4	*Philaenus spumarius* (Linnaeus, 1758)	3	0	8	6	5	44	0	0	1	1	0	0	0	2	0	70
Cicadellidae																			
	Agalliinae																		

	C5	*Agallia consobrina* Curtis, 1833	3	0	0	0	0	0	3	0	0	1	0	2	0	0	2	11
	C6	*Agallia* sp.	0	2	0	1	0	0	0	0	0	0	0	0	2	0	0	5
	C7	*Anaceratagallia glabra* Dmitriev, 2020 (*=A. laevis*)	2	8	5	2	2	35	0	7	1	21	13	16	0	0	0	112
	C8	*Anaceratagallia* sp.	1	4	8	1	0	1	0	1	1	1	0	0	0	0	4	22
	C9	*Anaceratagallia venosa (*de Fourcroy, 1785)	0	1	1	0	0	2	0	0	0	0	3	3	0	0	0	10
	C10	*Austroagallia sinuata* (Mulsant & Rey, 1855)	3	0	7	0	2	0	5	0	0	14	12	6	0	4	1	54
	C11	*Dryodurgades antoniae* (Melichar, 1907)	2	1	4	0	0	0	1	1	0	0	0	1	1	8	0	19
	C12	*Dryodurgades* sp.	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	4
Aphrodinae																		
	C13	*Aphrodes* sp.	1	1	0	0	0	0	1	3	0	1	0	1	0	0	0	8
Cicadellinae																		
	C14	*Cicadella viridis* (Linnaeus, 1758)	0	0	0	1	10	18	0	0	0	0	1	0	0	0	0	30
Deltocephalinae																		
	C15	*Allygus modestus* Scott, 1876	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	2
	C16	*Allygus* sp.	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2
	C17	*Anoplotettix* sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	C18	*Artianus manderstjernii* (Kirschbaum, 1868)	11	0	0	0	0	0	1	0	0	2	0	0	0	0	0	14
	C19	*Artianus* sp.	6	3	0	0	0	0	0	1	0	0	0	0	0	0	0	10
	C20	*Balclutha punctata* (Fabricius, 1775)	5	2	4	0	0	6	0	0	7	36	14	43	1	4	2	124
	C21	*Balclutha* sp.	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	C22	*Circulifer* sp.	0	0	0	0	0	0	0	0	0	0	0	2	13	0	0	15
	C23	*Circulifer tenellus* (Baker, 1896)	0	2	0	0	0	0	0	0	1	0	0	0	31	8	1	43
	C24	*Doratura homophyla* (Flor, 1861)	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	3
	C25	*Doratura* sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	C26	*Euscelidius* sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	C27	*Euscelis incisus* (Kirschbaum, 1858)	14	0	0	29	0	0	29	1	0	9	12	2	8	0	0	104
	C28	*Euscelis lineolatus Brullé. 1832*	0	0	0	0	0	0	1	0	0	4	4	0	1	0	0	10
	C29	*Euscelis* sp.	7	3	7	7	1	2	19	3	3	2	1	2	6	5	1	69
	C30	*Goniagnathus brevis* (Herrich-Schäffer, 1835)	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	3
	C31	*Goniagnathus guttulinervis* (Kirschbaum, 1868)	2	1	1	2	2	1	1	1	0	6	0	1	0	0	0	18
	C32	*Goniagnathus* sp.	0	0	2	1	0	0	0	2	0	0	0	0	0	0	0	5
	C33	*Hardya tenuis* (Germar, 1821)	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	8
	C34	*Mocydiopsis oranensis* (Matsumura, 1908)	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	6
	C35	Deltocephalinae sp.1	0	0	0	5	10	6	0	0	0	0	0	0	0	0	0	21
	C36	Deltocephalinae sp.2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
	C37	Deltocephalinae sp.3	0	0	0	2	8	0	0	0	0	0	0	0	0	0	0	10
	C38	Deltocephalinae sp.4	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3
	C39	*Neoaliturus fenestratus* (Herrich-Schäffer, 1834)	3	8	0	3	2	1	5	2	4	6	2	6	0	1	0	43
	C40	*Paradorydium* sp.	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	3
	C41	*Phlepsius ornatus* (Perris, 1857)	0	1	0	0	0	0	0	0	0	5	0	3	0	0	0	9
	C42	*Phlepsius* sp.	1	0	0	0	0	0	4	0	0	1	1	1	0	0	0	8
	C43	*Psammotettix* sp.	109	194	213	15	42	35	10	141	53	108	116	97	2	3	6	1144
	C44	*Recilia coronifer* (Marshall, 1866)	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	C45	*Sardius argus* (Marshall, 1866)	3	16	0	0	0	0	0	1	1	0	4	3	1	0	0	29
	C46	*Scaphoideus titanus* Ball, 193	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
	C47	*Selenocephalus conspersus* (Herrich-Schäffer, 1834)	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	3
	C48	*Selenocephalus sacarroi* Rodrigues, 1968	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	11
	C49	*Selenocephalus* sp.	0	0	0	0	0	0	0	0	0	0	0	0	6	1	0	7
	C50	*Sonronius binotatus* (Sahlberg, 1871)	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	2
Dorycephalinae																		
	C56	*Eupelix cuspidata* (Fabricius, 1775)	0	3	5	0	1	7	0	0	0	0	0	1	0	0	0	17
Typhlocybinae																		
	C57	*Empoasca* sp.	0	5	1	2	0	2	0	0	0	8	99	24	0	2	0	143
	C58	*Empoasca vitis* (Göthe, 1875)	0	0	0	0	0	0	0	0	0	101	517	188	0	0	0	806
	C59	*Eupteryx melissae* Curtis, 1837	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
	C60	*Eupteryx* sp.	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
	C61	*Fruticidia bisignata* (Mulsant & Rey, 1855)	47	43	15	0	0	0	0	0	0	0	0	0	0	0	0	105
	C62	*Fruticidia sanguinosa* (Rey, 1891)	0	80	7	0	0	0	0	0	0	0	0	0	0	0	0	87
	C63	*Jacobiasca lybica* (de Bergevin & Zanon, 1922)	0	0	0	0	0	0	0	0	0	4	4	4	0	0	0	12
	C64	*Zygina schneideri* (Günthart, 1974)	34	23	0	0	0	0	0	0	0	0	0	0	0	0	0	57
	C65	*Zygina* sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2
	C66	*Zyginidia scutellaris* (Herrich-Schäffer, 1838)	2	10	7	2	163	61	0	4	0	55	46	12	0	0	1	363

Ulopinae																			
	C67 *Uteca* sp.	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	4
Membracidae																			
	C68 *Centrotus cornuta* Linnaeus, 1758	0	0	0	0	0	0	1	0	0	0	0	0	16	0	0	17
Total		261	424	305	82	250	221	93	186	81	387	853	424	102	44	21	3734



Chapter 5

Olfactory responses to volatile organic compounds and movement parameters of *Philaenus spumarius* and *Cicadella viridis*

Chapter 5

Adapted from:

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Chapter 5

Olfactory responses to volatile organic compounds and movement parameters of *Philaenus spumarius* and *Cicadella viridis*

Abstract

Xylella fastidiosa, the causal agent of several diseases in crops of economic interest, could be rapidly transmitted and spread throughout the agroecosystem landscape by xylem sap-feeding insects. Chemical signals in the environment drive the behavior of insects. Attractive or repulsive responses to signals may affect insects' fitness, survival, and reproduction and elicit different movement patterns. Specific movement patterns derived from olfactory cues toward the selection of plants for feeding may trigger the transmission of the pathogen by the insect. Thus, understanding vectors' olfactory response and movement parameters are of utmost importance. This work aimed to assess the olfactory response of *Philaenus spumarius* and *Cicadella viridis* adults to two Volatile Organic Compounds (VOCs) (cis-3-hexenyl acetate and cis-3-hexen-1-ol) present in almond, olive, and vine leaves. Insects' behavioral responses were conducted in a four-arm olfactometer, and the two aforementioned VOCs were tested at different concentrations (5, 10, 20, and 30 µg/µL). At the lowest concentration, females of *P. spumarius* were significantly attracted by the two VOCs. At the highest concentrations, no significant differences were detected among treatments. *Cicadella viridis* individuals showed no significant differences in their choice at any concentration. Additionally, the walking movement of these insects was also studied in the absence of food. Females of *P. spumarius* and *C. viridis* can walk significantly more at a significantly higher velocity than males. Knowledge of the olfactory response of *X. fastidiosa* vectors and their movement parameters can be essential to develop new tools to limit the spread of this phytopathogen.

Keywords: behavior; cis-3-hexen-1-ol; cis-3-hexenyl acetate; vectors; videotracking, *Xylella fastidiosa*.

Chapter 5

5.1. Introduction

Insect-borne plant pathogens are an increasing concern in global agriculture (Huang et al., 2020). They are causal agents of devastating diseases that pose imminent threats to the global economy, diversity conservation, and public health (Anderson et al., 2004; Huang et al., 2020; Schneider et al., 2020; Tumber et al., 2014). *Xylella fastidiosa* Wells et al., 1987 (Xanthomonadales: Xanthomonadaceae) is one of the most important and severe insect-borne plant pathogens described in Europe (Schneider et al., 2020). This phytopathogenic bacteria is a xylem-limited Gram-negative gammaproteobacterium, and it is the causal agent of insidious diseases in important economic crops worldwide such as the Almond Leaf Scorch, the Olive Quick Decline Syndrome, and the Pierce's disease of vine (Hopkins & Purcell, 2002; Saponari et al., 2013).

Xylella fastidiosa is transmitted exclusively by xylem feeding insects (Almeida et al., 2005) belonging to the infraorder Cicadomorpha (superfamilies Cercopoidea) (spittlebugs and froghoppers) and Cicadoidea (cicadas) and the family Cicadellidae (subfamily Cicadellinae (sharpshooters) (Cornara et al., 2019; Morente et al., 2018; Redak et al., 2004). When *X. fastidiosa* reaches a new location, the spread throughout the landscape could be mediated by native insect vectors (Redak et al., 2004).

The spittlebug, *Philaenus spumarius* (Linnaeus, 1758) (Cercopoidea: Aphrophoridae), and the sharpshooter *Cicadella viridis* (Linnaeus, 1758) (Membracoidea: Cicadellidae) are the most common xylem sap-feeding insects in Europe (Jeger et al., 2018; Rodrigues et al., 2014). In the European continent, *P. spumarius* is considered the main vector of *X. fastidiosa* due to a higher bacterial transmission rate than other vectors (Cavalieri et al., 2019). On the other hand, little is known about the ability of *C. viridis* to transmit the bacteria. Nonetheless, Bodino et al. (2019a) found in laboratory conditions that *C. viridis* was able to acquire *X. fastidiosa* through an artificial diet and then transmit it to periwinkle plants. However, this occurred at a low rate, and transmission plant-to-plant was not observed. The epidemiology of insect-borne plant pathogens relies on the vectors' population dynamics, movement, host selection, and feeding behavior (Eigenbrode et al., 2018). Understanding these factors is fundamental to assessing these plant pathogens' potential spread and implementing measures to limit their damage to the ecosystem.

The behavior of the insects is mainly mediated by naturally occurring semiochemical cues (Tumlinson, 2014). These cues are detected by olfactory sensilla located on the insect

antennae (Bruce & Pickett, 2011). The stimuli may derive a positive behavior toward a food source or a suitable oviposition site, or repulsive behaviors against predators, or toxic substances (Depetris-Chauvin et al., 2015). Volatile Organic Compounds (hereafter VOCs), emitted by plants, provide important cues driving the behavior of many leafhoppers and froghoppers in their search for hosts (Ganassi et al., 2020; Germinara et al., 2017; Mazzoni et al., 2009; Riolo et al., 2012). Plants can produce VOCs in response to the attack of herbivorous insects, conferring protection to the plant by acting as signalling compounds at different trophic levels (Arimura et al., 2009; Dicke & Baldwin, 2010; Holopainen & Blande, 2013; Ye et al., 2018). For example, the cis-3-hexen-1-ol is responsible for the green aroma in the leaves and is one of the most important VOCs in plants (Cofer et al., 2018). It can directly affect the physiology and behavior of herbivores through its attractive or repelling properties (Wei & Kang, 2011). The cis-3-hexen-1-ol can be esterified by alcohol acyltransferases yielding the ester cis-3-hexenyl acetate (Akacha & Gargouri, 2009; Ameye et al., 2018; Ozawa et al., 2013). Cis-3-hexenyl acetate is a sweet, apple, and banana-tasting compound that might act as an insect's infochemical and play an important role in triggering plant communication.

Identifying the VOCs emitted by plants and disentangling their role on the response of insect vectors, can contribute to the implementation of approaches to manipulate the behavior of these insects and implement sustainable control strategies such as the push-pull (Cook et al., 2007), lure-and-kill (El-Sayed et al., 2009), or attract-and-kill (Gregg et al., 2018).

These approaches are based on the use of specific VOCs to aggregate the target pest population in a specific location. Then, the individuals are subsequently removed or repelled from the main crop. Therefore, they also can lure the natural enemies of the pest, enhancing biological control. Indeed, several studies related that cis-3-hexenyl acetate mediated plant communication and can attract parasitoids when emitted from infested plants (Ameye et al., 2018; Cofer et al., 2018; Ozawa et al., 2013). Altogether, these suggest that these VOCs might play an essential role in host location and selection by inducing attraction or repulsion behavior.

Movement is also fundamental to animal behavior, it is responsible for governing how animals use habitats, social interaction, avoid predators, obtain food, and even adapt to human-modified landscapes (Wilson et al., 2015). Therefore, insect movement is considered an element of survival (Dickinson et al., 2000) and the main way to spread insect-borne plant pathogens throughout the landscape (Finke, 2012).

The most common modes of locomotion of *P. spumarius* and *C. viridis* are flying, jumping, and walking (Burrows, 2003, 2007). The movement parameters related to flying and jumping have been deeply studied (Burrows, 2003, 2007; Clemente et al., 2017; Lago et al., 2021; Weaver & King, 1954); however, those related to walking were largely neglected.

Spittlebug adults are poor fliers, opting to walk or jump more often instead of flying (Cornara et al., 2018). During horizontal movements, *P. spumarius* walk with the hind legs usually dragged (Burrows, 2003, 2006). On the contrary, *C. viridis* uses all the legs to walk (Burrows, 2007). Weaver & King (1954) observed that adults of *P. spumarius* travelled more than 30 m in a single flight and moved as much as 100 m within one day, whereas Bodino et al. (2020) found that individuals were able to disperse up to 400 m during the population peak in Italy. Nevertheless, Lago et al. (2021), using a flight mill, concluded that spittlebugs were able to move up to 500 m in a 30 min flight. This insect can also jump up to 70 cm above the ground with an acceleration of 400 m s^{-2} (Burrows, 2003). Regarding *C. viridis*, adults move around the landscape, in response to visual or olfactory stimuli, by jumping or by performing short flights (Beok, 1972). On average, *C. viridis* can perform jumps with a take-off velocity of 0.88 m s^{-1} and a constant acceleration of 152 m s^{-2} (Bonsignori et al., 2013).

Philaenus spumarius and *C. viridis* are polyphagous insects that feed on a wide range of plant species (Bodino et al., 2020; Dmitriev & Nickel, 2006) due to the low nutritional value of their food (Ranieri et al., 2016). Once the insect acquires the bacteria, the transmission of *X. fastidiosa* to other plants is a fast process, and the inoculation can occur in 2 to 7 minutes after the first probe (Cornara et al., 2020b). Therefore, understanding the olfactory response of both vectors to VOCs and the extent to which the pathogen can be spread throughout the landscape according to their spatial dynamics (*e.g.,* velocity and distance moved) will help limit the spread or even prevent the diseases.

The main goal of this work was to characterize the volatile profile of the three most important crops in the Mediterranean region (olive, almond, and vine) and to study the olfactory response of adults *P. spumarius* and *C. viridis* to two VOCs common to the three crops (cis-3-hexenyl acetate and cis-3-hexen-1-ol). Additionally, the movement parameters (1) distance moved, (2) mean velocity, and (3) total time moving of both species' insects were also assessed.

5.2. Materials and methods

5.2.1. Volatile characterization

The volatile profile of almond tree (cv. Verdeal), olive tree (cv. Cobrançosa), and vine (cv. Touriga Nacional) leaves were evaluated through HS-SPME (headspace solid-phase microextraction) and GC/MS (gas chromatography with mass spectrometry detector).

HS-SPME

For each plant species, approximately 4 g of healthy leaves were placed in 50 mL individual vials, with the leaf petiole protected with aluminium foil, to avoid the potential registration of volatiles produced after cutting the petiole. The vials were sealed with a polypropylene cap with a silicon septum. After sealing, 5 µL of 4-metil-2-pentanol (0.127 mg/ mL) (Sigma Aldrich, USA) were added with a syringe to the vials as an internal standard. In a water bath, the volatiles were released at 40 ºC for 5 min. Then, a fiber coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS 50/30 µm) (Supelco, Bellefonte, USA), was exposed to the headspace for volatile adsorption. Vine leaves have a larger leaf area than olive and almond leaves. Therefore, there is a greater release of volatiles which could lead to sensor saturation. After some trial tests, the headspace of 3 min was the most adequate for the vine leaves and the headspace of 15 min for olive and almond leaves. For each plant species, five replicates of HS-SPME analysis were performed.

GC-MS conditions

Chromatographic analysis was performed using a Shimadzu GC-2010 Plus. The volatile compounds were eluted from the fiber by thermal desorption for 1 min in the injection port of the chromatography system (220 °C). The fiber was maintained for another 10 min in the injector port for cleaning and conditioning for further analyses. The gas chromatographer used was a Shimadzu GC-2010 Plus equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. A TRB-5MS (30 m × 0.25 mm × 0.25 µm) column (Teknokroma, Spain) was used. The injector was set at 220 °C, and the manual injections were made in splitless mode, with helium (Praxair, Portugal) at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min as mobile phase. The oven temperatures were: 40 °C (1 min); 2 °C/min until 220 °C (30 min). The ionization source was maintained at 250 °C with an ionization energy of 70 eV, and with an ionization current of 0.1 kV. All mass spectra were acquired by electron ionization in the m/z 35–500 range. The full scan MS spectra fragments were compared with those obtained from the NIST 69 Library

(National Institute of Standards and Technology, Gaithersburg, MD, USA) and those of commercial standards acquired from diverse producers. The areas of the chromatographic peaks were determined by integrating the re-constructed chromatogram from the full scan chromatogram using the ion base (m/z intensity 100 %) for each compound. For semi-quantification purposes, the amounts of the identified volatiles were calculated by the ratio of each base ion peak area to the area of the internal standard base ion peak area, without considering the response factors, and converted to mass equivalents based on the IS mass used.

5.2.2. Collection of the insects

For the olfactory and movement parameter assays, between April and July 2020, adults of *P. spumarius* and *C. viridis* were collected from the natural ground vegetation in the Campus of the Polytechnic Institute of Bragança (41º 47' 53.2" N, 6º 45' 51.5" W), with a standard entomological sweep net. The individuals were individually selected with a mouth aspirator. After collection, the insects were transferred onto Lavandula sp. plants in aerated cages (40 cm in height, 30 cm in length, 43 cm in width). The cages were then placed in rearing chambers at 18 °C aiming to reduce the activity of the individuals and subsequently maximize the survival, with 70 % relative humidity and a 16:8 h (L:D) photoperiod. After a week in these conditions, the insects were used in the olfactory and movement parameter assays.

5.2.3. Multiple-choice olfactory response assays

A four-arm olfactometer (Figure 5.1) was used to test the behavioral responses of *P. spumarius* and *C. viridis* adults to the VOCs cis-3-hexenyl acetate (Sigma Aldrich, Switzerland) and cis-3-hexen-1-ol selected (Sigma Aldrich, USA) (see section 5.1.). To avoid the rapid evaporation of these volatiles, they were dissolved in sunflower oil (VitaD'or, Portugal) and stored at -20 °C until utilization. Four different concentrations (5, 10, 20, and 30 µg/µL) of each volatile compound were prepared following Ganassi et al. (2020). For the test, each arm of the olfactometer arena was connected to a polypropylene tube (3 cm long and 1 cm in diameter) that worked as an odor source container. Each odor source container was connected to a gas washing bottle (250 mL) with activated charcoal (AppliChem, Panreac ITW ©) dissolved in 100 mL of distilled water to purify and humidify the airflow supplied by pumps with an airflow of 12 cm^3/min. Each volatile compound was applied (10 µg) separately to strips of filter paper (4 mm × 25 mm) placed in the odor source containers of two opposite olfactometer arms. The

remaining olfactometer arms operated as controls. A strip of filter paper soaked in 10 µg of sunflower oil was placed in one of them, whereas the opposite arm contained a blank strip of filter paper. The air streams containing the odors were directed towards a four-entry arena (25 cm × 25 cm). The arena was built with four 4 mm high overlapping acrylic layers. The bottom layer was a white opaline plate with a 25 × 25 cm, and the following two layers were white opaline triangles (12 cm × 12 cm × 17 cm) positioned in each corner of the bottom layer to create the arena for the insect. The top layer consisted of a transparent plate (25 cm × 25 cm) with a central hole (4 mm in diameter) to allow insect insertion and airflow. Before the olfactometer assay, insects were sexed using a binocular stereoscopic microscope and kept individually in 2 mL Eppendorf tubes to be starved for 4 h. After starvation, one insect each time was released into the central hole of the arena, and its behavior was recorded for 20 minutes with Computar® lens (H2Z0414C-MP, f=4-8 mm, F 1.4, ½", CCTV lens) mounted on a Basler®GigE HD Camera (acA1300-60gc with e2v EV76C560 CMOS sensor) (Noldus, 1991). The recording tool used was the Media Recorder 2.5 software (Noldus Media Recorder, 2013). The olfactometer was illuminated from below using 20 white cold LED lamps that provide uniform illumination (7500 ± 10 lux). A choice was considered valid if the individual accessed a designed area of 1.4 cm^2 around each air entry. Choice was codified through two behavioral parameters, (1) the frequency of visits (*i.e.,* the number of times the insect accessed an area) and (2) total length of stay in each area (*i.e.,* sum of the time in each area). For each VOC concentration tested, 30 females and 30 males of *P. spumarius* and *C. viridis* were used, respectively. The assays were conducted in a climate chamber at 21 °C and 70 %RH. This temperature was chosen to increase the activity of the insects (formerly maintained at 18 °C) and to avoid the rapid volatilization of VOCs. The olfactometer was rotated 180° after 15 tests to avoid directional bias.

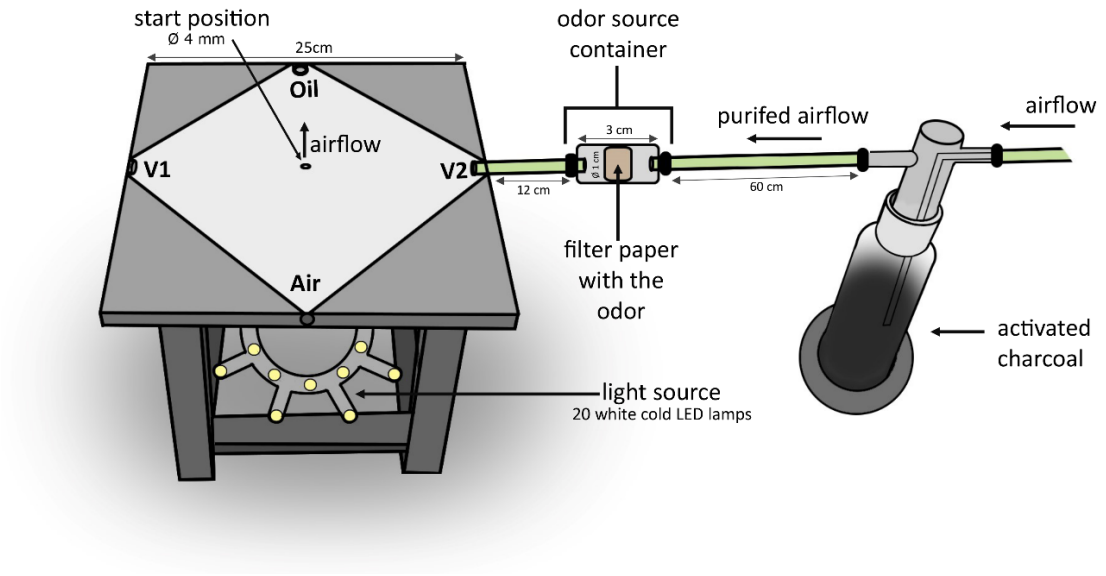

Figure 5.1. Schematic drawing of the four-arm olfactometer built to conduct the olfactometric assays (not drawn to scale).

5.2.4. Estimation of movement parameters

To estimate the movement parameters of *P. spumarius* and *C. viridis*, a total of 30 males and 30 females, respectively, were recorded individually in an arena (27 cm × 35 cm × 43 cm) for 10 minutes with the same camera and recording tool described in section 2.3. The arena was made of acrylic and illuminated from below (negative contrast) using a computer screen placed horizontally with a full white Microsoft PowerPoint slide loaded. Before each trial, the insects were maintained in the rearing plants and immediately transferred to the arena for the assay. The assays were conducted in a climate chamber at 21 °C 70 %RH and in the absence of olfactory stimuli.

5.2.5. Data analysis

Volatile characterization

Analysis of variance (ANOVA) was performed using the software PAST v.4.03 (Hammer et al., 2001) to compare the volatile profile between the crops. Subsequent multiple comparison posthoc tests were applied using the Tukey's test ($\alpha < 0.05$). Additionally, a Principal Component Analysis (PCA) was conducted in R software v.3.5.1 (R Core Team, 2020) using the function pca

from the "FactoMineR" package (Le et al., 2008). The correlation biplot of the two first PCs was drawn using the fviz_pca_biplot function from the "factoextra" package (Kassambara & Mundt, 2020).

Multiple-choice olfactory response assays

For each species, the behavior was analyzed with the Noldus Observer XT 11.5 software (Noldus et al., 2001). Generalized Estimating Equations (α = 0.05) with Poisson distribution were used to compare the frequency of visits and the total length of stay of the individuals in each delimited area. Sex and choice (air, oil, cis-3-hexenyl acetate, and cis-3-hexen-1-ol) selected were considered response variables and the interaction between the two terms was also used as explanatory variable. The frequency of visits and the total length of stay in each area was assessed and compared between the choices using posthoc Tukey's tests (α = 0.05) for males and females separately. Since all the insects exhibited activity, no individuals were excluded from the analysis. The model was developed in R using the geeglm function from packages "geepack".

Estimation of movement parameters

The total distance moved (m), mean velocity (cm/s), and the total time moving (s) were estimated using the Noldus Ethovision XT 11.5 software (Noldus et al., 2001). Each movement parameter was compared between females and males of *P. spumarius* and *C. viridis* using a Student's t-test (α = 0.05) in PAST.

5.3. Results

5.3.1. Volatile characterization

In total, 83 compounds were identified from the three plant species (Supplementary Table S5.1). The olive leaves presented the highest number of VOCs with 54 identified compounds, followed by almond and vine leaves with 40 and 16 identified compounds, respectively. The main components in olive leaves were cis-3-hexen-1-ol, cis-3hexenyl acetate, Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester and Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester. Almond leaves were also dominated by cis-3-hexen-1-ol, and cis-3hexenyl acetate, additionally, the compounds n-Hexane and D-Limonene also were present at a high percentage. The vine leaves presented the lowest number of VOCs, being cis-3hexenyl acetate, (Z)-, Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-

trimethylpentyl ester, Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester, and cis-3-hexen-1-ol the most frequent.

According to the PCA and ANOVA analyses, the volatile profiles significantly differed between plant species (Supplementary Figure S5.1 and Supplementary Table S5.1). PC1 and PC2 explained 82.1 % of the variation.

Among the five common VOCs to the three plants species (cis-3-hexen-1-ol; cis-3-hexenyl acetate; hexyl ester; D-Limonene, and Nonanal), cis-3-hexen-1-ol and cis-3-hexenyl acetate were found in a higher frequency. Their amounts were respectively for almond, olive, and vine leaves, of 42.07±10.38 μg/g, 29.87±4.83 μg/g, and 1.80±0.64 μg/g, for cis-3-hexen-1-ol; and 28.29±6.22 μg/g, 4.87±1.93 μg/g, and 31.77±7.11 μg/g, for cis-3-hexenyl acetate. Therefore, these two compounds were selected for the olfactory assay due to their frequency and quantity.

5.3.2. Multiple-choice olfactory response assays

At the lowest VOCs concentration (5 μg/μL), the females of *P. spumarius* were significantly attracted by the two volatile compounds (Table 5.1 and Figure 5.2). Females showed a significantly higher frequency of visits and a longer permanence in the areas with cis-3-hexenyl acetate and cis-3-hexen-1-ol only at the lowest concentration (Figure 5.3), whereas males of *P. spumarius* showed a significantly higher frequency of visits and longer stay only in the area with cis-3-hexen-1-ol compared to oil.

The frequency of visits significantly differed between the sexes except when the individuals were exposed to the volatile compounds at 10 μg/μL (Table 5.1). For this concentration, the stream of purified air was significantly more visited by females, whereas no significant choice was observed in the case of males (Figure 5.2). Regarding the total length of stay in each area, was not significantly influenced by treatment or sex for the highest concentrations (20 μg/μL and 30 μg/μL) (Table 5.2). At these concentrations (20 μg/μL and 30 μg/μL), no significant differences were detected in the choice of *P. spumarius*.

In the case of *C. viridis*, neither the frequency of visits nor the total length of stay significantly differed between treatments for any concentration (Table 5.1 and Table 5.2). However, the frequency of visits to each treatment varied significantly between sex for all the concentrations (Table 5.1).

Table 5.1. Results of the GEEs developed for the effect of tested treatments and sex as well as their interaction on the frequency of visits of *Philaenus spumarius* and *Cicadella viridis* in a 4-choice olfactometer.

Concentration	Independent variable	Response variable	*Philaenus spumarius*			*Cicadella viridis*		
			df	χ^2	P	df	χ^2	P
5 µg/µL	Choice	Frequency	3	40.50	<0.01	3	3.98	0.263
	Sex		1	5.90	0.015	1	10.63	<0.01
	Choice:Sex		3	28.10	<0.01	3	3.68	0.298
10 µg/µL	Choice	Frequency	3	9.32	0.524	3	6.42	0.093
	Sex		1	0.41	0.524	1	29.86	<0.01
	Choice:Sex		3	9.07	0.028	3	1.10	0.777
20 µg/µL	Choice	Frequency	3	3.33	0.343	3	4.79	0.188
	Sex		1	12.96	<0.01	1	6.80	<0.01
	Choice:Sex		3	3.98	0.263	3	1.74	0.628
30 µg/µL	Choice	Frequency	3	0.51	0.920	3	3.45	0.327
	Sex		1	29.98	<0.01	1	10.22	<0.01
	Choice:Sex		3	1.26	0.740	3	11.12	0.011

Table 5.2. Results of the GEEs developed for the effect of tested treatments and sex as well as their interaction on the length of stay of *Philaenus spumarius* and *Cicadella viridis* in a 4-choice olfactometer.

Concentration	Independent variable	Response variable	*Philaenus spumarius*			*Cicadella viridis*		
			df	χ^2	P	df	χ^2	P
5 µg/µL	Choice	Length of stay	3	50.30	<0.01	3	5.59	0.1332
	Sex		1	8.30	<0.01	1	8.70	<0.01
	Choice:Sex		3	20.40	<0.01	3	4.41	0.215
10 µg/µL	Choice	Length of stay	3	14.81	<0.01	3	3.05	0.383
	Sex		1	2.44	0.1181	1	20.47	<0.01
	Choice:Sex		3	15.41	<0.01	3	9.18	0.027
20 µg/µL	Choice	Length of stay	3	9.35	0.025	3	15.93	<0.01
	Sex		1	0.48	0.486	1	1.06	0.304
	Choice:Sex		3	1.34	0.72	3	3.42	0.332
30 µg/µL	Choice	Length of stay	3	5.03	0.170	3	3.00	0.390
	Sex		1	0.00	0.970	1	0.41	0.520
	Choice:Sex		3	1.54	0.670	3	4.71	0.190

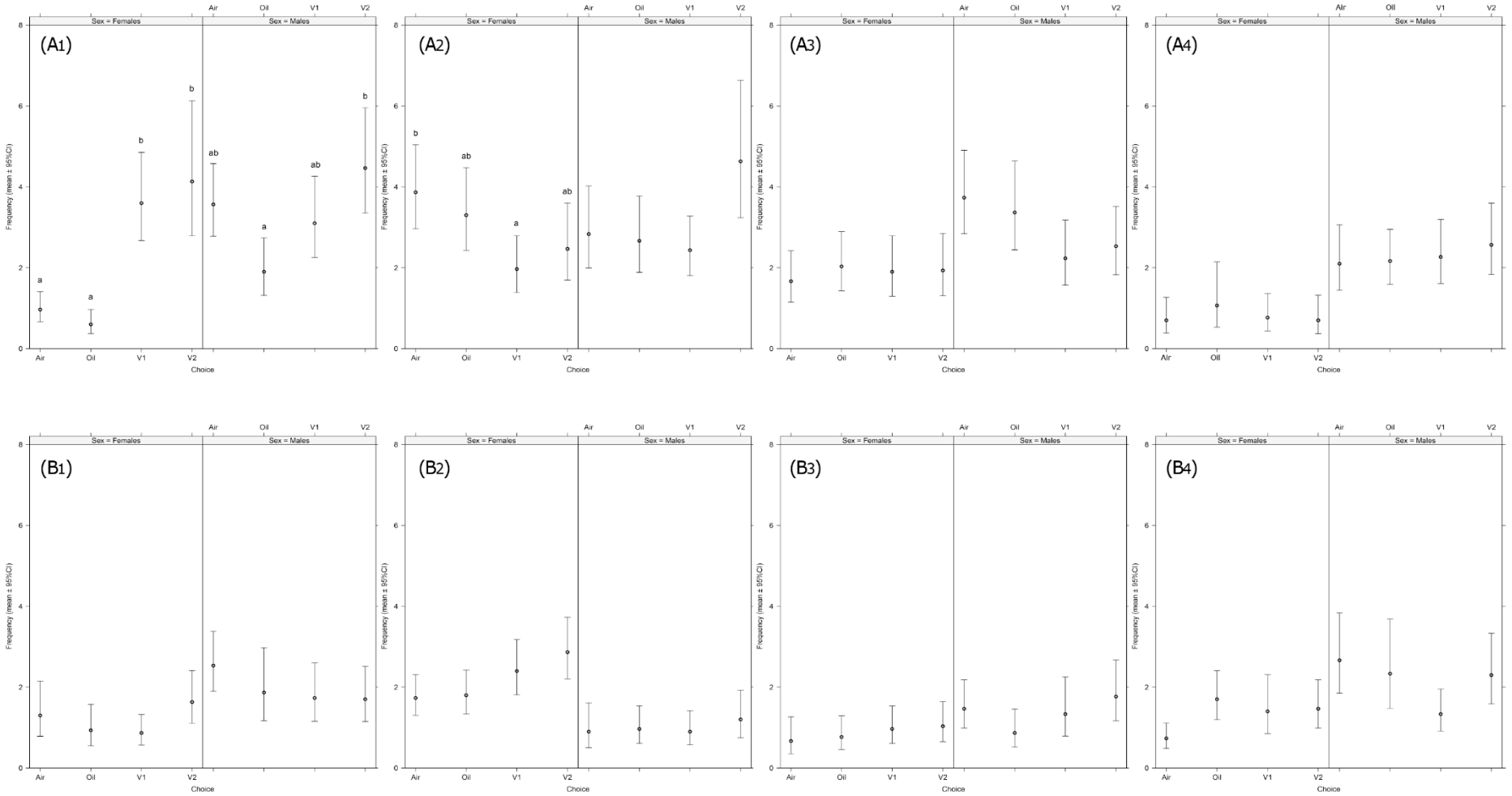

Figure 5.2. Number of visits (mean ± 95 %CI) of females and males of Philaenus spumarius (A) and Cicadella viridis (B) to cis-3hexenyl acetate (V1) and cis-3-hexen-1-ol (V2), Air and Oil in a 4-choice olfactometer at different concentrations of volatiles (5, 10, 20, and 30 µg/µL).

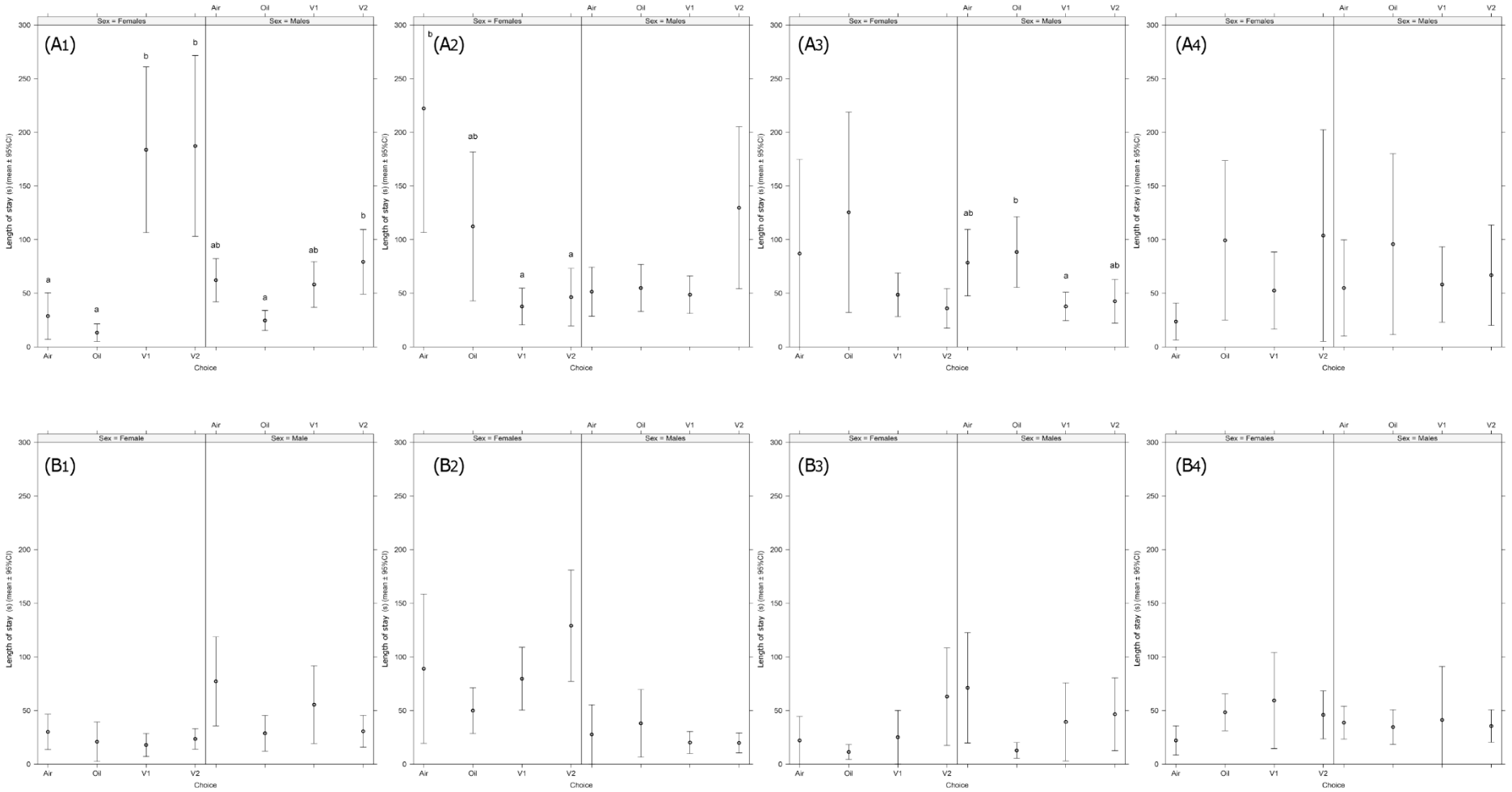

Figure 5.3. Total length of stay (mean ± 95%CI) of females and males of *Philaenus spumarius* (A) and *Cicadella viridis* (B) to cis-3-hexenyl acetate (V1) and cis-3-hexen-1-ol (V2), Air and Oil in a 4-choice olfactometer at different concentrations of volatiles (5, 10, 20, and 30 µg/µL).

5.3.3. Estimation of movement parameters

Females of *P. spumarius* were able to walk a total distance of 2.42 ± 0.20 m in 10 minutes, with a mean velocity of 0.43 ± 0.04 cm/s. Both movement parameters (total distance and mean velocity) were significantly higher compared with the males (P < 0.01) (Figure 5.4A1 and 5.4A2). Regarding the total time of moving, there were no significant differences between males and females (P = 0.81) (Figure 5.4A3). Females of *C. viridis* walked with mean velocities significantly higher (P < 0.01) than the males (Figure 5.4B1 and 5.4B2). In 10 minutes, females of *C. viridis* walked 3.13 ± 0.33 m with a mean velocity of 0.63 ± 0.07 cm/s. Females also were moving significantly more time than males (Figure 5.4B3).

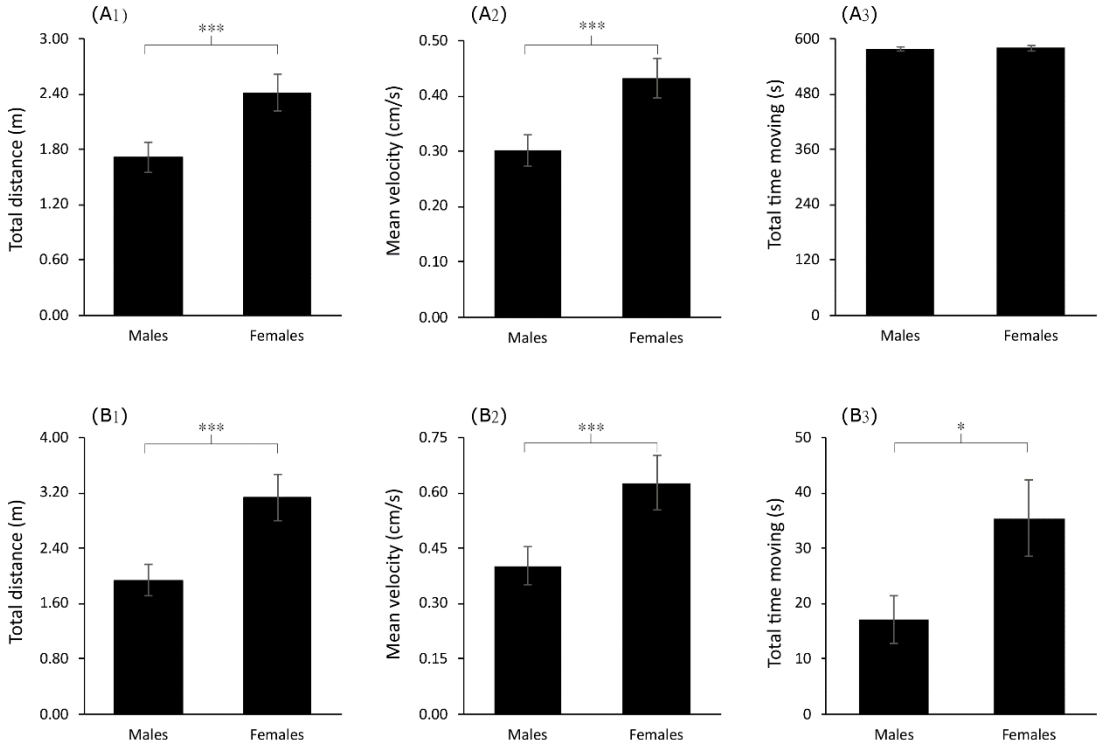

Figure 5.4. Movement parameters (walk) obtained for males and females of (A) *Philaenus spumarius* and (B) *Cicadella viridis*. (1) Distance walked (mean ± SE) in 10 minutes; (2) Velocity (mean ± SE); (3) Total time walking (mean ± SE). Significant differences are showed by asterisks (***P < 0.001; *P = 0.02).

5.4. Discussion

This work evaluated the olfactory response of adults of *P. spumarius* and *C. viridis* to cis-3-hexen-1-ol, and cis-3-hexenyl acetate, the main volatiles emitted by the leaves of the almonds,

olives, and vines. Also, movement parameters by walking of these individuals, without food stimulus, were analyzed. The development of innovative tools and approaches, as an alternative to synthetic pesticides, for the environmentally friendly control of *P. spumarius* and other potential vectors is essential to limit the dissemination of *X. fastidiosa*. Understanding the mechanisms of vectors of pathogens to locate and select host plants and their movement parameters are fundamental to the development of such approaches.

Philaenus spumarius and *C. viridis* spend most of their life cycle in the spontaneous ground cover vegetation. Nevertheless, they may shelter in the main crop plants such as the olive trees, where they feed in the late spring, due to mowing or a decrease in succulence of herbaceous plants (Cornara et al., 2019; Dongiovanni et al., 2019; Morente et al., 2018). *Xylella fastidiosa* can be transmitted to the insect from spontaneous ground cover vegetation such as *Lavandula* spp., *Hypericum perforatum* L., and *Artemisia* sp. (*e.g.,* Delbianco et al., 2022; DGAV, 2020, in Portugal) or from the main crop plants. Regardless of where the bacteria was acquired, once the individuals are infected, the transmission of *X. fastidiosa* to the crop plants may then occur (Cornara et al., 2017b).

Crops of economic importance, such as almond orchards, vineyards, and olive orchards, are key crops in the landscape across the Mediterranean basin (FAOSTAT, 2021). Our results showed that these crops have a very distinct volatile profile. In our work, the leaves of the olive tree, the main crop affected by *X. fastidiosa* in Europe (Saponari et al., 2019), showed the highest number of VOCs compared to the other two plants. These results agree with Malheiro et al., (2016), which found cis-3-hexen-1-ol and cis-3-hexenyl acetate the main components in the olive tree leaves. We also found these compounds to be abundant in the almond and vine leaves. Cis-3-hexen-1-ol and cis-3-hexenyl acetate are generated through the oxylipin pathway from C18-polyunsaturated fatty acids (FAs; α-linolenic acid (ALA) and linoleic acid (Kost & Heil, 2006). Both are naturally occurring VOCs in the plants, and their occurrence at a high percentage is related to tissue damage (Ameye et al., 2018). Since we used intact and healthy leaves from field plants, the high level of cis-3-hexen-1-ol and cis-3-hexenyl acetate could be due to abiotic stress such as high temperature or plant water deficit. In fact, according to Sofo et al. (2004), the synthesis of these compounds is more actively observed in stressed plants under drought conditions. Also, Malheiro et al. (2016) suggested that higher temperatures can mediate the emission of the two VOCs in the absence of tissue damage.

Spittlebugs interact socially by vibrational signals (Avosani et al., 2020), and pheromones are not common. Recent studies described that spittlebugs have a wide range of responses to VOCs, despite having a low number of antennal sensory structures (Ranieri et al., 2016). This fact indicates that spittlebugs use this type of stimuli to choose and locate host plants. Germinara et al. (2017) suggested that both sexes of spittlebugs have a general similarity in antennal sensitivity; However, we found a positive attraction to cis-3-hexen-1-ol and cis-3-hexenyl acetate only by females of *P. spumarius* for the lowest concentration tested. Furthermore, cis-3-hexenyl acetate can elicit an electroantennographic response (EAR) in other spittlebugs such as *Neophilaenus campestris* (Fallén, 1805) (Anastasaki et al., 2021). Although the main compounds found by Anastasaki et al. (2021) from olive plants were the terpenes trans-β-ocimene, α-pinene, and α-copaene, the VOC cis-3-hexenyl acetate was also present, nonetheless, it did not trigger an EAR in females of *P. spumarius*. On the other hand, they found a positive EAR when testing the VOC obtained from the extract of *Lolium arundinaceum* (Schreb.) Darbysh.

It has been shown that the antennae of male and female spittlebugs are sensitive to changes in stimuli concentration. Germinara et al. (2017) found a dose-dependent EAG response for both sexes through stimulation with increasing concentrations of cis-3-hexen-1-ol. In contrast, our results showed that the increase of the volatile concentration did not significantly influence the choices of *P. spumarius* which suggests that the increase in the concentration of volatiles can lead to sensilla saturation.

The sex significantly influenced the choice made by *P. spumarius* and *C. viridis* for the same volatiles except at 10 μg/μL in the case of *P. spumarius*. These results agree with those obtained by Ganassi et al. (2020), which observed different responses to odorant by males and females when individuals of *P. spumarius* were submitted to close (Y-tube) and long-range (wind tunnel) behavioral bioassays with essential oil and related plants.

In this work, the individuals of *C. viridis* presented no attraction to any volatile for any concentration. These results support that most species of the Cicadellidae detect suitable host plants based mainly on visual cues or with the combination of olfactory and visual stimulus (Bullas-Appleton et al., 2004; Cai et al., 2015; Grange et al., 2017; Todd et al., 1990). Since our study only focused on olfactory stimulation, it may explain why *C. viridis* did not make any choice: However other VOCs or blends could trigger a response. Future studies on the sensilla,

electrophysiological, olfactory, and visual behavior are essential to understanding how this leafhopper proceeds to locate and choose the host plants.

Several studies on the movement of spittlebugs and leafhoppers focused on jumping or flight performance (*e.g.,* Beok, 1972; Bodino et al., 2021a; Bonsignori et al., 2013; Burrows, 2003, 2007; Clemente et al., 2017; Goetzke et al., 2019; Lago et al., 2021). The body of *P. spumarius* and *C. viridis* is designed essentially for jumping, and they have long hind legs to increase leverage for jumping, wedge-shaped heads, and rigid front wings that form a continuous smooth structure to reduce drag when jumping (Burrows, 2003, 2007). However, they can also move by walking. The larger body size and longer legs of *C. viridis* (Cornara et al., 2018; Burrows, 2007) may allow it walking faster and longer distances than *P. spumarius*, although further studies are needed to compare the performance between both species. Beok (1972) suggested that *C. viridis* movements are trivial and confined within the habitat and individuals have been observed to stay on the same spot for days. This agrees with the low activity we found for the species in this work. On the contrary, *Philaenus spumarius* has a migratory behavior. Several authors described that *P. spumarius* begins his migratory journey at early summer, coinciding with the death of the vegetation cover until September, returning to the vegetation cover to lay eggs (Antonatos et al., 2020; Cruaud et al., 2018; Morente et al., 2018).

We found the females of both species being significantly faster and traveling longer than males. Although we did not study the flight, our results in terms of velocity and distance travelled, agree with Lago et al. (2021), which found better female performance in terms of distance traveled and flight duration.

5.5. Conclusions

In the present study, it was possible to verify that females of *P. spumarius* are attracted by the two VOCs under study, but only when they are in low concentration. Since the individuals of *C. viridis* did not present any significant choice, further tests on the sensilla and the electroantennographic responses may help better understand the role of olfactory cues in the selection of host plants by the species. The two VOCs studied occur naturally in olive, almond, and vine, crops that can host *X. fastidiosa*. Females of *P. spumarius* can play a key role in disseminating *X. fastidiosa* due to their ability to walk longer distances with higher speeds than males. Further research on the olfactory response of the vectors of *X. fastidiosa* and their

movement behavior is essential to design new techniques to limit the spread of this pathogen throughout Europe.

Acknowledgments

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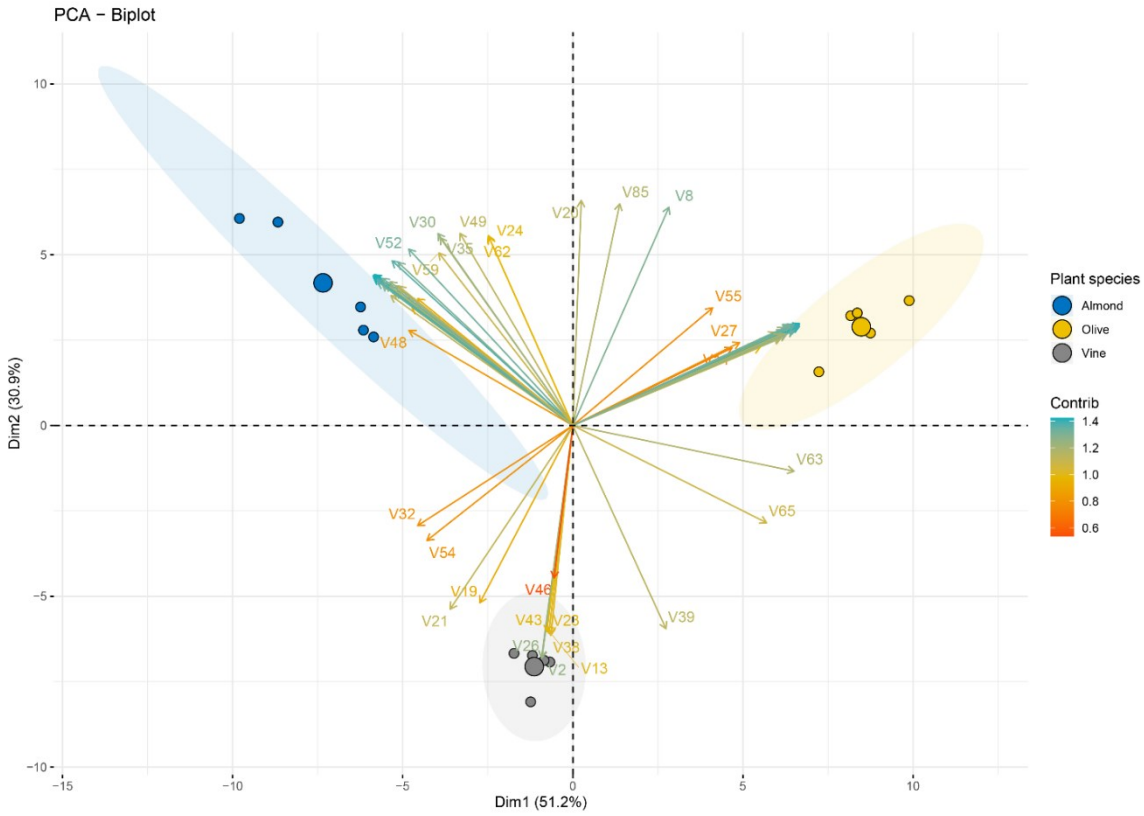


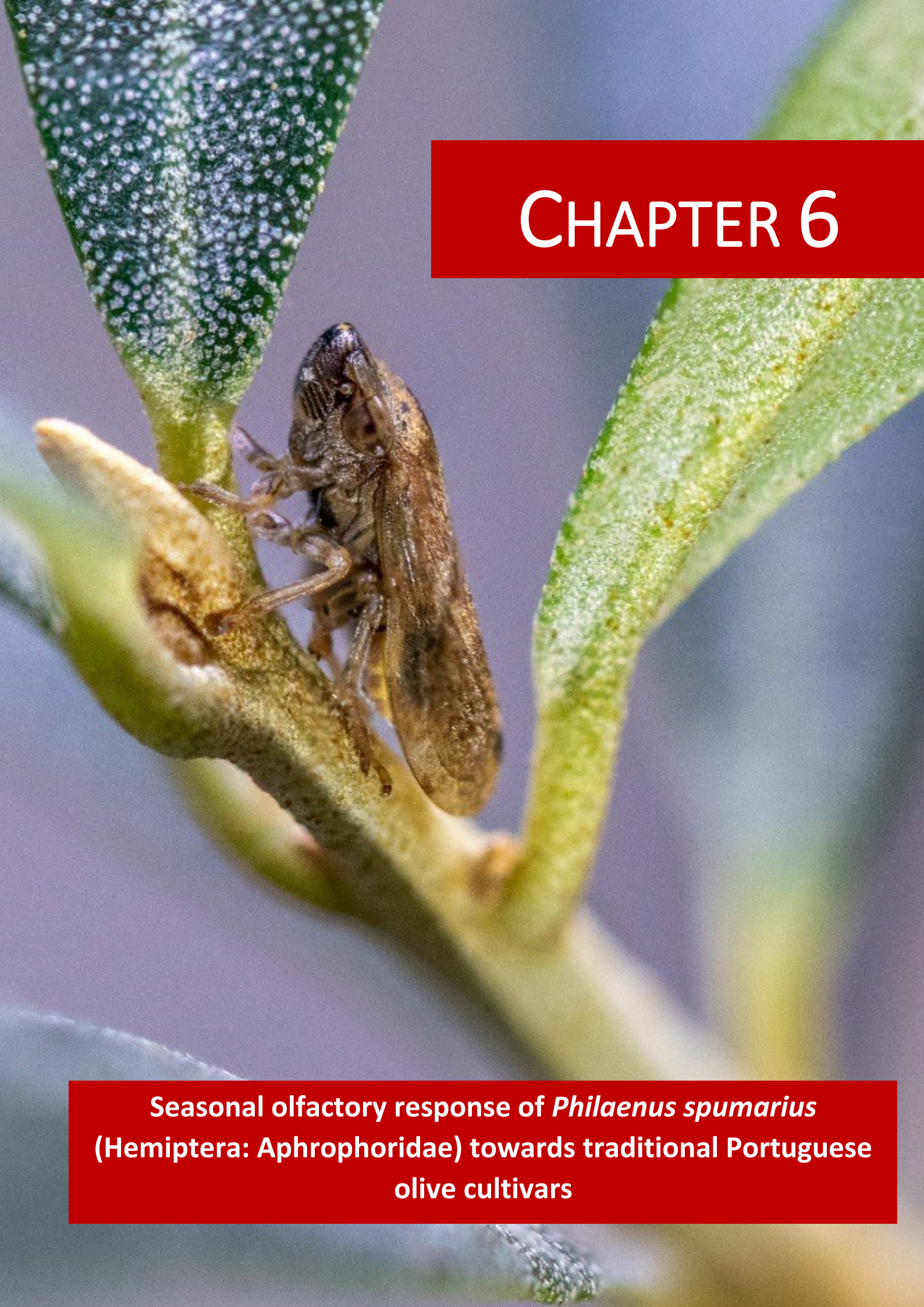
Supplementary Figure S5.1. Principal component analysis (PCA) biplot of volatile organic compounds (VOCs) emitted by the leaves of the almond, olive, and vine plants.

Chapter 5
Supplementary material

Supplementary Table S5.1. Volatile composition (mean percentage ± SE) of the almond, olive, and vine leaves. In the same line, mean values with different letters differ significantly (P < 0.05).

	Compound	Almond leaves	Olive leaves	Vine leaves	P
V1	(.+/-.)-Lavandulol, chlorodifluoroacetate	-	0.071 ± 0.005	-	-
V2	1,3,5-Cycloheptatriene, 3,7,7-trimethyl-	-	-	0.283 ± 0.040	-
V3	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	1.085 ± 0.272	-	-	-
V4	1,3-Pentanediol, 2,2,4-trimethyl-	-	0.503 ± 0.041	-	-
V5	1,4-Hexadiene, 5-methyl-3-(1-methylethylidene)-	0.143 ± 0.031	-	-	-
V6	1,5-Hexadien-3-ol	-	0.382 ± 0.029	-	-
V7	1,6-Octadien-3-ol, 3,7-dimethyl-	0.576 ± 0.049	-	-	-
V8	1-Hexanol	2.384 ± 0.212a	3.373 ± 0.266b	-	0.023
V9	1-Nonanol	-	0.094 ± 0.015	-	-
V10	2(3H)-Furanone, 5-ethyldihydro-	-	0.162 ± 0.019	-	-
V11	2(5H)-Furanone, 5-ethyl-	-	0.126 ± 0.051	-	-
V12	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	-	0.258 ± 0.039	-	-
V13	2,3-Pentanediol, 2,4-dimethyl-	-	-	0.294 ± 0.078	-
V14	2,4,4-Trimethyl-1-hexene	-	0.081 ± 0.012	-	-
V15	2,4-Dodecadienal, (E,E)-	-	0.129 ± 0.023	-	-
V16	2,4-Hexadienal, (E,E)-	-	0.463 ± 0.074	-	-
V17	2-Hexenal, (E)-	-	0.428 ± 0.118	-	-
V18	2-Hexenoic acid, methyl ester	0.279 ± 0.026	-	-	-
V19	3-Hexen-1-ol, (Z)-	41.099 ± 3.559b	36.987 ± 3.294b	4.091 ± 0.576a	<0.001
V20	3-Hexen-1-ol, acetate, (Z)-	28.604 ± 1.150b	9.512 ± 2.121a	53.604 ± 7.715c	<0.001
V21	3-Hexen-1-ol, formate, (Z)-	-	0.074 ± 0.015	-	-
V22	3-Hexenal	-	-	1.100 ± 0.275	-
V23	3-Hexenoic acid, methyl ester, (Z)-	1.911 ± 0.387	0.871 ± 0.255	-	0.053
V24	3-Pentanone	-	0.726 ± 0.067	-	-
V25	4-Hexen-1-ol, acetate	-	-	0.225 ± 0.030	-
V26	4-Tridecene, (Z)-	0.156 ± 0.025	-	-	-
V27	5-Hepten-2-ol, 6-methyl-	0.609 ± 0.253	-	-	-
V28	5-Hepten-2-one, 6-methyl-	0.166 ± 0.031b	0.051 ± 0.013a	-	0.009
V29	5-Octadecene, (E)-	-	0.089 ± 0.010	-	-
V30	Acetic acid, hexyl ester	0.788 ± 0.118ab	0.253 ± 0.032a	0.993 ± 0.226b	0.012
V31	Alloaromadendrene	0.390 ± 0.050	-	-	-
V32	Benzaldehyde	0.289 ± 0.077	-	-	-
V33	Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]-	0.097 ± 0.016b	0.031 ± 0.002a	-	0.04
V34	Benzocyclobutene	-	0.347 ± 0.054	-	-
V35	Benzoic acid, methyl ester	0.077 ± 0.022	-	-	-
V36	Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-methyl-6-vinyl-	-	-	0.447 ± 0.115	-
V37	Butanoic acid, 3-hexenyl ester, (E)-	0.093 ± 0.009a	0.433 ± 0.059b	0.755 ± 0.099c	<0.001
V38	Butanoic acid, 3-hexenyl ester, (Z)-	-	0.076 ± 0.015	-	-
V39	Butanoic acid, heptyl ester	-	0.243 ± 0.023	-	-
V40	Butanoic acid, hexyl ester	-	0.068 ± 0.016	-	-
V41	Butyric acid, 2,2-dimethyl-, vinyl ester	-	-	0.225 ± 0.068	-
V42	Caryophyllene	-	0.817 ± 0.108	-	-
V43	Cyclohexanone, 2-methyl-	0.232 ± 0.029	-	-	-
V44	Cyclopropanecarboxylic acid, dodec-9-ynyl ester	-	-	0.352 ± 0.207	-
V45	Decanal	-	0.087 ± 0.025	-	-
V46	D-Limonene	3.851 ± 1.560	0.528 ± 0.106	1.268 ± 0.274	0.05
V47	Dodecane	0.088 ± 0.010b	0.032 ± 0.008a	-	0.003
V48	Heptanal	-	0.129 ± 0.022	-	-
V49	Humulene	0.694 ± 0.136b	0.083 ± 0.012b	-	0.02

V50	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	0.209 ± 0.029b	0.043 ± 0.011a	-	0.001
V51	Neryl nitrile	1.101 ± 0.385	-	-	-
V52	n-Hexane	5.178 ± 2.118	-	7.354 ± 1.477	0.454
V53	Nonanal	0.457 ± 0.095ab	0.676 ± 0.062b	0.329 ± 0.058a	0.015
V54	Nonane, 5-(1-methylpropyl)-	-	0.080 ± 0.015	-	-
V55	Octanal	-	0.407 ± 0.059	-	-
V56	Octanoic acid, methyl ester	-	0.023 ± 0.007	-	-
V57	o-Cymene	0.593 ± 0.168b	0.156 ± 0.025a	-	0.05
V58	Oxime-, methoxy-phenyl-_	0.192 ± 0.054	-	-	-
V59	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	-	0.147 ± 0.019	-	-
V60	Phenylethyl Alcohol	0.223 ± 0.063	0.109 ± 0.033	-	<0.001
V61	Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	-	17.737 ± 1.335b	10.725 ± 2.921a	0.05
V62	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester	-	0.416 ± 0.049	-	-
V63	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	-	20.971 ± 1.537b	17.953 ± 4.589a	0.05
V64	Propanoic acid, 2-methyl-, anhydride	-	0.071 ± 0.019	-	-
V65	Propanoic acid, 5-hexen-1-yl ester	-	0.210 ± 0.010	-	-
V66	Sulfurous acid, cyclohexylmethyl hexadecyl ester	-	0.691 ± 0.051	-	-
V67	Sulfurous acid, dicyclohexyl ester	-	0.103 ± 0.010	-	-
V68	Tetradecane	0.105 ± 0.013	-	-	-
V69	Tridecane	0.090 ± 0.015	-	-	-
V70	Ylangene	0.871 ± 0.062	-	-	-
V71	α-Cubebene	0.786 ± 0.092	-	-	-
V72	α-Gurjunene	0.196 ± 0.054	-	-	-
V73	α-Muurolene	-	0.121 ± 0.018	-	-
V74	β-copaene	0.773 ± 0.108b	0.084 ± 0.023a	-	0.001
V75	β-Cubebene	-	0.051 ± 0.006	-	-
V76	β-Dihydroagarofurane	-	0.075 ± 0.009	-	-
V77	β-Elemene	0.675 ± 0.056	-	-	-
V78	β-Ocimene	-	0.108 ± 0.017	-	-
V79	β-ylangene	1.318 ± 0.093	-	-	-
V80	γ-Cadinene	1.492 ± 0.196	-	-	-
V81	γ-Muurolene	1.807 ± 0.296b	0.039 ± 0.011a	-	<0.001
V82	γ-Terpinene	0.177 ± 0.056	-	-	-
V83	δ-Cadinene	0.148 ± 0.018	0.177 ± 0.025	-	0.383



Chapter 6

Seasonal olfactory response of *Philaenus spumarius* (Hemiptera: Aphrophoridae) towards traditional Portuguese olive cultivars

Chapter 6

Adapted from:

Seasonal olfactory response of *Philaenus spumarius* (Hemiptera: Aphrophoridae) towards traditional Portuguese olive cultivars

Isabel Rodrigues, Jacinto Benhadi-Marín, Paula Baptista, José Alberto Pereira

Submitted

Chapter 6

Seasonal olfactory response of *Philaenus spumarius* (Hemiptera: Aphrophoridae) towards traditional Portuguese olive cultivars

Abstract

In Portugal, the strength of the olive sector relies on the great genetic heritage of traditional olive cultivars. However, this genetic heritage is threatened by the causal agent of olive quick decline syndrome (OQDS), the phytopathogenic bacteria *Xylella fastidiosa*, reported for the first time in 2019 in the country. This vector-borne pathogen is transmitted by xylem-feeding insects such as *Philaenus spumarius* which is considered the main European vector. Since there is no cure for this pathogen, the implementation of an integrated approach against the vectors should be considered to prevent and limit the spread of *X. fastidiosa*. In this sense, an in-depth assessment of the host plant preferences of the main European vector of *X. fastidiosa* is crucial to understand their seasonal dynamics towards olive cultivars to determine the most susceptible to vector attack. This work aimed to assess the olfactory response of *P. spumarius*, to five traditional Portuguese olive cultivars: "Cobrançosa," "Negrinha de Freixo," "Santulhana," "Madural," and "Verdeal Transmontana" in two separate seasons (Spring and Autumn). Our results showed that *P. spumarius* presented significantly different olfactory responses toward the different cultivars under study. In Spring, females and males were significantly attracted to "Negrinha de Feixo"; in Autumn, females were significantly attracted to "Cobrançosa". Also, in general, the olfactory response toward the five cultivars was sex-dependent. Our results suggest that in Spring, the cultivar "Negrinha de Freixo" can be more susceptible to *P. spumarius* and that the olfactory response towards the olive cultivars can vary throughout the life cycle of the vector. Our results can help future implementation of approaches to manage the vector and the spread of *X. fastidiosa*.

Keywords: Cobrançosa; Negrinha de Freixo; *Xylella fastidiosa*; Emerging plant diseases

Chapter 6

6.1. Introduction

The Olive Quick Decline Syndrome (OQDS) is a severe plant disease that has been spreading through southern Italy, where it is currently responsible for the death of thousands of olive trees, seriously affecting the olive production and economy of the country (Saponari et al., 2019). The etiological agent responsible for the disease is the plant pathogenic gram-negative bacterium native to the Americas, *Xylella fastidiosa* Wells et al., 1987 (Xanthomonadales: Xanthomonadaceae) (Saponari et al., 2013).

Once the bacterium infects the plant, it moves and multiplies within the xylem vessels, creating a biofilm, obstructing the vessels, blocking the passage of water, and, consequently, the transport of soluble mineral nutrients (Janse & Obradovic, 2010). Typical symptoms of infection are drying, scorching, wilting of the foliage, and, eventually, plant death (EFSA, 2013). However, these symptoms can vary according to the host plants, the bacteria subspecies involved, and the climatic conditions of the infected region (EFSA, 2013).

Xylella fastidiosa is transmitted to plants exclusively by xylem-feeding insects of the order Cicadomorpha (Almeida et al., 2005; Hill & Purcell, 1995; Krugner et al., 2019). In Europe, the meadow spittlebug, *Philaenus spumarius* (Linnaeus, 1758) (Hemiptera, Aphrophoridae), was identified as the main vector (Saponari et al., 2014; Cornara et al., 2017b). In addition to being widely abundant and distributed throughout Europe (Rodrigues et al., 2014), this insect also has higher bacterial transmission rates than other vectors such as *P. italosignus* Drosopoulos & Remane, 2000 and *Neophilaenus campestris* (Fallén, 1805) (Cavalieri et al., 2019). In olive groves, *P. spumarius* spend most of the life cycle in the herbaceous vegetation cover; however, at the end of the Spring/early Summer, due to mowing or a decrease in the succulence of herbaceous vegetation cover, the insect moves to the olive canopy for feeding (Cornara et al., 2019; Dongiovanni et al., 2019; Morente et al., 2018; Villa et al., 2020). Then, if infected with *X. fastidiosa*, the transmission of the bacteria to the olive tree may occur (Cornara et al., 2017a).

In Portugal, the presence of *X. fastidiosa* was first reported in 2019, and since then, new outbreaks have been identified (DGAV, 2021a 2023). The constant spread may lead to devastating economic and environmental problems, threatening olive tree cultivation in Portugal. Throughout mainland Portugal, the olive tree agroecosystem holds a remarkable ancient history and tradition, being one of the most emblematic and economically important crops, involving a great genetic heritage (Moreira & Veloso, 2009; Rodrigues et al., 2022c).

Since there is no cure for the OQDS, the only current approach to control the dissemination of the causal agent relies on destroying infected trees and managing the vector population (Commission Implementing Regulation (EU) 2020/1201 of 14 August 2020). Indeed, vector control is identified as the main tool to limit the spread of the disease (Schneider et al., 2020). Therefore, understanding the ecology and the seasonal olfactory response of the main European vector towards traditional Portuguese olive cultivars is crucial to protect the genetic heritage.

Philaenus spumarius is known to interact socially via substrate-borne vibrations (Avosani et al., 2020), whereas some studies suggest that it can also communicate via pheromones (Sevarika et al., 2022ab). Furthermore, electrophysiological assays demonstrated that *P. spumarius* respond to volatile organic compounds (Anastasaki et al., 2021; Germinara et al., 2017). Moreover, behavioral responses showed that different aromatic plants and their essential oils could elicit a repellent or attractive behavior (Ganassi et al., 2020). Moreover, Rodrigues et al. (2022b) described that females of *P. spumarius* were significantly attracted to lower concentrations of cis-3-hexenyl acetate and cis-3-hexen-1-ol, two volatile organic compounds commonly found in the olive tree. In addition, Cascone et al. (2022) reported that females of *P. spumarius* were attracted by the olive cultivars Ogliarola, Rotondella, and Frantoio, and repelled by FS-17, while males did not present any olfactory response. The results of these studies support that, although *P. spumarius* has significantly fewer antennal sensilla compared to other species (Ranieri al., 2016), it can use semiochemical cues to locate and choose host plants.

In this work, we evaluate the olfactory response of *P. spumarius*, to five different Portuguese olive cultivars: "Cobrançosa", "Negrinha de Freixo", "Santulhana", "Madural", and "Verdeal Transmontana" in Spring (after the emergence of the adults) and Autumn (end of the life cycle of the vector).

6.2. Material e methods

6.2.1. Plants

Three healthy olive trees belonging to five important traditional Portuguese olive cultivars were selected (*i.e.,* "Cobrançosa", "Negrinha de Freixo", "Santulhana", "Madural", and "Verdeal Transmontana") and grown in plastic pots (20 cm in height, 11 cm in length). The plants

were placed in rearing chambers at 25 °C, with 50 % relative humidity and a 16:8 h (L:D) photoperiod. All the olive trees were two-year-old and similar in size. No pesticides, fungicides, or fertilisers were used for growing the plants.

6.2.2. Origin and rearing of the insects

Adults of *P. spumarius* were collected, with an entomological sweep net, during the Spring and Autumn of 2020 in the herbaceous ground vegetation of an olive grove in Bragança (41°48'10.1"N, 6°44'50.9"W). The adults collected were sexed using a binocular stereoscopic microscope and transferred onto different cages (40 cm in height, 30 cm in length, and 43 cm in width) with one-year *Lavandula* sp. plants. The cages were then placed under controlled conditions (18 °C, 70 % relative humidity, and a 16:8 h (L:D) photoperiod).

6.2.3. Design/description of the olfactometric device

The seasonal olfactory response of *P. spumarius* to the different olive cultivars was assessed on a specifically designed eight-chamber olfactometer. The olfactometer was all made of transparent acrylic. The device encompassed two main areas, an outside square-shaped area (40 cm × 40 cm) and a central round area (18 cm in diameter) inside the latter. The circular arena was divided into eight radial chambers covered with white tape to minimise visual stimulus. Each chamber had an aperture on the base (1 cm × 1 cm) connecting the inner and outer areas to allow the insect to choose a chamber. The base of each chamber was individually connected to a gas washing bottle (250 mL) containing activated charcoal (AppliChem, Panreac ITW©) dissolved in 100 ml of distilled water to purify and humidify the airflow. The airflow was supplied by pumps providing a flow of 12 cm^3/min. This way, the continuous airflow entered each chamber from below, transporting the scents to each entry. The olfactometer has an acrylic lid with a removable triangular flap in each corner to facilitate the placing of insects into the outer squared area.

6.2.4. Olfactometric bioassays

The olfactory response of *P. spumarius* was evaluated in the Spring and Autumn. The seasons represented two life cycle stages: Spring (*i.e.,* after the emergence of the adults) and

Autumn (*i.e.,* end of the vector's life cycle). In each season, the selected olive cultivars were divided into two trials. In the first trial, the olfactory response of *P. spumarius* was tested towards the cultivars "Madural", "Cobrançosa", and "Verdeal Transmontana". In the second trial, the response was tested towards the cultivar significantly most chosen in the first trial, plus the cultivars "Negrinha de Freixo" and "Santulhana".

In each season and trial, three healthy leaves (4,29 ± 0.27 g) of the same cultivar were placed in two opposite chambers. Each leaf petiole was protected with aluminum foil to avoid the potential release of volatile compounds related to tissue damage. The remaining two chambers operated as control (purified airflow).

After placing the leaves of the cultivars in the respective chambers, the olfactometer was closed. Then, sixteen insects, previously kept for two hours in the absence of food and odours, were equally divided and released into each corner of the olfactometer arena (*i.e.,* four insects for each corner). The olfactory response of the insects was recorded 30 min after their release in the arena by recording the number of insects found in each chamber. Twenty replicates for males and females were performed. At each replicate, the olfactometer chambers were rotated one position each time to avoid directional bias.

6.2.5. Data analysis

Generalised Estimating Equations (GEEs) (Pekár & Brabec, 2018) with Poisson distribution were used to compare the olfactory response of *P. spumarius* towards the different olive cultivars per trial and season. Sex, cultivar (first trial: "Cobrançosa", "Madural", "Verdeal Transmontana", and "Control"; second trial: "Cobrançosa", "Negrinha de Freixo", "Santulhana", and "Control"), and the interaction between the two terms were used as explanatory variables. The number of individuals that chose the different cultivars was assessed per trial and season and compared using a post hoc pairwise *t*-test ($\alpha = 0.05$) for males and females separately. The models were developed in R (R Core Team, 2022) using the *geeglm* function from package 'geepack'.

6.3. Results

In all the seasons and trials, the olfactory response of *P. spumarius* significantly differed between cultivars (Table 6.1). As mentioned before, in each season, two consecutive trials were carried out; in the first trial, the olfactory response of *P. spumarius* adults was tested against the cultivars "Madural", "Cobrançosa", "Verdeal Transmontana", and a stream of purified air (control). Subsequently, in the second trial, the olfactory response of *P. spumarius* was tested towards the cultivar most significantly chosen in the first trial, which in general was "combrançosa", and the cultivars "Negrinha de Freixo" and "Santulhana". Therefore, in Spring, in the first trial, females and males were significantly more attracted by the cultivar "Cobrançosa" compared to the remaining cultivars (Figure 6.1a). On the contrary, during the second trial, when we assess the olfactory response of the insects towards the cultivars "Cobrançosa", "Santulhana", and "Negrinha de Freixo" and the control, the cultivar "Negrinha de Freixo" was the most significantly chosen (Figure 6.1b).

In Autumn, females were significantly more attracted to "Cobrançosa" in both trials (Figure 6.2a). Nonetheless, males were significantly attracted to "Cobrançosa" and "Verdeal Transmontana" in the first trial. Therefore, for statistical purposes, the cultivar "Cobrançosa" was used in the second trial, where the "Negrinha de Freixo" was the preferred cultivar compared to the "Santulhana" cultivar (Figure 6.2b).

Moreover, the interaction between cultivar and sex significantly affected the behavior of the insects, except in the Spring when the insects were exposed to the cultivars "Cobrançosa", "Santulhana", "Negrinho Freixo" and to the stream of purified air (Table 6.1).

Table 6.1. Results of the GEEs developed to assess the choosing behavior of *Philaenus spumarius* towards traditional Portuguese olive cultivars in an eight-chamber olfactometer. Cultivar:Sex stands for the interaction term.

Season	Independent variable	Response variable	First trial			Second trial		
			df	χ^2	P	df	χ^2	P
Spring	Cultivar	Number of individuals	3	195.4	<0.001	3	281.8	<0.001
	Sex		1	0.2	0.64	1	0.1	0.73
	Cultivar:Sex		3	13.3	0.004	3	0.9	0.83
Autumn	Cultivar	Number of individuals	3	125.4	<0.001	3	19.1	<0.001
	Sex		1	1.5	0.22	1	2.2	0.14
	Cultivar:Sex		3	60.6	<0.001	3	34.2	<0.001

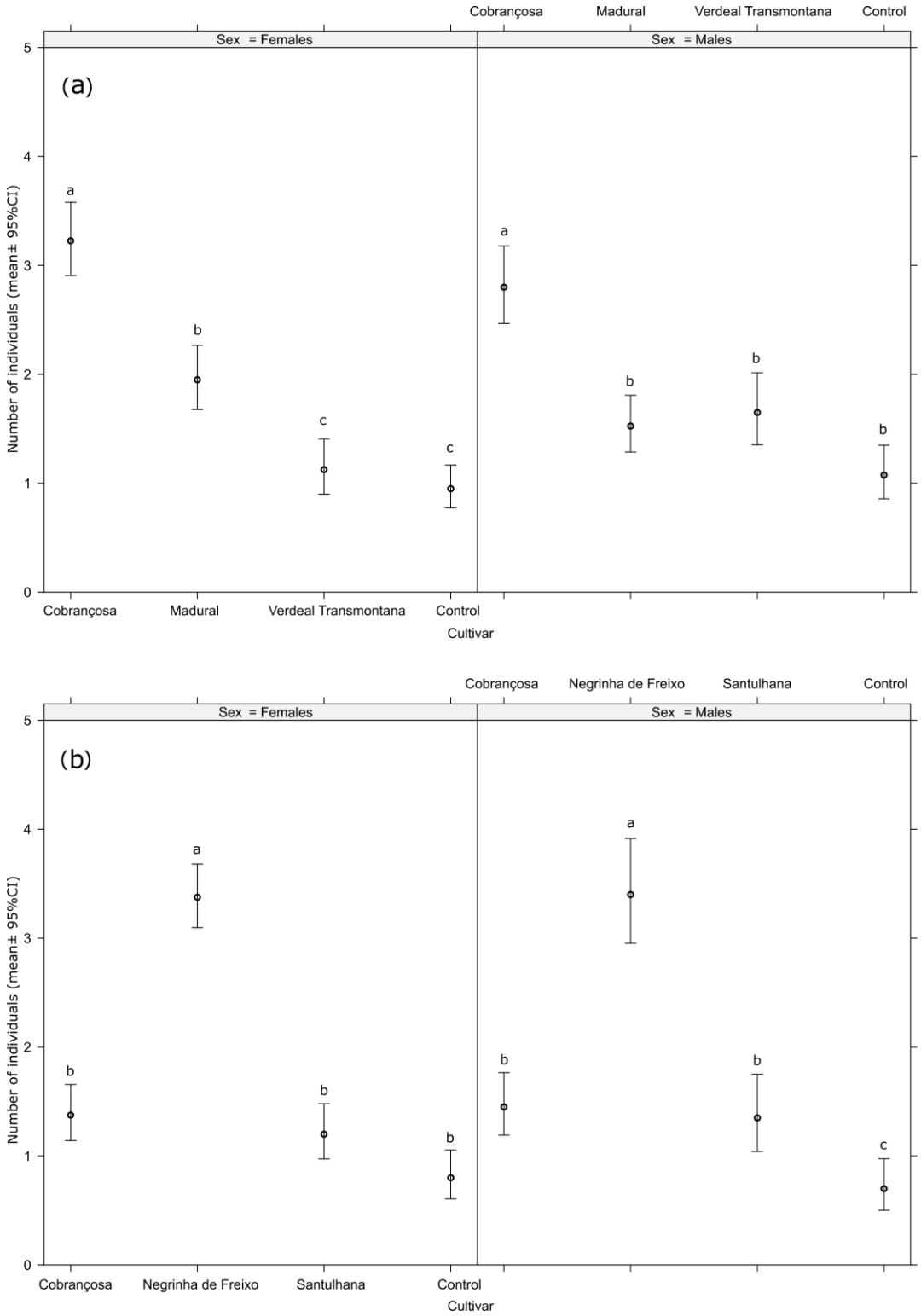


Figure 6.1**.** Olfactory response in Spring of females and males of *Philaenus spumarius* towards traditional Portuguese olive cultivars. (a) First trial (number of individuals (mean ± 95% CI) that chose the cultivars "Madural", "Cobrançosa", "Verdeal Transmontana" and control) and (b) Second trial (number of individuals (mean ± 95%CI) that chose "Cobrançosa", "Negrinha de Freixo", "Santulhana", and control).

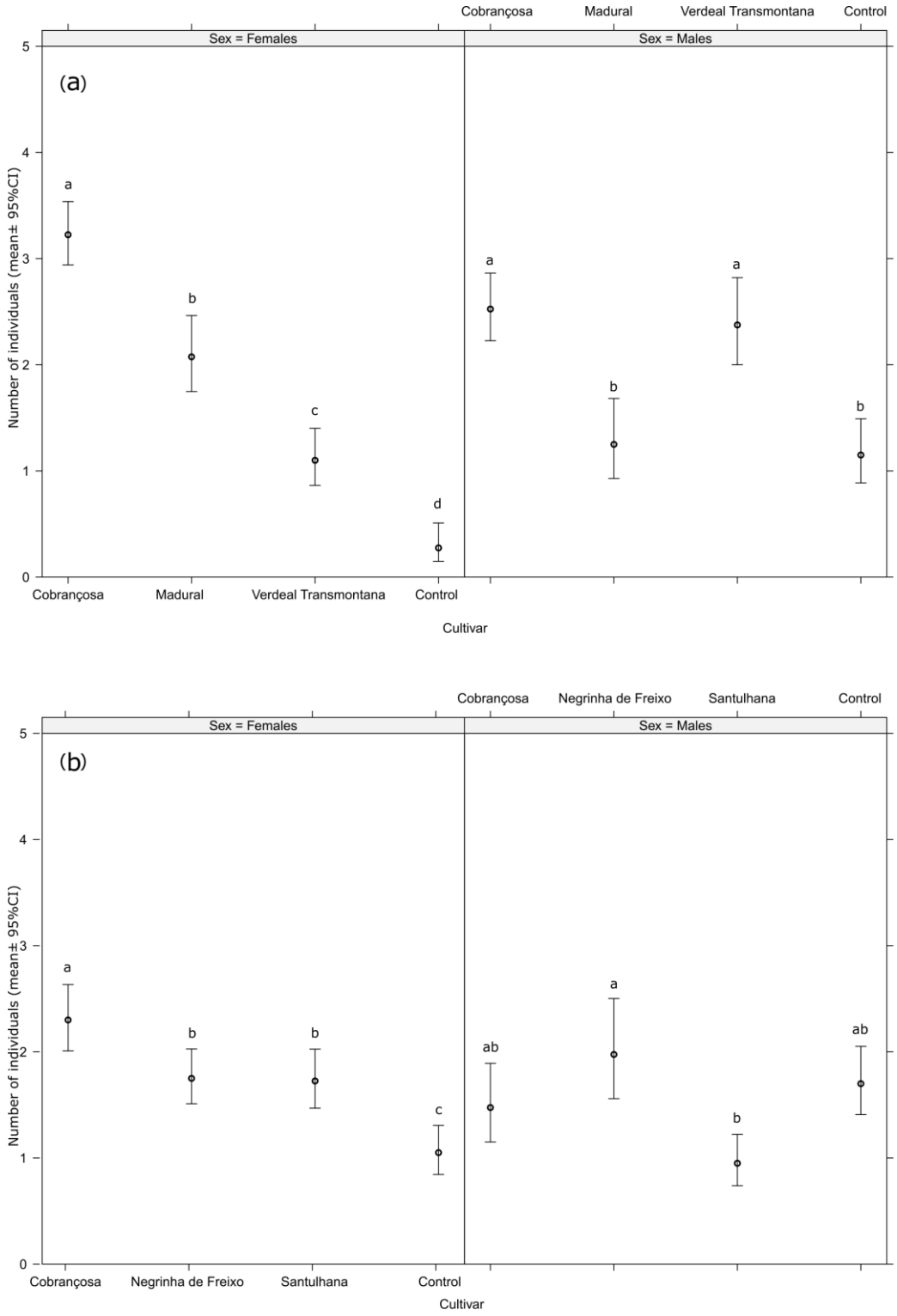


Figure 6.2. Olfactory response in Autumn of females and males of *Philaenus spumarius* towards traditional Portuguese olive cultivars. (a) First trial (number of individuals (mean ± 95% CI) that chose the cultivars "Madural", "Cobrançosa", "Verdeal Transmontana" and control) and (b) Second trial (number of individuals (mean ± 95% CI) that chose "Cobrançosa", "Negrinha de Freixo", "Santulhana", and control).

6.4. Discussion

In Portugal, the relevance of the olive sector relies on the high diversity of traditional and autochthonous cultivars (Rodrigues et al., 2020c, 2023). However, the introduction of the phytopathogenic bacterium *X. fastidiosa* in the country and its rapid spread threaten this diversity. The scenario observed in southern Italy represents an example of the detrimental impacts associated with the disease (Saponari et al., 2019).

Several studies highlighted the tolerance and resistance of olive cultivars *to X. fastidiosa*; however, these studies mainly focused on a few olive cultivars grown throughout the infected area in Italy (*e.g.,* Boscia et al., 2017; Giampetruzzi et al., 2016; Ranieri et al., 2016). So far, only the cultivars FS17 and Leccino showed resistance towards *X. fastidiosa* subspecies *pauca* ST53 (Boscia et al., 2017; EFSA et al., 2017). Moreover, the tolerance and resistance of autochthonous cultivars from countries other than Italy are still unknown. An in-depth assessment of the preferences of the main European vector of *X. fastidiosa* for host plants is crucial to understand the seasonal insect dynamics on a particular plant and to determining plants (in our case study, olive cultivars) more susceptible to vector attack. Consequently, this would allow the implementation of efficient prevention and control measures against the spread of *X. fastidiosa.*

Although *P. spumarius* is a polyphagous insect that feeds on the xylem sap of a wide range of host plants (Cornara et al., 2018). Several researches showed that polyphagous insects tend to show preferences for specific plant species or growth stages (*e.g.,* Barman et al., 2010; Jackson et al., 2008; Kennedy & Margolies, 1985; Liu et al., 2010; Rwomushana et al., 2008). This vector spends a large part of its life cycle in the herbaceous vegetation, which provides a wide range of host plants where they feed, mate, and lay eggs (Cornara et al., 2019; Dongiovanni et al., 2019; Morente et al., 2018). Nevertheless, few studies reported peaks of abundance in the olive tree canopy, in Spring, shortly after the emergence of adults (Bodino et al., 2019b, 2020; Sanna et al., 2021). In summer, adults tend to move from the olive groves (Antonatos et al., 2020; Ben Moussa et al., 2016; Bodino et al., 2019b; Tsagkarakis et al., 2018), returning in Autumn, after the first rains to start oviposition (Antonatos et al., 2020; Cruaud et al., 2018; Morente et al., 2018). Despite the lower abundance of *P. spumarius* in the canopy of olive trees in this season (Bodino et al., 2019b, 2020). Bodino et al. (2021c) reported that this vector has higher acquisition rates of *X. fastidiosa* in the Autumn. For these reasons, we choose to assess the olfactory response of *P. spumarius* adults to the different olive cultivars in two different seasons: Spring and Autumn.

Several studies have shown that *P. spumarius* can respond to olfactory stimuli (*e.g.,* Anastasaki et al., 2021; Cascone et al., 2022; Germinara et al., 2017; Rodrigues et al., 2022b), strongly suggesting that these may use chemical cues to choose and locate host plants. In this work, adults of *P. spumarius* presented significantly different olfactory responses toward the different cultivars under study. Cascone et al. (2022) reported significant differences in the olfactory responses of *P. spumarius* to the olive cultivars Leccino, FS-17, Ogliarola, Frantoio, and Rotondella, although the effect was sex-dependent. In general, our study agrees with these findings and sex also significantly influenced the choice of the cultivars, except in the Spring in the second trial. Similar results were also reported by Ganassi et al. (2020) and Rodrigues et al. (2022b).

In Spring, *P. spumarius* adults significantly preferred the "Negrinha de Freixo" cultivar. "Negrinha de Freixo" is a variety typically found in Trás-os-Monte's region, Portugal's second largest olive-growing region. This cultivar is essentially used to produce table olives since it has a very low oil yield (Albuquerque et al., 2019). In fact, this cultivar represents great economic importance in the region, the olive tables of this variety are recognised as Protected Denomination Origin (PDO) (Commission Regulation (EEC) 2081/92).

In Autumn, females significantly chose the "Cobrançosa" cultivar. This dual aptitude cultivar (*i.e.,* it can be used to produce olive oil and table olives) is widely distributed in the main Portuguese olive-producing regions (Reis, 2014). On the other hand, in Autumn, males presented a more random olfactory response. In the first trial, they significantly chose the cultivars "Cobrançosa" and "Verdeal Transmontana", but in the second trial, they showed no significant differences between the cultivars "Cobrançosa", "Negrinha de Freixo" and the control. According to Bodino et al., (2019b), males have shorter longevity than females, and their abundance tends to decrease through the season in the olive groves. Therefore, we suggest that since Autumn represents the end of the *P. spumarius* life cycle, females may rely on olfactory stimuli to find a suitable host plant for oviposition, while males no longer have an active role.

Our results suggest that *P. spumarius* chose different cultivars depending on seasons, whereas Cascone et al. (2022) found no differences in the olfactory response of *P. spumarius* to olive cultivars over time. Nevertheless, the volatile profile of olive cultivars tends to change according to their morphological state (Malheiro et al., 2016). Additionally, abiotic factors can also lead to changes in the volatile profile (Malheiro et al., 2016; Sofo et al., 2004), so it is expected that shifts in the volatile profile could induce different olfactory responses throughout

the season. Thus, further studies on the olfactory response to volatiles produced by olive cultivars in different seasons are needed to validate the results.

6.5. Conclusions

In the present work, *P. spumarius* showed different olfactory responses to the traditional Portuguese olive cultivars under study, evidencing that this insect can use olfactory stimuli to choose host plants. In Spring, the cultivar "Negrinha de Freixo" appears to be the most susceptible to vector attack, whereas in Autumn, the cultivar "Cobrançosa" was the most chosen. Further studies on feeding preference and monitoring the seasonal abundance of *P. spumarius* in mono-cultivar olive groves could provide stronger evidence of these behavioral patterns. Characterising the volatile profile of cultivars and understanding to which extent these volatile drives the olfactory response of *P. spumarius* can help future implementation of approaches to manage the vector. Additionally, our results may contribute to the design of olive groves with cultivars that are less subject to vector attack and consequently reduce the spread of the bacterium.

Acknowledgments

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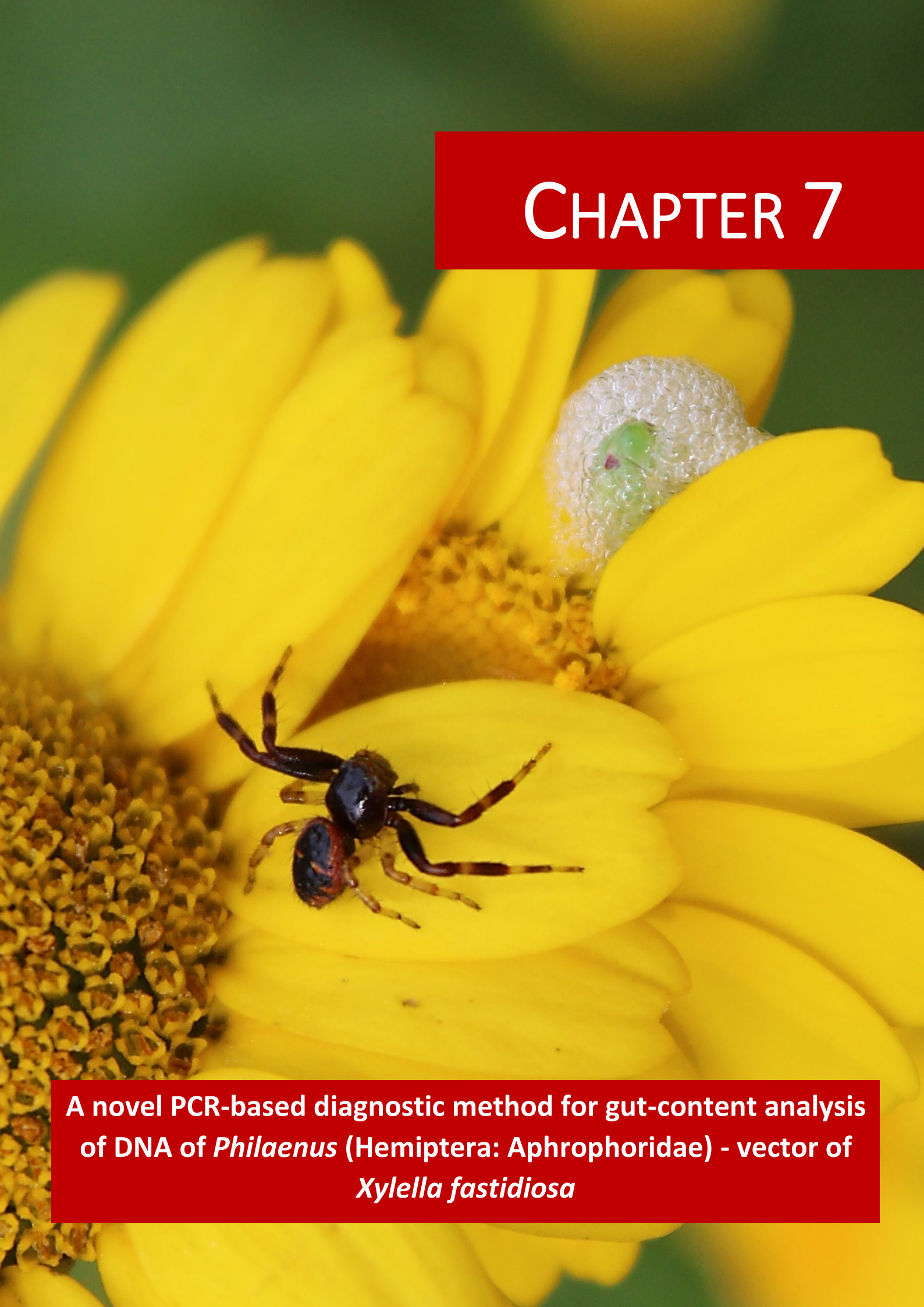

Chapter 7

A novel PCR-based diagnostic method for gut-content analysis of DNA of *Philaenus* (Hemiptera: Aphrophoridae) - vector of *Xylella fastidiosa*

Chapter 7

Adapted from:

A novel molecular diagnostic method for the gut content analysis of *Philaenus* DNA

Isabel Rodrigues, Vítor Ramos, Jacinto Benhadi-Marín, Aránzazu Moreno, Alberto Fereres, José Alberto Pereira, Paula Baptista

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Chapter 7

A novel PCR-based diagnostic method for gut-content analysis of DNA of *Philaenus* (Hemiptera: Aphrophoridae) - vector of *Xylella fastidiosa*

Abstract

Philaenus spumarius is a vector of *Xylella fastidiosa*, one of the most dangerous plants pathogenic bacteria worldwide. There is currently no control measure against this pathogen. Thus, the development of vector control strategies, like generalist predators, such as spiders, could be essential to limit the spread of this vector-borne pathogen. In this study, a polymerase chain reaction (PCR)-based approach was developed to principally detect DNA of *Philaenus spumarius* in the spider's gut. Accordingly, 20 couple of primers targeting the mitochondrial cytochrome oxidase I (COI) and cytochrome b (*cyt*B) genes were tested for specificity, sensitivity, and efficiency in detecting *P. spumarius* DNA. Overall, two primer sets, targeting the COI gene (COI_Ph71F/COI_Ph941R) and the *cyt*B gene (cytB_Ph85F/cytB_Ph635R), showed the highest specificity and sensitivity, being able to amplify 870 pb and 550 bp fragments, respectively, with *P. spumarius* DNA concentrations 100-fold lower than that of the DNA of non-target species. Among these two primer sets, the cytB_Ph85F/cytB_Ph635R was able to detect *P. spumarius* in the spider *Xysticus acerbus*, reaching 50% detection success 82 h after feeding. The feasibility of this primer set to detect predation of *P. spumarius* by spiders was confirmed in the field, where 20% of the collected spiders presented positive amplifications.

Keywords: cytochrome b; cytochrome oxidase I; insect–predator association; spiders; spittlebug.

Chapter 7

7.1. Introduction

The meadow spittlebug *Philaenus spumarius* (Linnaeus, 1758) (Hemiptera, Aphrophoridae) is the most common and widespread xylem-sap feeder insect in Europe, where it has never been considered as a pest (Rodrigues et al., 2014). However, since the first European outbreak of *Xylella fastidiosa* (Xanthomonadales: Xanthomonadaceae) in olive (Saponari et al., 2013), *P. spumarius* has become a serious threat to European agriculture to its recognized role in the transmission of this pathogen (Saponari et al., 2014). Up to date, in the south of Italy, thousands of olive trees in an area higher than 23,000 ha died due to *X. fastidiosa* (Saponari et al., 2019). *Philaenus spumarius* was identified as the main vector responsible for this severe outbreak (Cavalieri et al., 2019). Indeed, although all xylem-sap feeders are considered potential vectors of *X. fastidiosa* (Almeida et al., 2005), it is proven that species belonging to the genus *Philaenus* have transmission rates higher than other vectors (Cavalieri et al., 2019). Therefore, if proper prevention and control measures are not implemented, the continuous dispersion of *X. fastidiosa* over the course of 50 years could cause economic damage higher than 1.9 billion Euros (Schneider et al., 2020). In this regard, vector control is perceived as the main tool to limit the spread of *X. fastidiosa* (Schneider et al., 2020). Previous studies showed that vector control measures, based on chemical and physical strategies, can significantly reduce the population of *P. spumarius* and consequently decrease the spread of *X. fastidiosa* (Dongiovanni et al., 2018b; Fierro et al., 2019). However, there is a need for a more sustainable and ecological approach as an alternative to chemical control due to the known ability of pesticides to cause deleterious health and environmental effects.

Generalist predators, such as spiders, can play a significant role in the biological control of *P. spumarius*. Spiders are one of the most abundant and diverse arthropod orders (Nyffeler & Benz, 1987). There are more than 45,000 species of spiders described in the world, and in favorable conditions, they can reach population densities higher than 1000 individuals m^2 (Nyffeler & Birkhofer, 2017). In addition, they are considered one of the most important groups of natural insect enemies worldwide (Nyffeler & Benz, 1987). Spiders are polyphagous and opportunistic predators with different hunting strategies, capable of killing approximately 200 kg/ha of prey per year (Nyffeler, 2000). Although there are already reports of spiders preying on *P. spumarius* (Phillipson, 1960; Harper & Whittaker, 1976), knowledge about its antagonist's guild is scarce and outdated. Recently, a guild-based protocol to target spiders as potential natural enemies of *P. spumarius* was developed by Benhadi-Marín et al. (2020). The protocol focused on finding dominant guilds of spiders in olive groves and analyzing their functional

response towards *P. spumarius*. However, not always prey choice by predators in field conditions can sometimes be established by using direct observation. Thus, to achieve a more accurate identification of *P. spumarius* natural enemies, a gut analytical method enabling field assessment of predation is required.

Polymerase chain reaction (PCR)-based techniques are valuable tools in ecological studies, namely in the study of interactions between the pest and its natural enemies (King et al., 2008; Sint et al., 2011; Rejili et al., 2016; Albertini et al., 2018). By using taxon-specific primers, PCR-based techniques allow detecting specific ingested prey in the diet of predators since their DNA remains in the predator's gut before totally digested (Symondson, 2002). However, the effectiveness and sensitivity of such approach should take into account additional issues. The choice of target markers and the length of the sequence region to amplify are some aspects that need to be taken into account. Indeed, it is expected that degradation of the DNA of consumed preys will occur throughout the digestive process. In these conditions, approaches targeting cell-abundant, small multi-copy DNA fragments, like mitochondrial DNA, are preferable (Sousa et al., 2019). Another methodological aspect to be considered on PCR-based analysis of predation includes the type of predator tissue used to extract DNA (Albertini et al., 2018). The predator's digestive tract is the preferred source to extract DNA from ingested prey (*e.g.*, Juen & Traugott, 2006; Monzó et al., 2010; Rejili et al., 2016; Lantero et al., 2018). However, gut dissection in spiders is not possible. They have branching digestive tracts into highly complex diverticulum extending throughout the whole body, including their legs. Therefore, digestion takes place in different body parts (Cohen, 1995). Nevertheless, when performing molecular gut-content analyses in spiders, the extraction of the whole body or just the abdomen, which has a higher proportion of prey DNA, is necessary (Krehenwinkel et al., 2015). In this approach, the underrepresented and degraded DNA from the prey can be masked by the overabundant DNA of the predator (Kennedy et al., 2020). Aside from these issues, prey DNA digestion rates and the species of the predator (*e.g.,* Hoogendoorn & Heimpel, 2001; Eitzinger et al., 2014) can influence post-feeding prey detection periods in predators' gut.

The main goal of this work was to design and evaluate taxon-specific primers targeting the mitochondrial cytochrome oxidase I (COI) and cytochrome b (*cyt*B) genes, to be used for a PCR-based diagnostic method, to detect *P. spumarius* within spiders. Feeding experiments were performed to evaluate the effectiveness of this DNA-based diagnostic tool. Specifically, this work analyzed: (i) the suitability of the molecular marker selected regions and the specificity and sensitivity of the designed primers on *P. spumarius* detection; (ii) the prey detectability over

time in the spider *Xysticus acerbus* Thorell, 1872 (Thomisidae) using DNA extracts from their body; (iii) and the efficiency of the designed primers to detect *Philaenus* in *Oxyopes* sp. (Oxyopidae) spiders directly collected from the field.

7.2. Materials and methods

7.2.1. Collection and molecular identification of the arthropods

In order to evaluate the ability of the designed primer pairs to specifically amplify DNA from *Philaenus*, adults of *P. spumarius* were collected to act as a positive control. *Neophilaenus campestris* (Fallen, 1805) (Aphrophoridae), *Neophilaenus lineatus* (Linnaeus, 1758) (Aphrophoridae), *Lepyronia coleoptrata* (Linnaeus, 1758) (Aphrophoridae), *Aphrophora* sp. (Aphrophoridae), *Cicadella viridis* (Linnaeus, 1758) (Cicadellidae), *Cercopis* sp. (Cercopidae), and the spider *Xysticus acerbus* were also collected to be used as non-target species. Arthropod's collection was carried out in the natural ground vegetation with an entomological sweep net (38 cm diameter), in the *Campus* of Instituto Politécnico de Bragança (41º 47' 53.2" N, 6º 45' 51.5" W), between April and July of 2019. All the arthropods were initially identified to the genus/species level using a binocular stereoscopic, preserved in absolute ethanol, and stored at −20 °C, until subsequent DNA extraction. In order to confirm the identification of the arthropods, a molecular-based approach was followed. All the insects were homogenized in liquid nitrogen, and the genomic DNA was extracted using the SpeedTools tissue DNA extraction kit (Biotools, Spain) following the manufacturer's instructions. The barcode region of the mitochondrial COI gene was amplified using the universal primers LCO1490/HCO2198 (Folmer et al., 1994). Amplifications were run in a MyCycler™ Thermocycler (Bio-Rad) using 20 µL PCR reactions, which contained 1× buffer, 2.5 mM of $MgCl_2$, 200 µM of dNTPs, 0.2 µM of each primer, and 1.25 U of Taq DNA polymerase (BIORON, GmbH). Cycling conditions were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 10 min. PCR products (~710 bp) were run on a 1% (v/v) agarose gel stained with 1X Gel Red™ nucleic acid gel stain (Biotium, California, USA), and the amplified products were purified and sequenced at Macrogen Inc. (Madrid, Spain). The DNA sequences were analyzed and edited with MEGA v10.1.8 (Kumar et al., 2018), and the identification of each specimen was confirmed by querying the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLASTn) in NCBI's website (www.ncbi.nlm.nih.gov).

7.2.2. Design of Philaenus*-specific primers and development of diagnostic PCRs*

Publicly available full (extracted from mitochondrial genomes) or near-full sequences of COI and *cyt*B genes from *Philaenus* spp. and close-related taxa representatives (see Supplementary Figure S7.1) were reached from GenBank. Due to the small number of sequences present in the database, partial COI sequences from *Philaenus* specimens covering either the 5' or the 3' part of the gene were additionally retrieved from GenBank (Supplementary Figure S7.1). All sequences were then aligned in Geneious 8.1.8 (Biomatter, New Zealand) by using the algorithm ClustalW. Primer3 2.3.4. (Untergasser et al., 2012), a tool available in Geneious was then used for both datasets to identify potential gene regions suitable for the design of *Philaenus*-specific primers. Additional regions were also visually inspected for adequacy, being all candidate primers chosen or redesigned manually. Primers properties (*e.g.*, length, melting temperature, GC content) were evaluated with Geneious and OligoEvaluator, a Sigma-Aldrich accessible tool (http://www.oligoevaluator.com) and analyzed for secondary structures (including hairpins, self-dimers, and cross-dimers) formation in primer pairs with the online OligoAnalyzer™ tool (www.idtdna.com). The primer pairs' specificity was virtually assessed with Primer-BLAST (Ye et al., 2012). The shortlist of the best oligonucleotide candidates, seven targeting the COI gene and six targeting *cyt*B gene (Table 7.1 and Figure 7.1), were synthesized at Frilabo (Portugal), tested, and their PCR conditions optimized.

Table 7.1. Primers from COI and cytB genes designed to specifically amplify *Philaenus* and were tested in this study.

Primer names	Sequences (5'–3')
COI_Ph71F	CTGGAATAATTGGGACTACTC
COI_Ph307F	CTTCCTCCTTCGTTAACGC
COI_Ph515F	CAGGTATGAAAATAGATCG
COI_Ph553R	CGATCTATTTTCATACCTG
COI_Ph937R	CAGCAATAATTATTGTGGC
COI_Ph941R	GGTACAGCAATAATTATTGTGG
COI_Ph1018R	AGGAGAAGACAATTTG
cytB_Ph85F	GTCATAGGAGTAATAATTATACTGACAG
cytB_Ph91F	GGAGTAATAATTATACTGACAG
cytB_Ph204F	TCCTTACCTCGGAGAATC
cytB_Ph327R	GCTTCTTATAACTAACAC
cytB_Ph551R	TTAATGTGGGCAGGGGTG
cytB_Ph635R	GATATGATTAATGCAATTACCCC

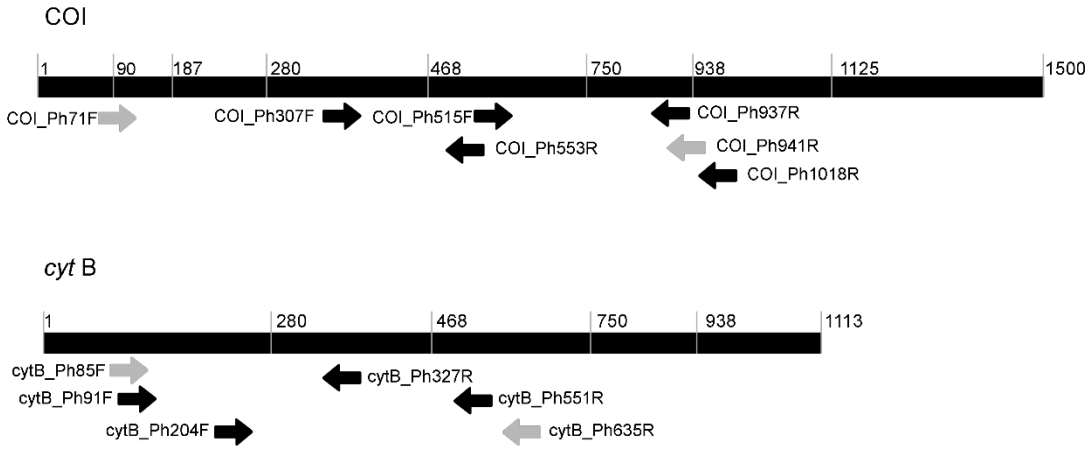

Figure 7.1. Schematic diagram of COI and cytB gene regions showing the binding sites of the PCR primers developed and tested in this study. Primers were designed on conserved regions of *P. spumarius* sequences. All possible combinations were tested (see also Supplementary Table S7.1 for additional details, *e.g.,* amplicon expected sizes). Nucleotide positions are according to full COI (NC_005944:1382-2915; 1534 nt) and cytB (NC_005944:10216-11348; 1133 nt) gene sequences retrieved from the complete mitochondrial genome of *P. spumarius*. White arrows represent primers selected for further experiments.

Evaluation of the primer sets' specificity and sensitivity and their efficacy as a diagnostic tool were performed for all possible primer pair combinations (Supplementary Table S7.1) in PCR assays. The specificity of the primers to *Philaenus* was evaluated by using as templates genomic DNA extracted from *P. spumarius* and from seven non-target species (including closely-related taxa of *Philaenus*). Primer sensitivity was assessed using different concentrations of *P. spumarius* DNA (*i.e*., at extraction concentration of 121.43 ng/µL, and diluted at 10 ng/µL and 0.1 ng/µL). For the evaluation of primers' efficiency, three different sample types were prepared: (i) a mock sample with a mixture of DNA of the seven non-target species at equal ratios and concentrations (10 ng/µL each); and mock samples spiked with DNA of *P. spumarius* at (ii) 10 ng/µL; (iii) and 0.1 ng/µL. For all PCR assays, each primer pair was used in 10 µL reactions, containing 1x buffer, 2.5 mM of $MgCl_2$, 1.5 mg/mL of bovine serum albumin (BSA, Promega), 200 µM of dNTP's, 0.2 µM of each primer, and 1.25 U Taq DNA polymerase (BIORON, GmbH). The PCR program was optimized by varying the annealing temperature (from 48 °C to 64 ˚C through gradient PCR; Supplementary Table S7.2). Primer sets showing good performance at higher annealing temperatures were then subjected to further tests and optimizations to improve the specificity and sensitivity by varying the time of denaturation, annealing, and extension

(Supplementary Table S7.3). Optimized cycling protocols for the selected primer pairs are indicated in the results section.

7.2.3. *Post feeding detection period of* Philaenus spumarius *in* Xysticus acerbus

Feeding trials were conducted to determine the time at which *Philaenus* DNA is detectable within the spider *X. acerbus* after feeding. Accordingly, live adults of *X. acerbus* were collected in natural ground vegetation with an entomological sweep net (38 cm diameter) in a meadow located in Rabal (41º 51' 30.8" N, 6º 44' 53.4" W), near Bragança (Northeastern Portugal), in July of 2019. Spiders were brought to the laboratory, where they were identified to species level and individually placed in Petri dishes (7 cm diameter) to be starved for seven days. During this period, the spiders were maintained at 21 °C with 55% relative humidity and 16:08 h (L:D) photoperiod, and water was supplied daily. At the end of the starvation period, one *P. spumarius* adult, provided by the Spanish National Research Council (CSIC, Madrid), was offered to each spider. Predators were observed until feeding started and ceased (8.2±0.22 h). At 0.5, 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, and 70 h post-feeding, specimens of *X. acerbus* were sacrificed and stored in 96% ethanol and frozen at -20 °C until subsequent molecular assay. At each post-feeding time, five replicates were conducted and processed independently. After being macerated in liquid nitrogen, DNA from each spider was extracted using the SpeedTools DNA Tissue Extraction kit (Biotools, Spain), according to manufacturer instructions. By using the whole body of the spiders for extraction, DNA of *P. spumarius* may be sticking on the legs or abdomen, which can lead to subsequent false positives. Therefore, before maceration, spiders were externally cleaned several times with 96% ethanol and dried on filter paper. Gut content PCR amplification of the *P. spumarius* DNA from the feeding trial was performed by using the two primer pairs COI_Ph71F/COI_Ph941R and cytB_Ph85F/cytB_Ph635R, and the respective optimized PCR condition, which is described in the results section. PCR reactions were performed with DNA at the extraction concentration (240.81 ± 117.80 ng/µL) and diluted in a proportion of 1:1. In each PCR assay, DNA extracted from *P. spumarius* specimens and a mix of DNA of *X. acerbus* and *P. spumarius*, in a proportion 3:1, was used as a positive control (C+). DNA extracted from *X. acerbus* starved for seven days was used as negative control (C−). PCR products were separated and visualized as previously described. Positive scores of PCR amplification were subjected to Probit analysis using MedCalc statistical software version 19.4.1 in order to

calculate the time-limit of *P. spumarius* detectability after consumption by *X. acerbus*. Chi-square (X^2) tests were performed to determine the fitting of data to the Probit model.

7.2.4. *Field assay*

The applicability of the PCR-based technique developed in this work was tested by screening 50 *Oxyopes* sp. spiders. A different spider was selected to corroborate the specificity of the primers. *Oxyopes* sp. spiders were collected in an olive grove under integrated production management located in the Mirandela region (Northeastern Portugal) (41º 29' 15.77" N, 7º 07' 52.11" W), in mid-July 2019. This sampling grove was selected due to the previously reported presence of *P. spumarius* (Morente et al., 2018). Adults of *Oxyopes* sp. were collected on ground cover vegetation with an entomological sweep net (38 cm diameter) and individually selected with a mouth aspirator. All collected spiders were immediately placed in falcon tubes with 96% ethanol, morphologically identified, and frozen at -20 °C for later DNA extraction. Total DNA was extracted from the whole spiders using the SpeedTools DNA tissue extraction kit (Biotools) following the manufacturer's guidelines. DNA diluted in a proportion of 1:1 from each spider was amplified using the cytB_Ph85F/cytB_Ph635R primer pair and its optimized PCR conditions to confirm the possible predation of *Philaenus* sp. in the field. Each reaction was checked by electrophoresis, and the positive samples were sequenced at Macrogen Inc. (Madrid, Spain) for taxonomic molecular-based identification, following the same procedure as mentioned above.

7.2.5. *Phylogenetic relationship among specimens*

COI and *cyt*B sequences from this study and all the GenBank-retrieved sequences used in the multiple alignments for the design of the primers were aligned using ClustalW, in MEGA v10.1.8 (Kumar et al., 2018). Using the same software, a Neighbor-Joining (NJ) tree with 5000 bootstrap replicates was constructed to each multiple-alignment. Phylogenetic trees were edited with Inkscape 0.92 (www.inkscape.org).

7.3. Results

7.3.1. Primer specificity and sensitivity

In this study, the specificity of the 20 primer pairs chiefly designed for *Philaenus spumarius* (Supplementary Table S7.1) was first tested. In total, 19 primer pairs successfully yielded DNA fragments of the expected size, although a few amplicons showed faint and/or double bands (Supplementary Table S7.2). Seven primer pairs gave clear single bands and high annealing temperatures and were further tested for specificity and sensitivity. Among them, the four primer pairs COI_Ph71F/COI_Ph937R, COI_Ph71F/COI_Ph941R, cytB_Ph85F/cytB_Ph551R, and cytB_Ph85F/cytB_Ph635R showed to be highly specific for *P. spumarius*, without non-specific amplifications (Supplementary Table S7.3). Experiments with diluted DNA concentrations from *P. spumarius* and with spiked mock samples indicated that the primer pairs COI_Ph71F/COI_Ph941R and cytB_Ph85F/cytB_Ph635R were the most sensitive and efficient (Supplementary Table S7.3). Both primer sets showed the capacity to amplify *P. spumarius* DNA at low concentrations of 0.1 ng/µL, including when mixed with large quantities of non-target species DNA (Figure 7.2).

Sequencing of the amplified fragments confirmed the specificity of amplification, showing 99% identity with *P. spumarius* sequences in GenBank. COI_Ph71F/COI_Ph941R set of primer generates a PCR product of 870 bp (Figure 7.2), and its high specificity and sensitivity were achieved with the following PCR cycling conditions: an initial denaturation for 3 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 40 s and a final extension at 72 °C for 7 min. The primer pair cytB_Ph85F/cytB_Ph635R generates an amplicon with 550 bp (Figure 7.2). Optimized amplification conditions were: initial denaturation step for 3 min at 94°C, followed by 30 cycles at 94 °C for 40 s, 64 °C for 40 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. Hence, these two pairs of primers were selected, and their suitability to detect *P. spumarius* DNA in feeding assays was further evaluated.

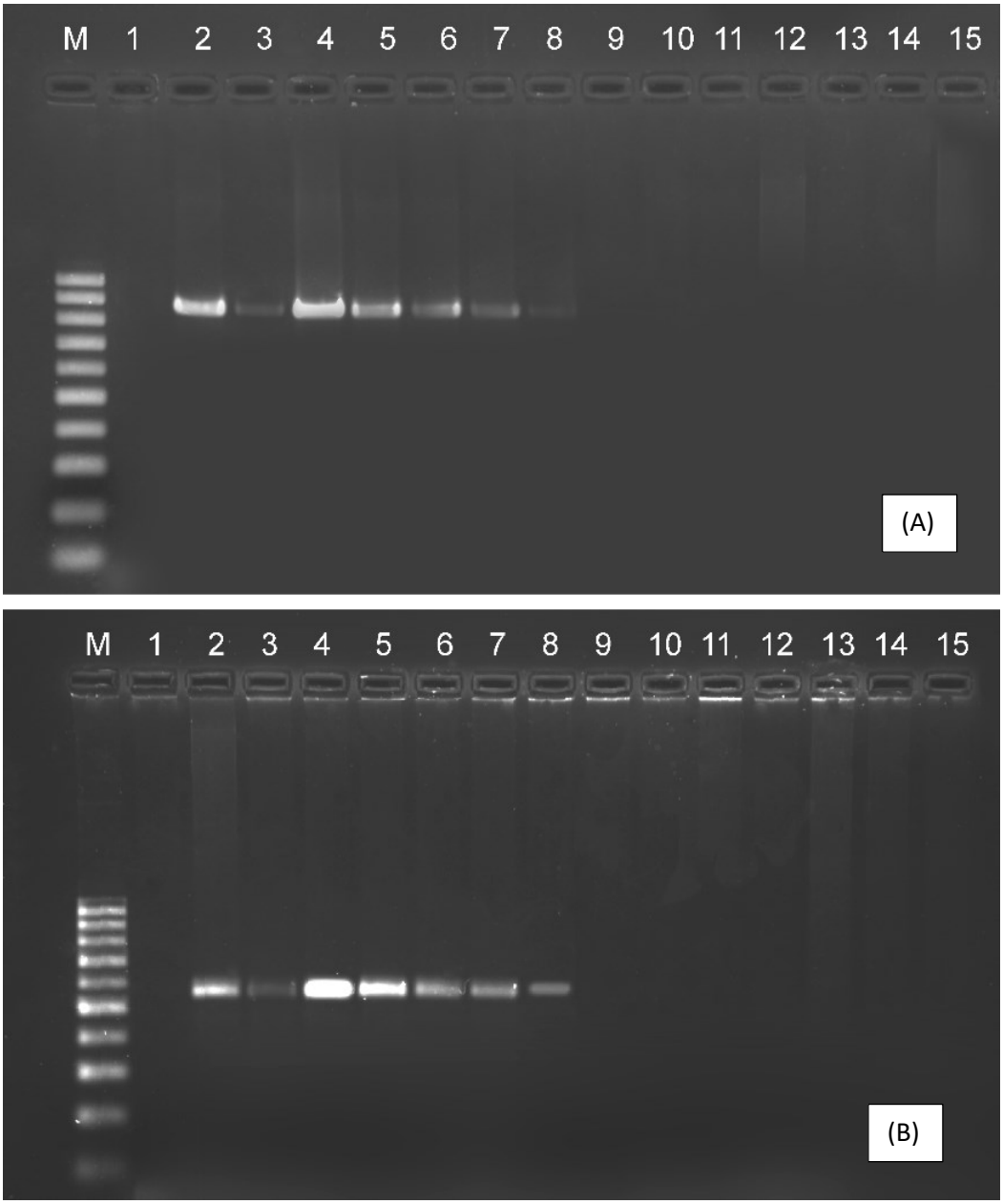

Figure 7.2. Primer's specificity and sensitivity for *Philaenus*. Agarose gel electrophoresis of amplification products with the primers pairs (A) COI_Ph71F / COI_Ph941R and (B) cytB_Ph85F/cytB_Ph635R. Lane M: Molecular Weight Marker 100 bp DNA Ladder (BIORON, GmbH); Lane 1: Mock community without DNA of *P. spumarius* added; lane 2: Mock community with 10 ng/µL DNA of *P. spumarius*; lane 3: Mock community with 0.1 ng/µL DNA of *P. spumarius*; lane 4: *P. spumarius* with DNA at the extracted concentration; lane 5 to 7: different specimens of *P. spumarius* at 10 ng/µL; lane 8: *P. spumarius* at 0.1 ng/µL; lane 9: *N. campestris* (10 ng/µL); lane 10: *N. lineatus* (10 ng/µL); lane 11: *Aphrophora* sp. (10 ng/µL); lane 12: *L. coleoptrata* (10 ng/µL); lane 13: *C. viridis* (10 ng/µL); lane 14: *Cercopis* sp. (10 ng/uL); lane 15: *X. acerbus* (10 ng/µL).

7.3.2. Feeding assay and digestion of Philaenus spumarius

The COI_Ph71F/COI_Ph941R and cytB_Ph85F/cytB_Ph635R primer pairs were used in the feeding assays to detect the presence of *P. spumarius* in the gut of *X. acerbus* specimens. The cytB_Ph85F/cytB_Ph635R primer pair proved to be the most efficient in detecting the presence of *P. spumarius* (Figure 7.3), since no positive amplifications were observed when the COI_Ph71F/COI_Ph941R primer pair was used in the feeding assays (data not showed). The detection of *P. spumarius* DNA with cytB_Ph85F/cytB_Ph635R primer pair was, however, higher with DNA dilution (1:1) than when used at the extracted concentration (Figure 7.4A). The detection of *P. spumarius* DNA following consumption by *X. acerbus* significantly decreased with the post-feeding time ($X^2 = 9.806$, df = 1, p = 0.0017 when no DNA dilution is done; and $X^2 = 4.59$, df = 1, p = 0.0321 when a DNA dilution, in a proportion 1:1, is made) (Figure 7.4B). According to Probit regression, in the diluted DNA samples, the *P. spumarius* DNA could be detected in 85% of cases within up to 20 h of digestion, decreasing to 50% after 82 h (Figure 7.4B).

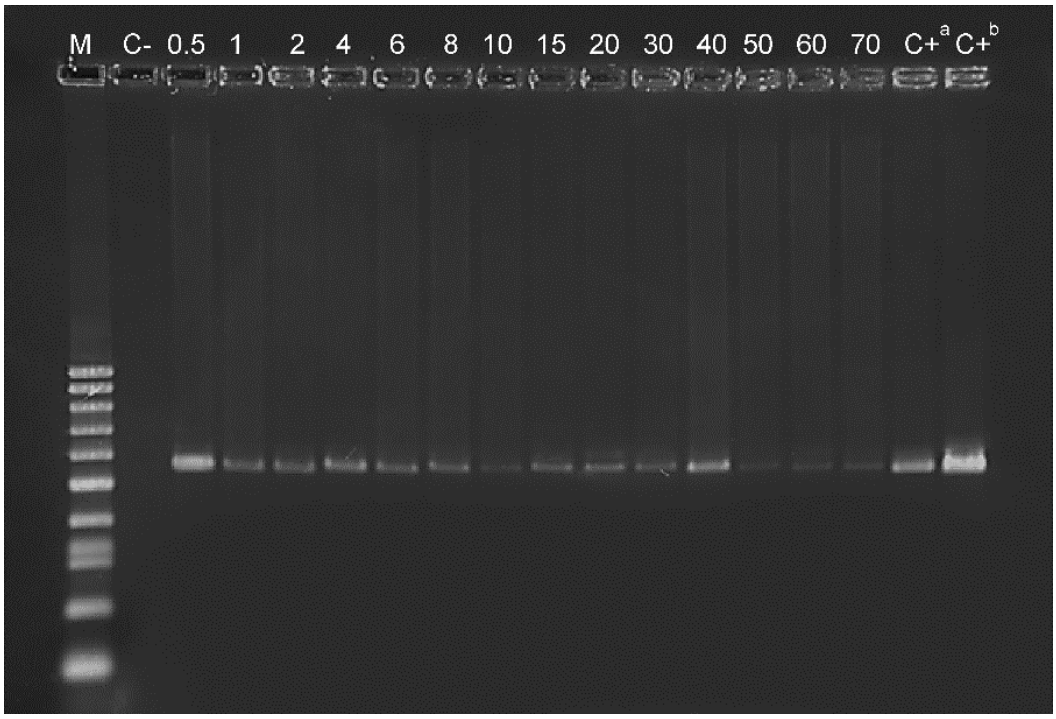

Figure 7.3. PCR amplification products obtained from DNA samples extracted in different post-feeding times, by using the primer pair cytB_Ph85F/cytB_Ph635R (expected size: 550 bp). Lane M: Molecular weight marker: 100 bp DNA Ladder (BIORON, GmbH); Lane C-: X. acerbus; Lanes 0.50, 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60 and 70 are the post-feeding times; Lane C+a mix of DNA of *X. acerbus* and *P. spumarius*, in a proportion 3:1; Lane C+b: *P. spumarius*.

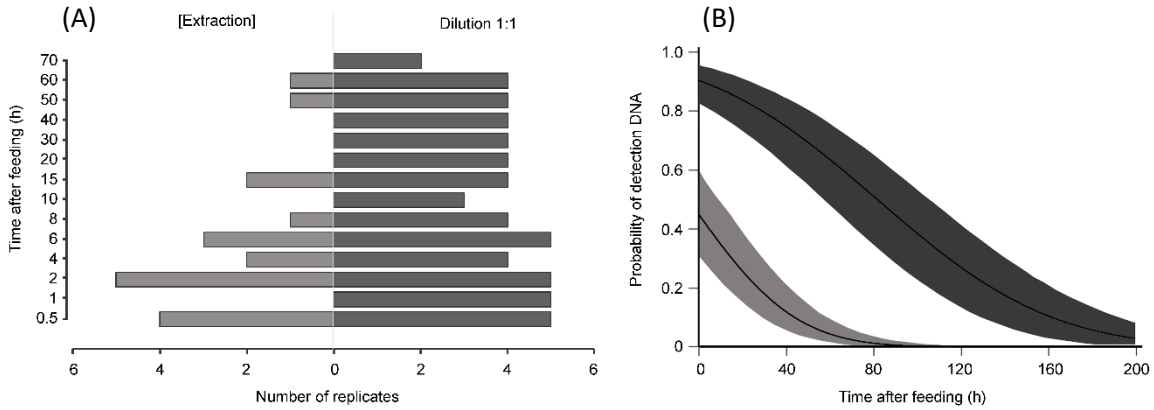

Figure 7.4. (A) Number of positive amplifications for detecting *P. spumarius* in the predator *X. acerbus* by comparing the use of gDNA at the extracted concentration (light gray bars) and gDNA diluted in 1:1 proportion (dark gray bars) at different post-feeding times (0.3 to 70 h). PCRs were performed using primer pair: cytB_Ph85F/cytB_Ph635R. (B) *P. spumarius* DNA detection probability curves in *X. acerbus* specimens after feeding. Lines are fitted Probit model, and the bands surrounding them represent the limits of the 95% confidence interval of the curves. Light gray curve: the probability of obtaining positive amplifications when no DNA dilution is done; dark gray curve: the probability of obtaining positive amplifications when the DNA is diluted in a 1:1 ratio.

7.3.3. Field assay

A total of 50 *Oxyopes* sp. individuals were analysed to confirm the possible predation of *Philaenus* in the field. This analysis was performed using the primer pair cytB_Ph85F/cytB_Ph635R. Twenty percent of the spiders tested positive. Sequence analysis of amplified products confirmed that all the positive spiders ingested *P. spumarius*.

7.3.4. Phylogenetic analyses

COI and cytB sequences from this study and all the GenBank-retrieved sequences used in the multiple-alignment for the primers design were subjected to phylogenetic analyses. The purpose of these analyses was to expose the relationship among the organisms and to compare the phylogenetic resolution of each DNA molecular marker. The COI sequences of the species specimens used in this study confirmed the initial identification based on morphological traits. Molecular analyses confirmed that used species effectively belonged to different clades and

were correctly identified (Supplementary Figure S7.2A). The three *P. spumarius* sequences obtained in this study cluster in a subclade, within a large clade encompassing all the *Philaenus* spp. sequences included in the alignment. The same pattern is observed for the *cyt*B phylogeny (Supplementary Figure S7.2B), with all the *P. spumarius* sequences from this study also grouped in a subclade. This molecular marker shows a higher level of genetic divergence of the *Philaenus* clade in relation to its relatives than that observed for COI.

7.4. Discussion

In this study, we successfully developed a PCR-based diagnostic assay for *Philaenus spumarius,* although its species-specific capability could not be tested. Among twenty primer sets tested, the primer pairs COI_Ph71F/COI_Ph941R and cytB_Ph85F/cytB_Ph635R showed sensitivity and specificity to DNA from *P. spumarius*. With both of them, it was possible to detect *P. spumarius* in a mixture of DNA from different organisms, at concentrations 100-fold lower than that of the DNA of non-target species (including species belonging to the same family of *Philaenus*). These primer pairs target regions from two mitochondrial protein-coding genes, *i.e.,* the standard barcode for invertebrates, cytochrome c oxidase subunit I (COI_Ph71F/COI_Ph941R), and the cytochrome b (cytB_Ph85F/cytB_Ph635R) genes. Currently, the COI gene is one of the most used markers for PCR-based gut-content analysis in arthropods (Monzó et al., 2010; Sint et al., 2011; Rejili et al., 2016; Unruh et al., 2016; Rowley et al., 2017; Macías-Hernández et al., 2018), including for the detection of *Philaenus* (Lantero et al., 2018). On the contrary, the *cyt*B has rarely been used for species identification of invertebrates but is widely used within vertebrates (King et al., 2008). In fact, no previous work has explored this gene for PCR-based detection and identification of prey consumed by arthropod predators. However, our results indicated that *cyt*B has a great potential for this type of diagnostic, even showing a higher discriminatory power to distinguish the *Philaenus* clade than COI (Supplementary Figure S7.2). More important, among the two primer pairs selected, only cytB_Ph85F/cytB_Ph635R was able to detect the presence of *P. spumarius* on the feeding assays. This result may be related with differences in the size of the sequence amplified by the two primer pairs and to differential DNA digestion, as observed in marine invertebrates (Troedsson et al., 2009). Overall, our results suggested that the size of the sequence amplified is crucial to successfully detect *P. spumarius* DNA in the gut of *X. acerbus*, as previously reported for other prey-predator combinations (Agustí et al., 1999, 2003b; Juen & Traugott, 2006; Aebi

et al., 2011). It is likely that during digestion, the prey DNA molecules are broken into smaller fragments, as also previously reported in other spider diet analyses (Hosseini et al., 2008; Macías-Hernández et al., 2018). Thus, the cytB_Ph85F/cytB_Ph635R primer pair that amplifies a shorter fragment (550 bp) would likely improve detection success when compared to the primer pair COI_Ph71F/COI_Ph941R that amplify longer fragments (870 pb) even if longer fragments (up to 600 bp) are readily amplified from predators' guts (Agustí et al., 1999, 2003b; Sint et al., 2011).

Prey detectability is, in most of the studies, focused on small-sized spiders by homogenizing the whole body or just the abdomen, in order to reduce the predator DNA (Agustí et al., 2003a; Monzó et al., 2010; Sint et al., 2011; Greenstone et al., 2014; Welch et al., 2016). Here, the digestion activity of *X. acerbus* specimens was successfully followed by applying the conceived diagnostic PCR assay in the DNA extracted from the entire body of the predator. However, the detection of *P. spumarius* from whole-body DNA extracts of *X. acerbus* showed to be greater in DNA samples diluted 1:1 compared with non-diluted ones. We hypothesized that this phenomenon is due to the reduction of PCR inhibitors, namely polysaccharides and proteins (which are main constituents of arthropods) (Vincent, 2002), through DNA dilution. It is possible that this procedure may also reduce the amount of non-target DNA of the predator, that at high concentrations can inhibit prey detection (Juen & Traugott, 2006; Macías-Hernández et al., 2018). Therefore, dilution of DNA to reduce the predator's DNA concentration seems to be an important procedure to enhance PCR amplification.

The present study showed that digestion time is an important aspect for the detectability of prey DNA, which is consistent with previous studies for other groups of predators and prey (Hoogendoorn & Heimpel, 2001; Agustí et al., 2003b; Hosseini et al., 2008). Understanding how quickly the level of prey DNA decreases inside a predator and identifying the digestion time where there is a 50% of detection success are essential to analyze predators sampled in the field (Hosseini et al., 2008). This information can be used together with knowledge of predator activity to plan the best hour in the day to collect them in the field. Spiders are generally nocturnal (Cardoso et al., 2008), so it is expected that specimens collected early in the day may lead to high prey detection rates. It is likely that the DNA in their guts is not digested since feeding the night before. Our diagnostic PCR assay allows the detection of *P. spumarius* in *X. acerbus* at least up to 70 h post-feeding, and based on the probit model, it can reach 50% detection success after 82 h. The capacity of spiders to store excess food in the branching of the midgut for extended periods (Harwood et al., 2001) can probably justify the long detection time

observed. Hosseini et al. (2008), Monzó et al. (2010), and Sint et al. (2011) also reported long detection periods in other spider species (49.6, 78.25, and 79.2 hours, respectively). Considering the fact that spiders are efficient predators and have higher average detection times when compared to other arthropods makes them prone to gut content analyses to accurately detect prey in specimens collected in the field. Indeed, this was corroborated by our field assay, which successfully confirmed the predation of Philaenus by the spider *Oxyopes* sp. naturally occurring in its ecosystem. Lantero et al. (2018) have previously designed specific primers for *P. spumarius* based on the COI sequence region. In their work, no feeding assay was developed to establish the prey detectability over time, and the DNA was extracted from the gut. By taking advantage of complete mitogenomes now available for Aphrophoridae species, we could explore others COI sequence regions (see Supplementary Figure S7.1) that could be suitable to design novel primers. Also, COI primers designed in our study were developed to amplify a larger fragment than those from Lantero et al. (2018) since an unequivocal identification of *Philaenus* at the species level may not be feasible by using small COI fragments (Seabra et al., 2020; see also Supplementary Figure S7.2). This is relevant for gut DNA derived from field samples whenever a confirmation by sequencing is required. Indeed, as in Lantero et al. (2018), we did not evaluate the primers on other *Philaenus* species. So, we cannot assure that the primers from this study are species-specific for *P. spumarius*, although the Primer-BLAST results indicated a good likelihood for it. Another advantage of our primers, and notably that of the primer set cytB_Ph85F/cytB_Ph635R (shown to amplify a 550 bp DNA fragment *P. spumarius* from the digestive tract of the two studied spider species), is that the size of the PCR products makes them feasible to be sequenced for molecular/phylogenetic analysis, allowing a further confirmation of the identification.

7.5. Conclusions

The *Philaenus* primers here designed (aiming to target principally *P. spumarius*) and the optimized PCR-based diagnostic assay can provide an effective and sensitive method for detecting potential predators of the main vector (or its very close phylogenetic relatives) of *X. fastidiosa*. This PCR-based diagnostic assay can help in the implementation of more sustainable measures to limit the spread of this vector-borne pathogen. We also reinforce the importance of spiders as predators, and particularly as a natural enemy of *Philaenus* in the field.

Acknowledgments

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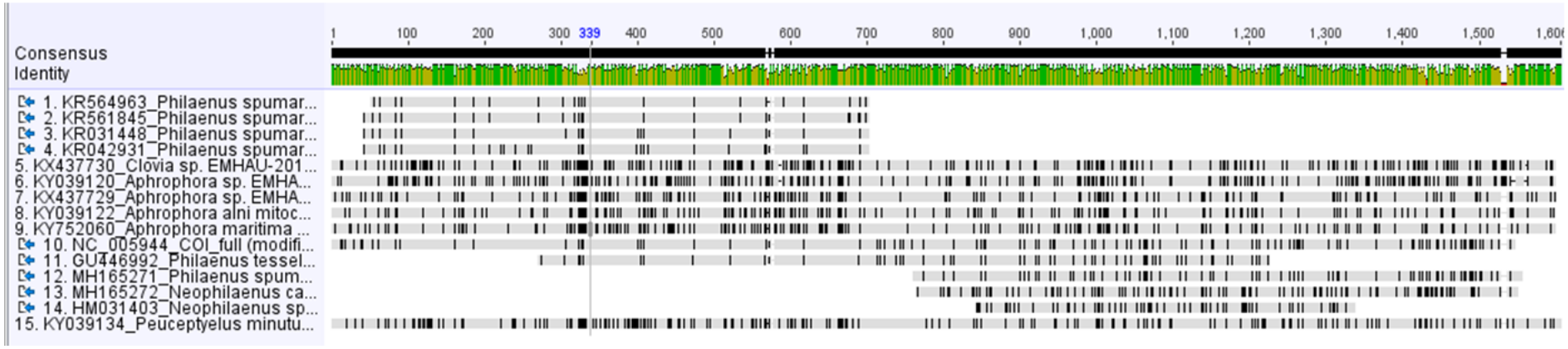
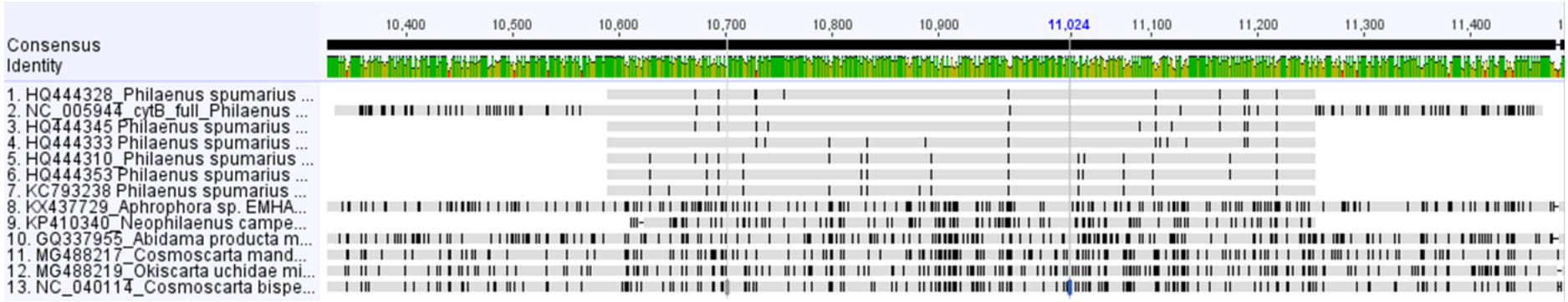
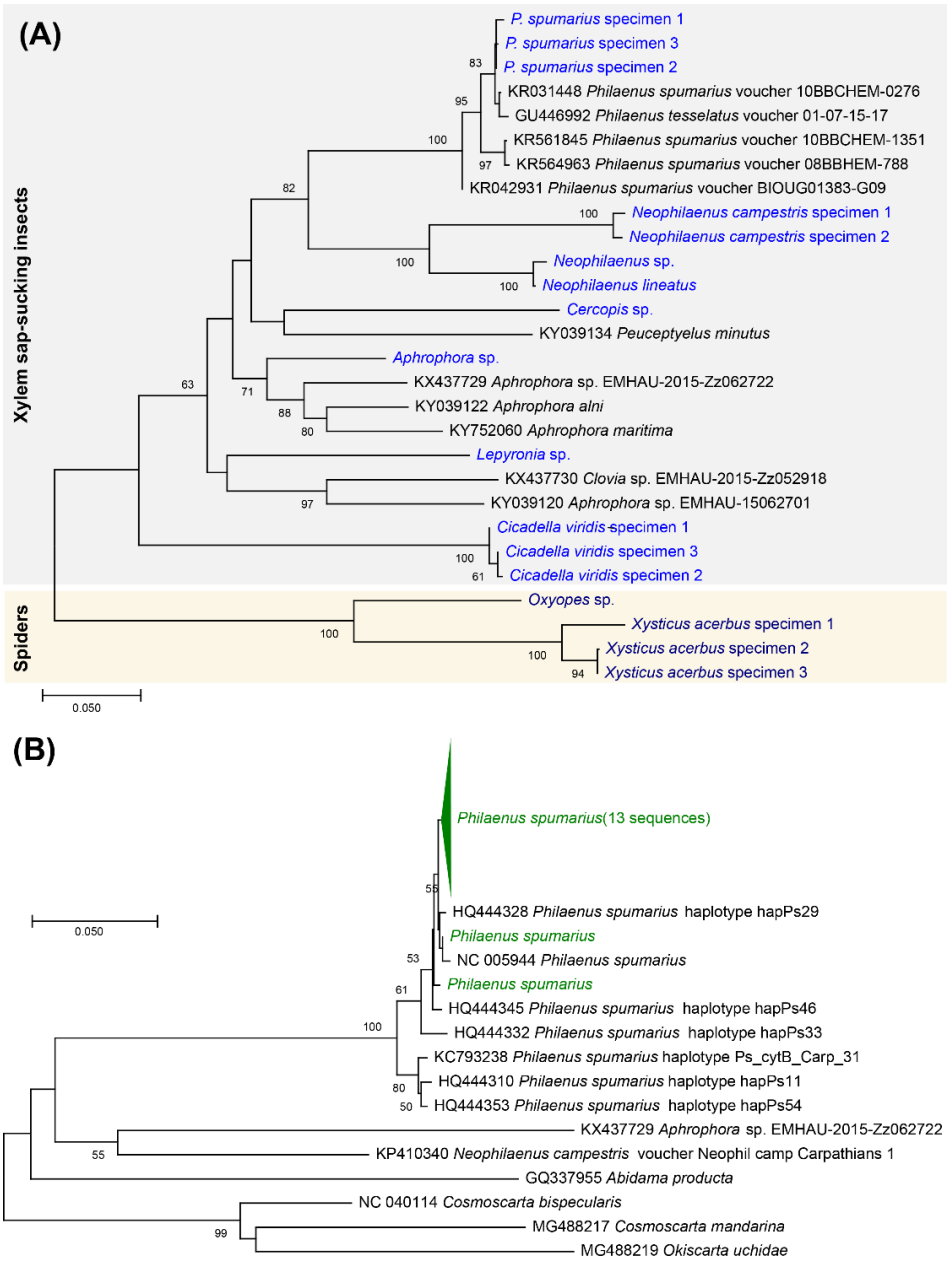
COI



*Cyt*B



Supplementary Figure S7.1. Alignments of COI and cytB gene sequences from different xylem feeding insects from the Aphrophoridae and Cercopidae families. *Positions according to the COI gene or the mitogenome sequence (for cytB) of P. spumarius (NC_005944). Primers were designed on conserved regions for P. spumarius.*



Supplementary Figure S7.2. NJ phylogenetic trees for species studied in this work (colored sequences) and their relation to species included in the primer designing (sequences in black, with accession numbers). (**A**) COI sequences PCR-amplified in this study with the universal primers from Folmer et al. (1994) are highlighted in blue. (**B**) *Cyt*B sequences obtained in this study (in green) were amplified with the newly developed primer pair cytB_Ph85F/cytB_Ph635R. There were a total of 681 (A) and 665 (B) positions in the final datasets. Bootstrap values (5000 replicates) are shown next to branch nodes. The scale bar indicates the number of base substitutions per site.

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Supplementary Table S7.1. Primer sets tested in this study and their estimated PCR product sizes. In bold are the selected primer pairs used in post feeding and field assays.

Forward	Reverse	Size (bp)
COI_Ph71F	COI_Ph553R	482
COI_Ph71F	COI_Ph937R	866
COI_Ph71F	**COI_Ph941R**	**870**
COI_Ph71F	COI_Ph1018R	947
COI_Ph307F	COI_Ph553R	246
COI_Ph307F	COI_Ph937R	630
COI_Ph307F	COI_Ph941R	634
COI_Ph307F	COI_Ph1018R	711
COI_Ph515F	COI_Ph937R	422
COI_Ph515F	COI_Ph941R	426
COI_Ph515F	COI_Ph1018R	503
cytB_Ph85F	cytB_Ph327R	242
cytB_Ph85F	cytB_Ph551R	466
cytB_Ph85F	**cytB_Ph635R**	**550**
cytB_Ph91F	cytB_Ph327R	236
cytB_Ph91F	cytB_Ph551R	460
cytB_Ph91F	cytB_Ph635R	544
cytB_Ph204F	cytB_Ph327R	123
cytB_Ph204F	cytB_Ph551R	347
cytB_Ph204F	cytB_Ph635R	431

Supplementary Table S7.2. Different annealing temperatures tested for each primer set to improve the specificity to *Philaenus*.

Primer set	PCR condition A					
	48 °C	49.7 °C	52.2 °C	55.5 °C	59.9 °C	64 °C
COI_Ph71F - COI_Ph553R	+	+	+	+*	+*	+*
COI_Ph71F - COI_Ph937R	+	+	+	+	+	+
COI_Ph71F - COI_Ph941R	+	+	+	+	+	+
COI_Ph71F - COI_Ph1018R	+	+	+	+$	-	-
COI_Ph307F - COI_Ph553R	+$*	+$*	+$*	+$*	+$*	-
COI_Ph307F - COI_Ph937R	+	+	+	+	+	+
COI_Ph307F - COI_Ph941R	+	+	+	+	+	+
COI_Ph307F - COI_Ph1018R	+$*	+$*	+$*	+$*	-	-
COI_Ph515F - COI_Ph937R	+	+	+	+	+$*	-
COI_Ph515F - COI_Ph941R	+	+	+	+$	+$*	-
COI_Ph515F - COI_Ph1018R	+	+	+	+$	-	-
cytB_Ph85F - cytB_Ph327R	+$*	+$*	+$*	+$*	+$	-
cytB_Ph85F - cytB_Ph551R	+	+	+	+	+	+
cytB_Ph85F - cytB_Ph635R	+	+	+	+	+	+
cytB_Ph91F - cytB_Ph327R	+	+*	+*	+*	-	-
cytB_Ph91F - cytB_Ph551R	+	+	+$	+$	-	-
cytB_Ph91F - cytB_Ph635R	-	-	-	-	-	-
cytB_Ph204F - cytB_Ph327R	+*	+*	+*	+*	-	-
cytB_Ph204F - cytB_Ph551R	+*	+*	+*	+*	+*	+*
cytB_Ph204F - cytB_Ph635R	+$	+$	+$	+$	+$	-

PCR condition A: initial denaturation for 3 min at 94 °C, followed by 30 cycles at 94 °C for 40 s, 48 to 64 °C for 40 s, 72 °C for 45 s and a final extension at 72 °C for 7 min.

+: positive amplification; - : negative amplification; $: faint band;*: double band.

: amplified products confirmed by sequencing.

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Supplementary Table S7.3. PCR condition optimization for each primer set and DNA template. Primer sets showing good performance at higher annealing temperatures were subjected to further tests and optimizations to improve the specificity and sensitivity to *Philaenus*.

	PCR condition B							PCR condition C												PCR condition D											
gDNA / Primer set	P. sp^{Ext}	P. sp^{10}	P. sp$^{0.1}$	N.ca^{10}	Mock 1	Mock 2	Mock 3	P. sp^{Ext}	P. sp^{10}	P. sp$^{0.1}$	N.ca^{10}	N. li^{10}	Aph^{10}	L. co^{10}	C. vi^{10}	Cer^{10}	X. ac^{10}	Mock 1	Mock 2	Mock 3	P. sp^{Ext}	P. sp^{10}	P. sp$^{0.1}$	N.ca^{10}	N. li^{10}	Aph^{10}	L. co^{10}	C. vi^{10}	Ce^{10}	X. ac^{10}	Mock 1	Mock 2	Mock 3
COI_Ph71F - COI_Ph937R	+	+$^{\$}$	+$^{\$}$	-	+$^{\$}$	+	+$^{\$}$	+	+ (3/3)$^{\$}$	-	-	-	-	-	-	-	-	-	+	+$^{\$}$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
COI_Ph71F - COI_Ph941R	+	+	+$^{\$}$	-	+$^{\$}$	+	+	+	+ (3/3)	+$^{\$}$	-	-	-	-	-	-	-	-	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
COI_Ph307F - COI_Ph937R	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
COI_Ph307F - COI_Ph941R	+	+$^{\$}$	-	-	+$^{\$}$	+	+$^{\$}$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
cytB_Ph85F - cytB_Ph551R	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+$^{\$}$	+ (3/3)$^{\$}$	-	-	-	-	-	-	-	-	-	-	-
cytB_Ph85F - cytB_Ph635R	+	+	+$^{\$}$	-	+$^{\$}$	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+ (3/3)	+$^{\$}$	-	-	-	-	-	-	-	+	+$^{\$}$
cytB_Ph204F - cytB_Ph635R	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

PCR condition B: initial denaturation for 3 min at 94 °C, followed by 30 cycles at 94 °C for 40 s, 64 °C for 40 s, 72 °C for 45 s and a final extension at 72 °C for 7 min; PCR condition C: initial denaturation for 3 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 40 s and a final extension at 72°C for 7 min; PCR condition D: initial denaturation for 3 min at 94 °C, followed by 30 cycles at 94 °C for 40 s, 64 °C for 40 s, 72 °C for 30 s and a final extension at 72 °C for 7 min.

P.sp: *Philaenus spumarius;* N.ca: *Neophilaenus campestris;* N.li: *Neophilaenus lineatus*; Aph: *Aphrophora* sp.; L.co: *Lepyronia coleoptrata*; C.vi: *Cicadella viridis*; Cer: *Cercopis* sp.; X.ac: *Xysticus acerbus*. Mock 1: mix with gDNA of the non-target species in the same ratio; Mock 2: mix with gDNA of the non-target species in the same ratio and gDNA of *P. spumarius* at 10 ng/µL; Mock 3: mix with gDNA of the non-target species in the same ratio and gDNA of *P. spumarius* at 0.1 ng/µL.

Ext: [gDNA] at the extracted concentration (121.43 ng/µL.); 10: [gDNA] at 10 ng/µL; 0.1: [gDNA] at the 0.1 ng/µL.

+: positive amplification; - : negative amplification; $: faint band; n.t. not tested

In parentheses number of positive *vs* tested specimens.

amplified products confirmed by sequencing.

Supplementary Table S7.4. Nucleotide Basic Local Alignment Search Tool (BLASTn) best-hit results for the different COI sequences (obtained with the universal primers) from specimens studied in this work.

Scientific name	Score	Query cover	E-value	Identity (%)	Accession
Cicadella viridis	1155	99%	0.0	99.68%	FR775764.1
Lepyronia coleoptrata	1062	100%	0.0	98.35%	KF919404.1
Neophilaenus campestris	856	98%	0.0	97.25%	MK188544.1
Neophilaenus lineatus	1046	99%	0.0	97.09%	KF920371.1
Oxyopes sp.	1027	94%	0.0	98.62%	MK644596.1
Philaenus spumarius	1140	99%	0.0	99.68%	NC_005944.1
Philaenus spumarius	822	100%	0.0	99.56%	HQ444308.1
*Philaenus spumarius**	383	93%	4.00E-10[2]	88.46%	MG406615.1
Xysticus acerbus	983	98%	0.0	95.60%	KY268806.1

* bad quality sequence

CHAPTER 8

General conclusions and future perspectives

8. General conclusions and future perspectives

- Five xylem-feeding insects, namely, *Philaenus spumarius*, *Neophilaenus campestris*, *N. lineastus*, *Lepyronia coleoptera*, and *Cicadella viridis* were captured in the Portuguese vineyards; *Cicadella viridis* was the most abundant followed by *P. spumarius;*

- All the vectors and potential vectors of *X. fastidiosa* and most species of the infraorder Cicadomorpha collected in Portuguese vineyards showed a positive correlation with the inter-row vegetation;

- In the sampled vineyards, individuals of Cicadomorpha that cause direct damage to vines and can act as vectors of grapevine yellows' phytoplasmas were also collected and identified;

- Almond, olive, and citrus orchards, vineyards and scrublands can harbour a great diversity and abundance of Cicadomorpha. This diversity and abundance may vary with the agrosystem and the time of year;

- The confirmed *X. fastidiosa* vectors, *P. spumarius* and *N. campestris* were captured in all sampled agroecosystems, generally, with higher abundance in Autumn;

- Females of *P. spumarius* are attracted to cis-3hexenyl acetate and cis-3-hexen-1-ol, but only when they are in low concentration;

- *Cicadella viridis* did not present attraction to cis-3hexenyl acetate and cis-3-hexen-1-ol, in any concentration;

- An increase in the volatile concentration did not significantly influence the choices of *P. spumarius;*

- Females of *P. spumarius* can play a key role in *X. fastidiosa* dissemination due to their ability to walk longer distances at higher speeds than males;

- In Spring, males and females of *P. spumarius* were significantly attracted by "Negrinha de Freixo".

- The olfactory response towards the olive cultivars can vary throughout the life cycle of the vector;

- In Autumn, females of *P. spumarius* were significantly attracted by the cultivar "Cobrançosa";

- The olfactory response toward the traditional cultivars was sex-dependent;

- The primer sets, COI_Ph71F/COI_Ph941R, targeting the COI gene, and the *cyt*B gene (cytB_Ph85F/cytB_Ph635R), showed the highest specificity and sensitivity, being able to amplify 870 pb and 550 bp fragments;

- The cytB_Ph85F/cytB_Ph635R was able to detect *P. spumarius* in the spider *Xysticus acerbus*, reaching 50% detection success 82 h after feeding;

The results presented in this thesis are only the first steps towards finding and implementing sustainable measures to reduce the spread of *X. fastidiosa*.

More research on how the landscape, agricultural practices and vegetation cover composition shape the Cicadomorpha community, focusing essentially on the vectors and potential vectors of *X. fastidiosa*, is essential to understand better what contributes to the variations in the abundance e diversity of these insects.

More studies focused on *Cicadella viridis*, the most abundant xylem-feeding insect in the sampled vineyards, to better understand its role in transmitting and disseminating *X. fastidiosa* in agrosystems other than olive groves are also urgently needed.

Complementary studies on the feeding preferences of the vectors are necessary, as well as monitoring the seasonal abundance of *P. spumarius* to deepen the knowledge of its behaviour patterns and increase the protection of the olive tree against the bacteria. As well as the study of the volatile profile of the different olive cultivars to understand to what extent these volatiles drive the olfactory response of *P. spumarius*.

And finally, studies to test the primers designed in this present thesis in other insects to find other potential predators are also essential.

Resumen

Resumen

Xylella fastidiosa es una bacteria patógena, gram-negativa, limitada al xilema, de crecimiento lento, descrita por primera vez en 1987 (Wells et al., 1987). Es nativa del continente americano y está muy extendida por todas las zonas del mismo (América del Norte, Central y del Sur) (Almeida & Nunney, 2015; EPPO, 2022a). En Europa, fue descrita por primera vez en 2013 en olivares de Apulia (sur de Italia) (Saponari et al., 2013), donde este patógeno se propagó y mató a millones de olivos en Apulia, causando problemas socioeconómicos sin precedentes (Saponari et al., 2019). Desde este primer informe, se ha detectado *X. fastidiosa* en otros países europeos, incluido Portugal.

En Portugal, este patógeno se describió por primera vez en enero de 2019 en un seto ornamental de *Lavandula dentata* L. en Oporto (norte de Portugal) (EPPO, 2019b). A finales de 2022 se establecieron diez nuevas áreas afectadas por este patógeno en este país (DGAV, 2022d), lo que demuestra que la bacteria se está propagando progresivamente. Actualmente, se han identificado 80 plantas hospedantes en el país, entre las cuales, plantas de importancia económica como el olivo (*Olea europaea* L.), los cítricos (*Citrus* spp.), el alcornoque (*Quercus suber* L.) y el almendro (*Prunus dulcis* (Mill.) D.A.Webb) (DGAV, 2022; EFSA et al., 2023).

Este patógeno es un endófito obligado con alta variabilidad genómica y plasticidad, lo que le permite tener una amplia gama de hospedantes. Actualmente, se sabe que *X. fastidiosa* infecta a 655 especies de plantas pertenecientes a 88 familias diferentes (EFSA et al., 2022a). De entre ellas, se incluyen varias especies de importancia económica que son responsables de enfermedades de gran perjuicio económico como el chamuscado de la hoja del almendro, el síndrome de declive rápido del olivo, la enfermedad de Pierce de la vid (*Vitis vinifera* L.), clorosis variegada de los cítricos, quemado de la hoja del café (*Coffea spp*.), el "*phony peach*" del melocotonero (*P. persica* (L.) Batsch), escaldado del ciruelo (*P. domestica* L.), quemado de la hoja (*Nerium oleander* L., *Ulmus spp*., *Platanus spp*. y *Acer spp*.) y enanismo de la alfalfa (*Medicago sativa* L.) (Hopkins & Purcell, 2002; Saponari et al., 2013).

Xylella fastidiosa, además de colonizar el xilema de la planta huésped, también coloniza las piezas bucales de sus insectos vectores (Martelli et al., 2016; Sabella et al., 2019). El insecto adquiere la bacteria cuando se alimenta del xilema de una planta infectada; una vez dentro del insecto, *X. fastidiosa* permanece restringida a las partes del intestino anterior del canal alimentario del insecto, donde la bacteria se adhiere, se multiplica y persiste (Almeida et al., 2005; EFSA et al., 2018; Overall & Rebek, 2017). Una vez adquirida, la bacteria no requiere un período de latencia antes de la transmisión, lo que significa que el vector puede transmitir el

patógeno a plantas sanas inmediatamente después de la adquisición (Janse & Obradovic, 2010). Además, los vectores adultos pueden transmitir la bacteria durante toda su vida (Almeida et al., 2005; Chatterjee et al., 2008a,b; EFSA et al., 2018); sin embargo, la bacteria no se transmite a la descendencia (EFSA et al., 2018; Krugner et al., 2019; Redak et al., 2004). Los vectores pueden tener una eficiencia de transmisión diferente, que puede depender de la especie del vector, la preferencia de la planta huésped y el tejido, el genotipo del patógeno, la temperatura y otros factores (Sicard et al., 2018).

Como bacteria limitada al xilema, *X. fastidiosa* es transmitida exclusivamente por insectos chupadores de savia del xilema (EFSA et al., 2019). Por tanto, en Europa todos los xilema-alimentadores de savia son considerados vectores potenciales hasta que se demuestre lo contrario (EFSA et al., 2019). Estos insectos pertenecen al suborden Auchenorrhyncha (Hemiptera) infraorden Cicadomorpha, Superfamilias Cercopoidea (familias: Aphrophoridae y Cercopidae) y Cicadoidea, y la familia Cicadellidae (subfamilia: Cicadellinae) (Cornara et al., 2019; Redak et al., 2004). En Europa, están descritas más de 90 especies como exclusivas en la alimentación de savia bruta (EFSA, 2013,2015; Cornara et al., 2019). Sin embargo, solo *Philaenus spumarius* (Linnaeus, 1758), *Philaenus italosignus* Drosopoulos & Remane, 2000, y *Neophilaenus campestris* (Fallén, 1805) (Cicadomorpha: Aphrophoridae) están confirmados como vectores de *X. fastidosa* (Cavalieri et al., 2019). *Cicadella viridis* (Linnaeus, 1758) (Cicadomorpha: Cicadellidae: Cicadellinae), un cicadélido abundante en Europa, ha demostrado ser un vector específico de *X. fastidiosa* en condiciones de laboratorio; sin embargo, las tasas de adquisición y en especial las de inoculación son significativamente inferiores a las registradas en otros vectores (Bodino et al., 2022). No existe información sobre la competencia de otros insectos chupadores de savia bruta en Europa que transmiten *X. fastidiosa*. Aunque sea probable que la alimentación por la savia bruta permita la adquisición y transmisión de la bacteria, es importante demostrar que las especies no identificadas a día de hoy como vectores, sean también otros posibles vectores y puedan transmitir la bacteria de una planta a otra.

Philaenus spumarius y *N. campestris* tienen ciclos biológicos similares; son especies univoltinas con una única generación al año (Bodino et al., 2021b; Cornara et al., 2018). Pasan el invierno en forma de huevo hasta principios de primavera, cuando se produce la eclosión (Bodino et al., 2021b). Una vez eclosionadas, las ninfas comienzan a producir un tenue mucílago en la planta hospedante (Bodino et al., 2021b; Villa et al., 2020). Los adultos empiezan a emerger entre mayo y julio (Antonatos et al., 2021; Bodino et al., 2021b). El apareamiento ocurre después de la aparición del adulto y continúa a lo largo del año; sin embargo, la maduración de los huevos

se retrasa hasta final del verano (Witsack, 1973). En otoño, las hembras depositan sus huevos en restos de plantas. Los huevos son puestos en masa adheridos y cubiertos por una sustancia cementosa (Weaver, 1951; Whittaker, 1973; Yurtsever, 2000). Mientras que el Cicadellidae más abundante en Europa, *C. viridis,* puede tener una o más generaciones al año. En las partes más frías de Europa, este insecto puede tener solo una generación (Beok, 1972), en las partes más cálidas puede tener dos y en otras regiones puede tener tres generaciones al año. Los huevos invernantes eclosionan en abril, dando inicio a la primera generación, pasan por cinco estados ninfales y entre mayo y junio comienzan a aparecer los primeros adultos (Beok, 1972). La oviposición se produce siete semanas después de la aparición de las hembras; sus huevos los depositan en la cubierta vegetal (Hasbroucq et al., 2020). La segunda generación surge en agosto (Hasbroucq et al., 2020). Las hembras de la segunda generación ponen sus huevos en la cubierta vegetal y plantas leñosas, causando frecuentemente daños de importancia económica a los árboles (Beok, 1972).

Philaenus spumarius, *N. campestris*, y *C. viridis* se alimentan únicamente del xilema durante los estados ninfal y adulto, pudiendo alimentarse de una amplia gama de plantas hospedantes (Bodino et al., 2020; Weaver & King, 1954; Yurtsever, 2000). Los ensayos electrofisiológicos demostraron que *P. spumarius* y *N. campestris* pueden responder a compuestos orgánicos volátiles (Anastasaki et al., 2021; Germinara et al., 2017). Además, Cascone et al. (2022) informaron que las hembras de *P. spumarius* fueron atraídas por los cultivares de olivo Ogliarola, Rotondella y Frantoio, y repelidas por FS-17. Estos resultados respaldan que, aunque estos vectores tienen significativamente menos sensibilidad en las antenas que otras especies (Ranieri et al., 2016), pueden recurrir a señales semioquímicas para localizar y elegir plantas hospedantes. Sin embargo, no existe información sobre cómo *C. viridis* elige su planta huésped. Las ninfas de *P. spumarius* se encuentran fundamentalmente en especies de plantas de las familias Asteraceae, Fabaceae, Apiaceae, Geraniaceae y Asparagaceae (Bodino et al., 2021; Dongiovanni et al., 2019; Morente et al., 2018; Villa et al., 2020). Las ninfas de *N. camprestris* se encuentran mayoritariamente en plantas monocotiledóneas, principalmente de la familia Poaceae (Bodino et al., 2021b; Dongiovanni et al., 2019; Morente et al., 2018; Villa et al., 2020). Por otro lado, las ninfas de *C. viridis* se alimentan y se refugian en la base de plantas como *Polygonum fagopyrum* L., *Phragmites* spp., *Cyperus* spp., y *Juncus* spp. (Cornara et al., 2019).

En cuanto a los adultos, se ha descrito en la bibliografía que *P. spumarius* se alimenta de olivos, vides y otras plantas de importancia económica. *Neophilaenus camprestris* se puede

encontrar en coníferas (*Pinus* spp.) durante el período de estivación y normalmente se alimenta de *Bromus madritensis* L. (Poaceae) (Cornara et al., 2021). Los adultos de *C. viridis* se pueden encontrar alimentándose de las mismas plantas que las ninfas (Cornara et al., 2019).

Philaenus spumarius, N. campestris, y *C. viridis* se desplazan volando, saltando y caminando (Cornara et al., 2018). Weaver & King (1954) refieren que *P. spumarius* puede viajar más de 30 m en un solo vuelo y desplazarse hasta 100 m en 24 horas desde el punto de liberación, mientras que Bodino et al. (2021a) observaran que los individuos pueden dispersarse hasta 400 m durante el pico de población en Italia. Además, Lago et al. (2021a), usando un molino de vuelo, concluyeron que estos insectos podían moverse hasta 500 m en un vuelo de 30 min. *Philaenus spumarius* también puede saltar hasta 70 cm sobre el suelo con una aceleración de 400 m/s^2 (Burrows, 2003). *Neophilaenus campestris*, puede volar largas distancias, alcanzando casi 1,4 km en 82 minutos en un solo vuelo (Lago et al., 2021b). En cuanto a C. *viridis*, en promedio puede realizar saltos con una velocidad de despegue de 0,88 m/s y una aceleración constante de 152 m/s^2 (Bonsignori et al., 2013). En comparación con *P. spumarius* (Burrows, 2003,2007), el salto de *C. viridis* es más lento, lo que permite al insecto conservar energía (Bonsignori et al., 2013). En cuanto a la capacidad de vuelo, Beok (1972) observó que, en promedio, las hembras de *C. viridis* pueden volar durante cuatro minutos, mientras que los vuelos de los machos duran menos de cuatro segundos.

Actualmente, no existe una cura para *X. fastidiosa*, por lo que el control de vectores se percibe como la principal herramienta para limitar la propagación de esta bacteria (Schneider et al., 2020). Estudios anteriores mostraron que las medidas de control de vectores basadas en estrategias químicas pueden reducir significativamente la población de *P. spumarius* (*e.g.,* Dáder et al., 2019; Dongiovanni et al., 2018a,b). Sin embargo, el uso extensivo de plaguicidas en la agricultura puede presentar serios riesgos para la biodiversidad, los servicios ecosistémicos y la salud humana (Sharma et al., 2019). Por lo tanto, se necesitan enfoques sostenibles y ecológicos como alternativas al control químico. Las prácticas culturales también podrían ayudar a reducir la actividad de los vectores y la densidad de población. Sanna et al. (2021) afirman que las prácticas de laboreo del suelo pueden reducir la densidad de *P. spumarius* en un 60%, mientras que la siega frecuente puede reducir la densidad en un 20%. Sin embargo, las prácticas culturales como el laboreo pueden tener impactos ambientales negativos más fuertes (Karamahouna et al., 2019).

La cubierta vegetal proporciona refugio y alimento a una amplia gama de fauna beneficiosa que realiza servicios ecosistémicos esenciales en los agroecosistemas, como la

polinización, descomposición, regulación del ciclo de nutrientes y control de plagas y enfermedades (Silva et al., 2010). Las plantas trampa pueden ser alternativas útiles a las prácticas culturales agresivas. Esta práctica implica el cultivo de especies de plantas que son muy atractivas para la plaga y diferentes del cultivo principal para atraer a la plaga objetivo y reducir su población. Por lo tanto, identificar las especies de plantas preferidas y menos preferidas por *P. spumarius* es esencial para diseñar cultivos de cobertura o plantas en márgenes de cultivos para reducir las poblaciones de *P. spumarius*. Además, identificar los compuestos orgánicos volátiles (VOCs) emitidos por las plantas y desentrañar su papel en la respuesta de los insectos vectores también puede contribuir a la implementación de enfoques para manipular el comportamiento de estos insectos e implementar estrategias de control sostenibles como el *push-pull* (Cook et al., 2007), y atracción y muerte (El-Sayed et al., 2009; Gregg et al., 2018). Con respecto al control biológico, algunos trabajos muestran que aves, arácnidos y otras especies de insectos se alimentan ocasionalmente de *P. spumarius* (Benhadi-Marín et al., 2020; Halkka et al., 1967; Harper & Whittaker, 1976; Phillipson, 1960). Los depredadores generalistas, como las arañas, también pueden desempeñar un papel importante en el control biológico de *P. spumarius*. Las arañas son uno de los órdenes de artrópodos más abundantes y diversos y se consideran uno de los grupos más importantes de enemigos naturales (Nyffeler, 2000; Nyffeler & Birkhofer, 2017). Aunque se ha descrito que las arañas se alimentan de *P. spumarius*, el conocimiento sobre la actuación de los arácnidos como antagonistas es escaso y está desactualizado (Harper & Whittaker, 1976; Phillipson, 1960).

Objetivos

La aparición de *X. fastidiosa* en Europa y su presencia en otros países europeos, como Francia, España y Portugal, ha aumentado enormemente la concienciación sobre la amenaza que supone este patógeno para la agricultura europea. De hecho, esta bacteria infecta los tejidos del xilema de una amplia gama de cultivos de importancia agrícola, como la planta de vid, los cítricos, el almendro y el olivo, causando elevadas pérdidas. Tras su introducción, la única vía de propagación natural de *X. fastidiosa* son los insectos vectores, en particular los que se alimentan de la savia bruta, del infraorden Cicadomorpha, de las superfamilias Cercopoidea (familias: Aphrophoridae y Cercopidae) y Cicadoidea, y de la familia Cicadellidae (subfamilia: Cicadellinae).

En la actualidad, los métodos de control disponibles contra este patógeno mortal y altamente invasivo son ineficaces. Hasta ahora, el conocimiento sobre los vectores de la bacteria y los

vectores potenciales en Portugal es escaso. También es incipiente la respuesta de los vectores o potenciales vectores europeos a diferentes estímulos de Compuestos Orgánicos Volátiles (COVs). Estos COVs podrían influir en el comportamiento de los insectos y en la preferencia de cultivares, lo que es particularmente importante para los cultivares tradicionales de olivo producidos bajo sistemas de producción sostenibles. Los olivares sostenibles son agroecosistemas ricos en biodiversidad; sin embargo, se desconoce el papel de los depredadores potenciales en la supresión de los vectores de *X. fastidiosa*.

En este contexto, el objetivo general de esta tesis fue estudiar aspectos de la diversidad y abundancia de vectores y potenciales vectores en diferentes agroecosistemas (viñedo, cítricos, almendro y olivar) y en los bordes de parcela del olivar (matorral). Así como esclarecer el papel de los COVs y de los cultivares tradicionales de olivo en el comportamiento olfativo de los vectores o vectores potenciales, y el potencial de los depredadores generalistas en la limitación natural de los vectores confirmados *de X. fastidiosa*.

Los objetivos específicos fueron:

(1) Estudiar la abundancia, diversidad y riqueza de individuos de Cicadomorpha en la línea y vegetación de las calles de cultivo de los viñedos portugueses;
(2) Estudiar la abundancia, diversidad y riqueza de la comunidad Cicadomorpha en diferentes agroecosistemas: almendro, olivar y cítricos, viñedo y matorral;
(3) Evaluar la respuesta olfativa de *Philaenus spumarius* y *Cicadella viridis* a los COV emitidos por las hojas de plantas de olivo, almendro y vid;
(4) Estimar los parámetros de movimiento de *Philaenus spumarius* y *Cicadella viridis*;
(5) Evaluar la respuesta olfativa de *Philaenus spumarius* a cinco cultivares tradicionales portugueses de olivo muy difundidas;
(6) Diseñar y probar cebadores específicos de taxón para aplicar un método de diagnóstico basado en PCR para detectar *Philaenus spumarius* entre los depredadores.

Estructura de la tesis

La tesis se estructuró en ocho capítulos diferentes, atendiendo a las diferentes áreas de conocimiento a desarrollar que se exponen a continuación:

Capítulo 1. Una breve descripción de *Xylella fastidiosa*, importancia y distribución. Un resumen de la diversidad de vectores potenciales y vectores de este patógeno, así como su ciclo de vida,

preferencia de plantas hospederas, distribución geográfica, movimiento, comportamiento de dispersión y las respectivas estrategias de control.

Capítulo 2. Se presentaron los objetivos generales y la estructura de la tesis.

Capítulo 3. En este capítulo, se estudió la abundancia, diversidad y riqueza de individuos de Cicadomorpha en la línea de cultivo y en la vegetación de las calles de 35 viñedos portugueses durante dos años consecutivos.

Capítulo 4. En este capítulo se presenta la caracterización de la abundancia, diversidad y riqueza de la comunidad Cicadomorpha asociada a almendros, olivos y cítricos, viñedos y matorrales cerca a los olivares en diferentes periodos de muestreo.

Capítulo 5. En este capítulo se caracterizó el perfil volátil de las hojas de olivo, almendro y vid, los cultivos más importantes de la región mediterránea, y la respuesta olfativa de los adultos *Philaenus spumarius* y *Cicadella viridis* a dos COVs comunes a los tres cultivos (cis-3-hexenil acetato y cis-3-hexen-1-ol). También se evaluaron los parámetros de movimiento mostrados durante el desplazamiento de los insectos de ambas especies.

Capítulo 6. La respuesta olfativa del principal vector de *Xylella fastidiosa*, *Philaenus spumarius*, frente a cinco cultivares tradicionales de olivo portuguesas "Cobrançosa", "Negrinha de Freixo", "Santulhana", "Madural", y "Verdeal Transmontana" se evaluó en primavera (después de la emergencia de los adultos) y otoño (fin del ciclo de vida del vector).

Capítulo 7. En este capítulo, se diseñaron y probaron varios cebadores específicos de taxón dirigidos a los genes mitocondriales de la citocromo oxidasa I (COI) y del citocromo b (cytB) para usarlos en un método de diagnóstico basado en PCR para detectar *Philaenus spumarius* en la araña *Xysticus acerbus* Thorell, 1872 Se realizaron experimentos de alimentación para evaluar la eficacia de esta herramienta de diagnóstico basada en el ADN.

Capítulo 8. Se presentaron las conclusiones generales del trabajo.

Capítulo 3. Comunidad de Cicadomorpha (Hemiptera: Auchenorrhyncha) en viñedos portugueses: con referencia a vectores y vectores potenciales de *Xylella fastidiosa*

Los insectos Cicadomorpha (Hemiptera) son actualmente responsables de un creciente impacto negativo en la economía agrícola debido a su capacidad de dañar directamente los cultivos o por su capacidad de actuar como vectores de patógenos de plantas. El objetivo de

este trabajo fue estudiar la comunidad Cicadomorpha, centrándose en los vectores y vectores potenciales de *X. fastidiosa* en la línea y en la vegetación de las calles de los viñedos portugueses. Para ello, se muestrearon adultos de Cicadomorpha en 35 viñedos distribuidos en Portugal continental en 2018 y 2019 (20 viñedos en los dos años y 15 adicionales en el segundo año) en tres períodos diferentes (finales de primavera, verano y otoño). Los adultos se colectaron en el la línea y calle de cultivo de los viñedos con una red de barrido entomológica estándar (38 cm). En la calle de los viñedos se recolectaron diez muestras de 10 barridas consecutivas distribuidas aleatoriamente en 1 ha y en la línea se recolectaron diez muestras de 50 barridas sucesivas. Se identificaron todos los adultos del infraorden Cicadomorpha recolectados. Para la identificación de especies, se diseccionaron los genitales masculinos y se colocaron en una solución calentada de hidróxido de potasio (KOH) al 10% y la clasificación taxonómica se basó en claves e ilustraciones apropiadas (Biedermann & Niedringhaus, 2009; Dietrich, 2005; Dmitry, 2006; Le Quesne & Payne, 1981; Nielson, 1968). Las hembras se identificaron al nivel taxonómico más bajo posible. La estructura de la comunidad se evaluó en términos de abundancia, riqueza y diversidad de especies/morfoespecies. Los datos de cada año del estudio, 2018 y 2019, se trataron de forma independiente debido a la variabilidad interanual. Para analizar el efecto del estrato muestreado en la comunidad de Cicadomorpha, se realizó un análisis de varianza multivariante permutacional (PERMANOVA) y para evaluar el esfuerzo de muestreo se trazaron curvas de acumulación de especies. Adicionalmente, se realizó un análisis de coinercia (CIA) para determinar la relación entre especie/morfoespecies de Cicadomorpha y el año de muestreo y estrato.

En total se recolectaron 11834 individuos, de los cuales 3003 en 2018 y 8831 en 2019. Durante los dos años de estudio se identificaron 81 especies/morfoespecies. Psammotettix sp.1 (3314 individuos), *Empoasca vitis* (Göthe, 1875) (2866 individuos) y *Zyginidia scutellaris* (Herrich-Schäffer, 1838) (1079 individuos) fueron las especies/morfoespecies más abundantes. En la línea de los viñedos se recuperaron un total de 4262 individuos. La subfamilia Typhlocybinae predominó en este estrato, representando el 92% del total de individuos muestreados en la copa de las plantas de vid. *Empoasca vitis* y *J. lybica*, fueron las especies más abundantes de esta subfamilia. Estas especies, comúnmente conocidas como mosquitos verdes, son una plaga clave en varias regiones vitivinícolas europeas (Decante & van Helden, 2006; Fornasiero et al., 2016).

En la vegetación de las calles del viñedo, se observó una mayor abundancia de individuos de Cicadomorpha, se recuperaron un total de 7572 individuos. Además, la riqueza específica y el índice de diversidad de Shannon Wiener (H') también fueron significativamente mayores en la vegetación de las calles de cultivo del viñedo ($P < 0,01$). Además, según el análisis NMDS y el análisis PERMANOVA ($df = 1$; $F = 2$; $p = 0,001$ para 2018, y $df = 1$; $F = 5$; $p = 0,001$ para 2019), la comunidad de Cicadomorpha difirió significativamente entre los estratos muestreados, las líneas de vides y la vegetación entre líneas participantes. De hecho, el análisis de coinercia mostró que la mayoría de las especies de Cicadomorpha, incluidos los vectores y los vectores potenciales de *X. fastidiosa*, mostraron una correlación positiva con la vegetación de las calles de cultivo, lo que va en concordancia con la literatura (Antonatos et al., 2021; Carpio et al., 2020; Elbeadaino et al., 2014; Morente et al., 2018; Tsagkarakis et al., 2018; Villa et al., 2020).

Se capturaron un total de cinco especies de insectos que se alimentan de savia de la xilema, a saber: *Cicadella viridis* (Linnaeus, 1758) (307 individuos), *Philaenus spumarius* (Linnaeus, 1758) (112 individuos), *Neophilaenus campestris* (Fallén, 1805) (62 individuos), *Lepyronia coleoptera* (Linnaeus, 1758) (7 individuos), y *N. lineatus* (Linnaeus, 1758) (3 individuos), y la mayor abundancia de individuos se observó en la vegetación de las calles del viñedo en el año 2019. *Philaenus spumarius* fue el más abundante en los viñedos muestreados, en consonancia con otros estudios realizados en viñedos europeos y californianos (Beal et al., 2021; Bodino et al., 2021b; López-Mercadal et al., 2021). Además, varios estudios han demostrado que *P. spumarius* puede transmitir eficientemente *X. fastidiosa* a los viñedos (Beal et al., 2021; Cornara et al., 2016; Severin, 1950). La comunidad de Cicadomorpha recuperada de los viñedos muestreados está dominada por insectos que se alimentan del floema. Algunos estudios han demostrado que algunos insectos que se alimentan del floema pueden infectarse con la bacteria (Ben Moussa et al., 2016; Chuche et al., 2017; Elbeadaino et al., 2014). Sin embargo, no hay evidencia de que puedan transmitir el patógeno (Cavalieri et al., 2019; Purcell, 1980). Adicionalmente, algunas de las especies recolectadas en los viñedos muestreados también son consideradas vectores o vectores potenciales de enfermedades fitoplasmáticas de la vid (*grapevine yellow* GY) y son responsables de importantes daños en el viñedo. Entre estos, *Scaphoideus titanus* Ball, 1932, principal vector del fitoplasma de la Flavescencia dorada (Chuche & Thiéry, 2014). *Euscelidius variegatus* (Kirschbaum, 1858) es otra especie potencialmente importante; una vez que demostró la capacidad de adquirir y transmitir el fitoplasma que causa la Flavescencia dorada en condiciones de laboratorio (Picciau et al., 2020) y también dio positivo a 'Candidatus Phytoplasma solani' (Laviña et al., 2006; Quaglino et al., 2019). Se ha demostrado

que *Neoaliturus fenestratus* (Herrich-Shäffer, 1834) es portador del '*Candidatus* Phytoplasma solani' (Batlle et al., 2000; Riolo et al., 2007; Minuz et al., 2013). *Anaceratagallia glabra* Dmitriev, 2020 (=*A. laevis*), *Austroagallia sinuata* (Mulsant & Rey, 1855) y *Z. scutellaris* también se han establecido como vectores potenciales de las enfermedades fitoplasmáticas de la vid (Batlle et al., 2000).

En conclusión, nuestros resultados demuestran que los vectores y potenciales vectores de *X. fastidiosa* están presentes en los viñedos portugueses. *Cicadella viridis* fue el vector potencial más abundante en los viñedos muestreados. Se necesitan más estudios sobre las tasas de transmisión para comprender mejor el papel de este insecto en la epidemiología de *X. fastidiosa*. *Philaenus spumarius*, el principal vector europeo, fue el más abundante. Además, se recolectaron e identificaron vectores de enfermedades fitoplasmáticas de la vid así como especies que pueden dañar físicamente las plantas de vid. Los vectores y potenciales vectores de *X. fastidiosa* y gran parte de la población de Cicadomorpha mostraron una correlación positiva con la vegetación de las calles de cultivo. Un mayor número de investigaciones sobre cómo el paisaje, las prácticas agrícolas, la aplicación de tratamientos fitosanitarios, la variedad presente en el sitio de muestreo y las condiciones ambientales afectan a la comunidad Cicadomorpha son necesarios para diseñar nuevas técnicas para prevenir la propagación de este patógeno en los viñedos portugueses.

Capítulo 4. Comunidad de Cicadomorpha (Hemiptera: Auchenorrhyncha) en distintos agroecosistemas en el norte de Portugal

En Portugal, el conocimiento de vectores potenciales de *Xylella fastidiosa* es todavía muy incipiente. Sin embargo, existe una importante necesidad de conocer la diversidad y abundancia de vectores potenciales en los agroecosistemas portugueses, ya que este patógeno fue descrito por primera vez en 2019 en el norte del país, habiéndose reportado varios focos nuevos principalmente en el norte y centro de Portugal (DGAV, 2022). Por tanto, el objetivo del presente estudio fue caracterizar la abundancia y diversidad de la comunidad Cicadomorpha asociada a almendros, olivos, cítricos, viñedos y espacios naturales aledaños a los olivares. Se realizaron estudios de campo en cinco plantaciones de almendros, cinco viñedos, cinco olivares, cinco matorrales (alrededores de los olivares) en dos años consecutivos (2018 y 2019) y cinco limonares (en adelante, plantaciones de cítricos), solo en 2019, ubicados en el norte de Portugal.

Resumen

Todos los agroecosistemas se encontraban bajo sistemas productivos sostenibles, y la cobertura vegetal herbácea se mantuvo en las líneas de cultivo durante los periodos de muestreo. En cada año y agroecosistema se muestrearon adultos del infraorden Cicadomorpha en tres periodos diferentes: principios de primavera verano y otoño. El muestreo de adultos se realizó en el matorral y en la copa de los árboles con una manga entomológica de barrido (38 cm de diámetro). En cada fecha de muestreo en todos los sitios de muestreo, distribuidos aleatoriamente en 1 ha, se recolectaron diez muestras de diez barridos consecutivos en la vegetación del suelo. Cada barrido se realizó moviendo la manga entomológica 180 grados. En la copa de las plantas de todas las plantaciones se realizaron diez muestreos de dos barridos en seis árboles, seleccionados al azar. Además, se recogieron diez muestras de 50 barridos en la copa de los viñedos. Cada muestra se realizó en un transecto de 40 metros. Se identificaron todos los adultos del infraorden Cicadomorpha recolectados. Para la identificación de especies, se diseccionaron los genitales masculinos y se colocaron en una solución calentada de hidróxido de potasio (KOH) al 10% y la clasificación taxonómica se basó en claves e ilustraciones descritas en la bibliografía especializada (Biedermann & Niedringhaus, 2009; Dietrich, 2005; Dmitry, 2006; Le Quesne & Payne, 1981; Nielson, 1968).

Los datos de cada año del estudio, 2018 y 2019, se trataron de forma independiente debido a la variabilidad interanual. Se calculó la abundancia total de Cicadomorpha (N), la riqueza de especies (S) y el índice de Shannon (H') por período de muestreo. El efecto del agroecosistema y el período de muestreo sobre la abundancia y diversidad de Cicadomorpha se investigó utilizando modelos mixtos lineales generales (GLMM). Se realizó un escalamiento multidimensional no métrico (NMDS) y un análisis de varianza multivariante permutacional (PERMANOVA) para evaluar más a fondo la variabilidad en la comunidad de Cicadomorpha a lo largo de los agroecosistemas y los períodos de muestreo. Finalmente, para explorar las especies de Cicadomorpha asociadas a cada agroecosistema y período de muestreo y evaluar la contribución de estos aspectos a la estructura de la comunidad de Cicadomorpha, se realizaron análisis de coinercia.

Se recolectaron un total de 6056 individuos del infraorden Cicadomorpha, de los cuales 2322 en 2018 y 3734 en 2019. En las plantaciones de almendros, se recogieron un total de 1691 individuos (701 en 2018 y 990 en 2019) pertenecientes a 42 especies (28 especies en 2018 y 36 en 2019). En 2018, *Fruticidia sanguinosa* (Rey, 1891) fue la especie más abundante, mientras que, en 2019, la especie: *Psammotettix* sp. y *F. bisignata* (Mulsant & Rey, 1855) fueron las más

abundantes. En los viñedos, se recuperaron un total de 2908 individuos (1244 en 2018 y 1664 en 2019) pertenecientes a 44 especies (36 especies en 2018 y 32 en 2019). *Psammotettix* sp., *Empoasca vitis* (Göthe, 1875) y *Empoasca* sp. fueron las especies más abundantes en los dos años de estudio. En los olivares, se recuperaron un total de 568 individuos (208 en 2018 y 360 en 2019) pertenecientes a 41 especies (26 especies en 2018 y 34 en 2019), *Psammotettix* sp. fue la especie más abundante en los dos años de estudio. En las zonas de matorrales, se recuperaron un total de 336 individuos (169 en 2018 y 167 en 2019) pertenecientes a 31 especies (20 especies en 2018 y 27 en 2019). *Circulifer tenellus* (Baker, 1896), *Selenocephalus sacarroi* Rodrigues, 1968 y *Centrotus cornuta* Linnaeus, 1758 fueron las especies más abundantes. En las plantaciones de cítricos, se recuperaron un total de 553 individuos pertenecientes a 23 especies. *Zyginidia scutellaris* (Herrich-Schäffer, 1838) fue la especie más abundante, seguida de *Psammotettix* sp (92 individuos) y *Philaenus spumarius* (Linnaeus, 1758) (55 individuos). La abundancia y riqueza de Cicadomorpha difirieron significativamente entre agroecosistemas por periodos de muestreo (generalmente todos $P < 0,01$). En 2018, el agroecosistema del viñedo mostró la mayor abundancia y riqueza a principios de verano y otoño. Sin embargo, en verano, las plantaciones de almendros mostraron más abundancia de Cicadomorpha que los olivares. En 2019, el agroecosistema del viñedo tuvo mayor abundancia de Cicadomorpha que los cítricos y olivares a principios de verano y otoño y mayor abundancia que los matorrales en verano. El NMDS y el análisis PERMANOVA ($df = 6$; $F = 2,15$; $P < 0,01$ para 2018, y $df = 8$; $F = 1,46$; $P < 0,01$ para 2019) sugirieron un cambio en la comunidad de Cicadomorpha entre agroecosistemas por período de muestreo en ambos años en estudio. Los resultados obtenidos en el análisis de coinercia corroboran con lo observado previamente en los análisis NMDS y PERMANOVA. Nuestros resultados indican que los agroecosistemas del norte de Portugal pueden albergar conjuntos abundantes y diversos de insectos Cicadomorpha. El agroecosistema de integración y el período de muestreo juegan un papel esencial en la configuración de la estructura y composición de la comunidad Cicadomorpha. Esto podría estar asociado a sus plantas hospedantes herbáceas, que dependen del manejo y la estación del agroecosistema (Aguyoh et al., 2004; Carpio et al., 2020; Villa et al., 2020).

Dentro de estas especies identificadas, fue posible observar varias especies consideradas plagas y vectores de patógenos. *Scaphoideus titanus* Ball, 1932, conocido por ser el vector del agente fitoplasmático de la Flavescencia dorada, una enfermedad de gran perjuicio económico para la planta de vid (Chuche & Thiéry, 2014), fue capturado en los viñedos muestreados. Además, algunos de los géneros de insectos recogidos en el viñedo, como

Austroagallia, Anaceratagallia, Euscelidius, Euscelis, Circulifer, Neoaliturus y Zyginidia, fueron previamente descritos como vectores potenciales causantes de enfermedades fitoplasmáticas de la vid (Batlle et al., 2000; Laviña et al., 2006; Minuz et al., 2013; Picciau et al., 2020; Quaglino et al., 2019; Riolo et al., 2007). Los vectores confirmados de *X. fastidiosa* (*P. spumarius* y *Neophilaenus campestris* (Fallén, 1805)) fueron capturados en todos los agroecosistemas muestreados y, en general, con mayor abundancia en otoño. Según la bibliografía después de las primeras lluvias, estos insectos tienden a regresar a los principales agroecosistemas en otoño, cuando hay un rebrote de la vegetación herbácea donde estos individuos realizan las ovoposiciones (Antonatos et al., 2021; Bodino et al., 2020; Cruaud et al., 2018; Morente et al., 2018; Tsagkarakis et al., 2018). Nuestros resultados sugieren que los olivares y matorrales tienen una comunidad Cicadomorpha similar. Los matorrales pueden actuar como reservorios de artrópodos proporcionando alimento y refugio opcional del cultivo principal (Kubiak et al., 2022).

En conclusión, comprender y conocer la diversidad y abundancia de insectos que pueden constituir plagas importantes o jugar un papel importante en la diseminación de patógenos es el primer paso para implementar las medidas adecuadas para combatir los efectos negativos de estos insectos. Con el presente trabajo se pudo comprobar que los agroecosistemas en estudio pueden albergar una gran diversidad y abundancia de Cicadomorpha. Dentro de esta diversidad, fue posible observar varias especies consideradas plagas y vectores de patógenos. Además, esta diversidad y abundancia puede variar con el agroecosistema y la época del año. Los vectores confirmados de *X. fastidiosa* (*P. spumarius* y *N. campestris*) se recuperaron en todos los agroecosistemas muestreados en diferentes abundancias. Futuras investigaciones sobre cómo el paisaje, las prácticas agrícolas y la composición de la cubierta vegetal afectan a las comunidades de los Cicadomorpha es esencial para comprender mejor qué factores contribuyen a la composición de estas comunidades de insectos. Esto permitirá implementar estrategias para reducir la propagación de patógenos transmitidos por estos insectos si se introducen en los agroecosistemas.

Capítulo 5. Respuestas olfativas a compuestos orgánicos volátiles y parámetros de movimiento de *Philaenus spumarius* y *Cicadella viridis*

Resumen

Las señales químicas del entorno determinan el comportamiento de los insectos. Las respuestas atractivas o repulsivas a las señales pueden afectar a la aptitud, supervivencia y reproducción de los insectos y provocar diferentes patrones de movimiento. Los patrones de movimiento específicos derivados de las señales olfativas hacia la selección de plantas para alimentarse pueden desencadenar la transmisión del patógeno por el insecto. Así pues, comprender la respuesta olfativa y los parámetros de movimiento de los vectores es de suma importancia. El principal objetivo de este trabajo fue caracterizar el perfil volátil de los tres cultivos más importantes de la región mediterránea (olivo, almendro y vid) y estudiar la respuesta olfativa de adultos de *P. spumarius* y *C. viridis* a dos VOCs comunes a los tres cultivos (cis-3-hexenil acetato y cis-3-hexen-1-ol). Además, también se evaluaron los parámetros de movimiento (1) la distancia recorrida, (2) la velocidad media y (3) el tiempo total de movimiento de los insectos de ambas especies.

El perfil de volátiles de hojas de almendro (cv. Verdeal), olivo (cv. Cobrançosa) y vid (cv. Touriga Nacional) se evaluó mediante HS-SPME (microextracción en fase sólida con espacio de cabeza) y GC/MS (cromatografía de gases con detector de espectrometría de masas). Se utilizó un olfatómetro de cuatro brazos para probar las respuestas conductuales de los adultos de *P. spumarius* y *C. viridis* a los VOCs cis-3-hexenil acetato y cis-3-hexen-1-ol. Se prepararon cuatro concentraciones diferentes (5, 10, 20 y 30 µg/µL) de cada compuesto volátil. Para la prueba, cada brazo del olfatómetro funcionó como un contenedor de fuente de olor. Cada contenedor fuente de olor se conectó a una botella de lavado de gases (250 mL) con carbón activado disuelto en 100 mL de agua destilada para purificar y humidificar el flujo de aire suministrado por las bombas. Cada compuesto volátil se aplicó (10 µg) por separado sobre tiras de papel de filtro colocadas en los contenedores de fuente de olor de dos brazos opuestos del olfatómetro. Los brazos restantes del olfatómetro operaron como controles. En uno de ellos se colocó una tira de papel de filtro empapada en 10 µg de aceite de girasol, mientras que el brazo opuesto contenía una tira de papel de filtro en blanco. Antes del ensayo con el olfatómetro, los insectos se sexaron usando un microscopio estereoscópico binocular y se mantuvieron individualmente en tubos Eppendorf de 2 ml para dejarlos en ayunas durante 4 h. Después de la inanición, se soltó un insecto cada vez en el orificio central de la caja de arena y se registró su comportamiento durante 20 minutos con una lente Computar® (H2Z0414C-MP, f = 4-8 mm, F 1.4, ½", lente CCTV) montado en una cámara Basler®GigE HD (acA1300-60gc con sensor e2v EV76C560 CMOS) (Noldus, 1991) Se utilizaron ecuaciones de estimación generalizadas ($\alpha = 0,05$) con distribución de Poisson para comparar la frecuencia de las visitas y la duración total de la

estancia de los individuos en cada área delimitada. Además, para estimar los parámetros de movimiento de *P. spumarius* y *C. viridis*, se registraron individualmente un total de 30 machos y 30 hembras, respectivamente, de la arena (27 cm × 35 cm × 43 cm) durante 10 minutos La distancia total recorrida (m), la velocidad media (cm/s) y el tiempo total de movimiento (s) se estimaron utilizando el software Noldus Ethovision XT 11.5 (Noldus et al., 2001).

En total, se identificaron 83 compuestos de las tres especies de plantas. Nuestros resultados mostraron que estos cultivos tienen un perfil volátil muy distinto. Según los análisis ACP y ANOVA, los perfiles volátiles diferían significativamente entre las especies de plantas. PC1 y PC2 explicaron el 82,1% de la variación. En nuestro trabajo, las hojas del olivo, principal cultivo afectado por *X. fastidiosa* en Europa (Saponari et al., 2019), presentaron el mayor número de VOCs en comparación con las otras dos plantas (en las hojas del olivo se identificaron 54 compuestos, en la almendra 40 y en las hojas de vid 16. El cis-3-hexen-1-ol y el cis-3-hexenil acetato se encontraron en todas las especies de plantas y en mayor frecuencia, estos resultados concuerdan con Malheiro et al. (2016), que encontraron al cis-3-hexen-1-ol y al acetato de cis-3-hexenilo como componentes principales en las hojas del olivo.

A la concentración más baja de VOCs (5 µg/µL), las hembras de *P. spumarius* se sintieron significativamente atraídas por los dos compuestos volátiles. Las hembras mostraron una frecuencia de visitas significativamente mayor y una permanencia más prolongada en las áreas con cis-3-hexenil acetato y cis-3-hexen-1-ol solo en la concentración más baja, mientras que los machos de *P. spumarius* mostraron una frecuencia significativamente mayor de visitas y estancia más larga en la zona con cis-3-hexen-1-ol en comparación con el aceite. La frecuencia de las visitas difirió significativamente entre los sexos excepto cuando los individuos estuvieron expuestos a los compuestos volátiles a 10 µg/µL. Estos resultados concuerdan con los obtenidos por Ganassi et al. (2020), que observaron diferentes respuestas al olor por parte de machos y hembras cuando los individuos de *P. spumarius* se sometieron a bioensayos de comportamiento cerrados (tubo en Y) y de largo alcance (túnel de viento) con aceites esenciales y plantassimilares.

En el caso de *C. viridis*, ni la frecuencia de las visitas ni la duración total de la estancia difirieron significativamente entre los tratamientos para cualquier concentración. Estos resultados respaldan que la mayoría de las especies de Cicadellidae detectan plantas hospedantes adecuadas basándose principalmente en señales visuales o con la combinación de estímulos olfativos y visuales (Bullas-Appleton et al., 2004; Cai et al., 2015; Grange et al., 2017;

Todd et al., 1990). Dado que nuestro estudio solo se centró en la estimulación olfativa, puede explicar por qué *C. viridis* no tomó ninguna decisión: sin embargo, otros VOCs o mezclas podrían desencadenar una respuesta. Los estudios futuros sobre el comportamiento de sensilla, electrofisiológico, olfativo y visual son esenciales para comprender cómo procede este insecto para localizar y elegir las plantas hospedantes.

Las hembras de *P. spumarius* pudieron desplazarse una distancia total de 2,42 ± 0,20 m en 10 minutos, con una velocidad media de 0,43 ± 0,04 cm/s. Ambos parámetros de movimiento (distancia total y velocidad media) fueron significativamente mayores en comparación con los machos ($P < 0,01$). Las hembras de *C. viridis* también se desplazaron con velocidades medias (0,63 ± 0,07 cm/s) significativamente mayores ($P < 0,01$) que los machos. Aunque no estudiamos el vuelo, nuestros resultados en términos de velocidad y distancia recorrida, concuerdan con los de Lago et al. (2021a), que encontró un mejor desempeño femenino en términos de distancia recorrida y duración del vuelo.

En conclusión, con este trabajo fue posible verificar que las hembras de *P. spumarius* son atraídas por los dos VOCs en estudio, pero solo cuando se encuentran en baja concentración. Dado que los individuos de *C. viridis* no presentaron ninguna opción significativa, estudios complementarios y más pruebas sobre la sensibilidad y las respuestas electroantenográficas pueden ayudar a comprender mejor el papel de las señales olfativas en la selección de plantas huésped por parte de la especie. Las hembras de *P. spumarius* pueden desempeñar un papel clave en la diseminación de *X. fastidiosa* debido a su capacidad para recorrer distancias más largas a mayor velocidad que los machos. Futuras investigaciones sobre la respuesta olfativa de los vectores de *X. fastidiosa* y su comportamiento al movimiento son esenciales para diseñar nuevas técnicas que limiten la propagación de este patógeno por toda Europa.

Capítulo 6. Respuesta olfativa estacional de *Philaenus spumarius* (Hemiptera: Aphrophoridae) respecto a los cultivares tradicionales portugueses de olivo

En Portugal, la riqueza del sector olivícola se basa en el gran patrimonio genético de los cultivares de olivo tradicionales. Sin embargo, este patrimonio genético está amenazado por el agente causal del síndrome de decaimiento rápido del olivo (OQDS), la bacteria fitopatógena *Xylella fastidiosa,* notificada por primera vez en 2019 en el país. Una evaluación en profundidad de las preferencias de plantas hospedantes del principal vector europeo de *X. fastidiosa* es crucial para entender su dinámica estacional con relación a los cultivares de olivo y determinar

los más susceptibles al ataque del vector. En este trabajo, evaluamos la respuesta olfativa de *P. spumarius* a cinco cultivares tradicionales de olivo portugueses: "Cobrançosa", "Negrinha de Freixo", "Santulhana", "Madural" y "Verdeal Transmontana" en primavera (después de la emergencia de los adultos) y otoño (fin del ciclo de vida del vector).

La respuesta olfativa estacional de *P. spumarius* a los diferentes cultivares de olivo se evaluó en un olfatómetro de ocho cámaras diseñado específicamente. El olfatómetro está hecho de plástico acrílico transparente. El dispositivo abarcaba dos zonas principales, una zona exterior de forma cuadrada (40 cm × 40 cm) y una zona central redonda (18 cm de diámetro) en el interior de esta última. La caja circular con arena se dividió en ocho cámaras radiales cubiertas con cinta blanca para minimizar el estímulo visual. Cada cámara tenía una abertura en la base (1 cm × 1 cm) que conectaba las áreas interior y exterior para permitir que el insecto eligiera una cámara.

Se evaluó la respuesta olfativa de *P. spumarius* en primavera y otoño. Las estaciones representaron dos etapas del ciclo de vida: primavera (es decir, después de la emergencia de los adultos) y otoño (es decir, final del ciclo de vida del vector). En cada temporada, los cultivares de olivo seleccionados se dividieron en dos ensayos. En el primer ensayo se probó la respuesta olfativa de *P. spumarius* hacia los cultivares "Madural", "Cobrançosa" y "Verdeal Transmontana". En el segundo ensayo, se probó la respuesta hacia el cultivar significativamente más elegido en el primer ensayo, más los cultivares "Negrinha de Freixo" y "Santulhana". Dos cámaras del olfatómetro funcionaron como control (flujo de aire purificado). Dieciséis insectos, previamente mantenidos durante dos horas en ausencia de comida y olores, se dividieron por igual y se liberaron en cada rincón de la arena del olfatómetro (es decir, cuatro insectos por cada rincón). La respuesta olfativa de los insectos se registró 30 min después de su liberación en la arena registrando el número de insectos encontrados en cada cámara. Se realizaron veinte repeticiones para machos y hembras. Se utilizaron ecuaciones de estimación generalizadas (GEE) (Pekár & Brabec, 2018) con distribución de Poisson para comparar la respuesta olfativa de *P. spumarius* hacia los diferentes cultivares de olivo por ensayo y temporada. Sexo, cultivar (primer ensayo: "Cobrançosa", "Madural", "Verdeal Transmontana" y "Control"; segundo ensayo: "Cobrançosa", "Negrinha de Freixo", "Santulhana" y "Control"), y la interacción entre los dos términos se utilizaron como variables explicativas. En primavera, los adultos de *P. spumarius* prefirieron significativamente el cultivar "Negrinha de Freixo". La "Negrinha de Freixo" es una variedad típica de la región de Trás-os-Montes, la segunda región olivarera más grande de Portugal. Este cultivar se utiliza fundamentalmente para producir aceitunas de mesa ya que tiene un rendimiento de aceite bajo (Albuquerque et al., 2019). De hecho, este cultivo

representa una gran importancia económica en la región, las aceitunas de mesa de esta variedad están reconocidas como Denominación de Origen Protegida (DOP) (Reglamento de la Comisión (CEE) 2081/92). En otoño, las hembras eligieron significativamente el cultivar "Cobrançosa". Este cultivar de doble aptitud (es decir, se puede utilizar para producir aceite de oliva y aceitunas de mesa) está ampliamente distribuido en las principales regiones olivareras portuguesas (Reis, 2014). En cambio, en otoño, los machos presentaron una respuesta olfativa más aleatoria. En el primer ensayo, eligieron significativamente los cultivares "Cobrançosa" y "Verdeal Transmontana", pero en el segundo ensayo no mostraron diferencias significativas entre los cultivares "Cobrançosa", "Negrinha de Freixo" y el testigo. Según Bodino et al. (2019), los machos tienen una longevidad más corta que las hembras, y su abundancia tiende a disminuir a lo largo de la temporada en los olivares. Por lo tanto, sugerimos que las hembras pueden seguir los estímulos olfativos para encontrar una planta huésped adecuada para la oviposición dado que el otoño representa el final del ciclo de vida de *P. spumarius*. A diferencia de los machos, que ya no tienen un papel activo en la reproducción. Nuestros resultados sugieren que *P. spumarius* eligió diferentes cultivares según las estaciones, mientras que Cascone et al. (2022) no encontraron diferencias en la respuesta olfativa de *P. spumarius* a los cultivares de olivo a lo largo del tiempo. No obstante, el perfil volátil de los cultivares de olivo tiende a cambiar según su estado fenológico (Malheiro et al., 2016). Además, los factores abióticos también pueden provocar cambios en el perfil volátil (Malheiro et al., 2016; Sofo et al., 2004), por lo que se espera que los cambios en el perfil volátil puedan inducir diferentes respuestas olfativas a lo largo de la temporada.

En conclusión, *P. spumarius* mostró diferentes respuestas olfativas a los cultivares de olivo tradicionales portugueses bajo estudio, lo que demuestra que este insecto puede usar estímulos olfativos para elegir las plantas hospedantes. En primavera, el cultivar "Negrinha de Freixo" fue el más susceptible al ataque del vector, mientras en otoño, el cultivar "Cobrançosa" fue el más elegido. Estudios adicionales sobre la preferencia de alimentación y el seguimiento de la abundancia estacional de *P. spumarius* en olivares monocultivares podrían proporcionar una evidencia más sólida de estos patrones de comportamiento. Caracterizar el perfil volátil de los cultivares y comprender hasta qué punto estos volátiles impulsan la respuesta olfativa de *P. spumarius* puede ayudar a la implementación futura de enfoques para manejar el vector. Además, nuestros resultados pueden contribuir al diseño de olivares con cultivares menos sujetos al ataque del vector y, en consecuencia, reducir la propagación de la bacteria.

Capítulo 7. Un nuevo método de diagnóstico basado en PCR para el análisis en el contenido estomacal de ADN de *Philaenus* (Hemiptera: Aphrophoridae) - vector de *Xylella fastidiosa*

Dado que actualmente no existe ninguna medida eficiente frente a *Xylella fastidiosa,* el desarrollo de estrategias de control integrado combinando distintos medios de lucha contra los vectores, utilizando depredadores generalistas, como las arañas, podría ser esencial para limitar la propagación de este patógeno.

El objetivo principal de este trabajo fue diseñar y evaluar cebadores específicos dirigidos a los genes mitocondriales del citocromo oxidasa I (COI) y del citocromo b (cytB), que se utilizarán para un método de diagnóstico basado en PCR, para detectar *P. spumarius* dentro de las arañas. Se realizaron experimentos de alimentación para evaluar la eficacia de esta herramienta de diagnóstico basada en el ADN. En concreto, este trabajo analizó: (i) la idoneidad de las regiones seleccionadas del marcador molecular y la especificidad y sensibilidad de los cebadores diseñados en la detección de *P. spumarius*; (ii) la detectabilidad de la presa a lo largo del tiempo en la araña *Xysticus acerbus* Thorell, 1872 (Thomisidae) usando extractos de ADN de su cuerpo; (iii) y la eficiencia de los cebadores diseñados para detectar *Philaenus* en *Oxyopes* sp. (Oxyopidae) arañas recolectadas directamente del campo.

Se diseñaron un total de veinte pares de cebadores. La evaluación de la especificidad y la sensibilidad de los conjuntos de cebadores y su eficacia como herramienta de diagnóstico se realizó para todas las posibles combinaciones de pares de cebadores. La especificidad de los cebadores para *Philaenus* se evaluó utilizando como molde ADN genómico extraído de *P. spumarius* y de siete especies no diana (incluidos taxones estrechamente relacionados de *Philaenus*). La sensibilidad del cebador se evaluó utilizando diferentes concentraciones de ADN de *P. spumarius* (es decir, a una concentración de extracción de 121,43 ng/µL y diluido a 10 ng/µL y 0,1 ng/µL). Para la evaluación de la eficacia de los cebadores, se prepararon tres tipos de muestras diferentes: (i) una muestra simulada con una mezcla de ADN de las siete especies no diana en proporciones y concentraciones iguales (10 ng/µL cada una); y muestras simuladas enriquecidas con ADN de *P. spumarius* a (ii) 10 ng/µL; (iii) y 0,1 ng/µL. Los conjuntos de cebadores que mostraban un buen rendimiento a temperaturas de hibridación más altas se sometieron luego a más pruebas y optimizaciones para mejorar la especificidad y la sensibilidad variando el tiempo de desnaturalización, hibridación y extensión. Se realizaron pruebas de alimentación para determinar el momento en que el ADN de *Philaenus* es detectable dentro de la araña *X. acerbus* después de la alimentación. A cada araña se le ofreció un adulto de *P.*

spumarius. Se observaron los depredadores desde que comenzó hasta que terminó su alimentación (8,2 ± 0,22 h). A las 0,5, 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60 y 70 h después de la ingesta de los insectos, se sacrificaron especímenes de *X. acerbus* y se almacenaron en etanol al 96% y se congelaron a -20 °C hasta el posterior ensayo molecular. En cada momento posterior a la alimentación, se realizaron y procesaron cinco réplicas de forma independiente. Todo el cuerpo de las arañas se utilizó para la extracción de ADN. La amplificación por PCR del ADN de *P. spumarius* del ensayo de alimentación se realizó utilizando los dos pares de cebadores COI_Ph71F/COI_Ph941R y cytB_Ph85F/cytB_Ph635R, y las respectivas condiciones de PCR optimizadas. Las reacciones de PCR se realizaron con ADN a la concentración de extracción (240,81 ± 117,80 ng/ µL) y diluido en una proporción de 1:1. En cada ensayo de PCR, se utilizó como control positivo (C+) ADN extraído de especímenes de *P. spumarius* y una mezcla de ADN de *X. acerbus* y *P. spumarius*, en una proporción de 3:1, y ADN extraído de *X. acerbus* privado de alimento. durante siete días se utilizó como control negativo (C-). Para calcular el tiempo límite de detectabilidad de *P. spumarius* después del consumo por *X. acerbus*. Se realizaron pruebas de chi-cuadrado (X2) para determinar el ajuste de los datos al modelo Probit. La aplicabilidad de la técnica basada en PCR desarrollada en este trabajo se probó mediante el cribado de 50 arañas (*Oxyopes* sp.) Se seleccionó una araña diferente para corroborar la especificidad de los cebadores. Adultos de *Oxyopes* sp. Las arañas fueron recogidas sobre la vegetación de cobertura del suelo con una manga entomológica de barrido (38 cm de diámetro) y seleccionadas individualmente con un aspirador bucal recolectadas en un olivar bajo manejo de producción integrada ubicado en la región de Mirandela (Noreste de Portugal) (41º 29' 15.77" N, 7º 07' 52.11" W), a mediados de julio de 2019. Esta arboleda de muestreo fue seleccionada debido a la presencia previamente reportada de *P. spumarius* (Morente et al., 2018). El ADN diluido en una proporción de 1:1 de cada araña se amplificó utilizando el par de cebadores cytB_Ph85F/cytB_Ph635R y sus condiciones de PCR optimizadas para confirmar la posible depredación de *Philaenus* sp. en el campo. Cada reacción se comprobó por electroforesis.

Desarrollamos con éxito un ensayo de diagnóstico basado en PCR para *P. spumarius*, aunque no se pudo probar su capacidad específica a nivel de especie. Entre veinte pares de cebadores probados, los pares COI_Ph71F/COI_Ph941R y cytB_Ph85F/cytB_Ph635R mostraron sensibilidad y especificidad al ADN de *P. spumarius*. El conjunto de cebadores COI_Ph71F/COI_Ph941R genera un producto de PCR de 870 pb, y su alta especificidad y sensibilidad se lograron con las siguientes condiciones de ciclo de PCR: una desnaturalización inicial durante 3 min a 94 °C, seguida de 30 ciclos a 94 °C durante 30 s, 64 °C por 30 s, 72 °C por

40 s y una extensión final a 72 °C por 7 min. El par de cebadores cytB_Ph85F/cytB_Ph635R genera un amplicón de 550 pb. Las condiciones de amplificación optimizadas fueron: paso de desnaturalización inicial de 3 min a 94 °C, seguido de 30 ciclos a 94 °C durante 40 s, 64 °C durante 40 s, 72 °C durante 30 s y una extensión final a 72 °C durante 7 min. Los pares de cebadores COI_Ph71F/COI_Ph941R y cytB_Ph85F/cytB_Ph635R se utilizaron en los ensayos de alimentación para detectar la presencia de *P. spumarius* en el intestino de los especímenes de *X. acerbus*. El par de cebadores cytB_Ph85F/cytB_Ph635R demostró ser el más eficaz para detectar la presencia de *P. spumarius*, ya que no se observaron amplificaciones positivas cuando se utilizó el par de cebadores COI_Ph71F/COI_Ph941R en los ensayos de alimentación.

Sin embargo, la detección de ADN de *P. spumarius* con el par de cebadores cytB_Ph85F/cytB_Ph635R fue mayor con la dilución de ADN (1:1) que cuando se usó en la concentración extraída. Presumimos que este fenómeno se debe a la reducción de los inhibidores de la PCR, pudiendo ser debido a los polisacáridos y las proteínas (que son los principales constituyentes de los artrópodos), a través de la dilución del ADN. Es posible que este procedimiento también pueda reducir la cantidad de ADN no diana del depredador, que en altas concentraciones puede inhibir la detección de presas (Juen & Traugott, 2006; Macías-Hernández et al., 2018). Por lo tanto, la dilución del ADN para reducir la concentración de ADN del depredador parece ser un procedimiento importante para mejorar la amplificación por PCR.

La detección de ADN de *P. spumarius* después del consumo por *X. acerbus* disminuyó significativamente con el tiempo posterior a la alimentación ($X2 = 9,806$, $df = 1$, $p = 0,0017$ cuando no se realiza ninguna dilución de ADN; y $X2 = 4,59$, $df = 1$, $p = 0.0321$ cuando se hace una dilución de ADN, en proporción 1:1). Comprender qué tan rápido disminuye el nivel de ADN de la presa dentro de un depredador e identificar el tiempo de digestión donde hay un 50% de éxito en la detección, son esenciales para analizar los depredadores muestreados en el campo (Hosseini et al., 2008). Esta información se puede utilizar junto con el conocimiento de la actividad de los depredadores para planificar la mejor hora del día para recolectarlos en el campo.

Según la regresión Probit, en las muestras de ADN diluido, el ADN de *P. spumarius* pudo detectarse en el 85% de los casos hasta 20 h después de la digestión, disminuyendo al 50% después de 82 h. La capacidad de las arañas para almacenar el exceso de comida en la ramificación del intestino medio durante períodos prolongados (Harwood et al., 2001) probablemente justifique el largo tiempo de detección observado. Hosseini et al. (2008), Monzo

et al. (2010) y Sint et al. (2011) también observaran largos períodos de detección en otras especies de arañas (49,6, 78,25 y 79,2 h, respectivamente). El veinte por ciento de las *Oxyopes* sp., las arañas recolectadas en el campo dieron positivo. El análisis de secuenciación de los productos amplificados confirmó que todas las arañas positivas ingirieron *P. spumarius*. Este hecho confirmó que la depredación de *Philaenus* por arañas puede ocurrir de forma natural en el ecosistema.

En conclusión, los cebadores diseñados en este estudio para detectar *Philaenus* (con el objetivo de llegar a nivel de especie *P. spumarius*) y el ensayo de diagnóstico optimizado basado en PCR pueden proporcionar un método eficaz y sensible para detectar posibles depredadores del vector principal (o sus parientes filogenéticos muy cercanos) de *X. fastidiosa*. Este ensayo de diagnóstico basado en PCR puede ayudar en la implementación de medidas más sostenibles para limitar la propagación de este patógeno transmitido por vectores. También reforzamos la importancia de las arañas como depredadores, y particularmente como enemigos naturales de *Philaenus* en el campo.

Conclusiones y perspectivas futuras

- Fueron identificados en los viñedos portugueses cinco especies de insectos que se alimentan del xilema, los cuales son *Philaenus spumarius*, *Neophilaenus campestris*, *N. lineastus*, *Lepyronia coleoptera* y *Cicadella viridis*; *Cicadella viridis* fue el más abundante seguido de *P. spumarius*;

- Todos los vectores y potenciales vectores de *X. fastidiosa* y la mayoría de las especies del infraorden Cicadomorpha recolectadas en viñedos portugueses mostraron una correlación positiva con la vegetación de las calles de cultivo de los viñedos;

- En los viñedos muestreados también se recolectaron e identificaron individuos de Cicadomorpha que causan daños directos a las plantas y que pueden actuar como vectores de fitoplasmas causando enfermedades fitoplasmáticas de la vid;

- Viñedos y matorrales de plantaciones de almendros, olivos, cítricos pueden albergar una gran diversidad y abundancia de Cicadomorpha. Esta diversidad y abundancia puede variar con el agroecosistema y la época del año;

- Los vectores confirmados de *X. fastidiosa*, *P. spumarius* y *N. campestris* fueron capturados en todos los agroecosistemas muestreados, generalmente, con mayor abundancia en otoño;

- Las hembras de *P. spumarius* se sienten atraídas por el acetato de cis-3hexenilo y el cis-3-hexen-1-ol, pero solo cuando están en baja concentración;

- *Cicadella viridis* no presentó atracción por el cis-3hexenil acetato y el cis-3-hexen-1-ol, en ninguna concentración;

- Un aumento en la concentración de volátiles no influyó significativamente en la elección de *P. spumarius*;

- Las hembras de *P. spumarius* pueden desempeñar un papel clave en la diseminación *de X. fastidiosa* debido a su capacidad para caminar distancias más largas a mayor velocidad que los machos;

- En primavera, machos y hembras de *P. spumarius* fueron atraídos significativamente por el cultivar "Negrinha de Freixo";

- La respuesta olfativa hacia los cultivares de olivo puede variar a lo largo del ciclo de vida del vector;

- En otoño, las hembras de *P. spumarius* fueron atraídas significativamente por el cultivar del olivo "Cobrançosa";

- La respuesta olfativa hacia los cultivares tradicionales fue dependiente del sexo;

- Los conjuntos de cebadores, COI_Ph71F/COI_Ph941R, dirigidos al gen COI, y al gen cytB (cytB_Ph85F/cytB_Ph635R), mostraron la mayor especificidad y sensibilidad, pudiendo amplificar fragmentos de 870 pb y 550 pb;

- El cytB_Ph85F/cytB_Ph635R fue capaz de detectar *P. spumarius* en la araña *Xysticus acerbus*, alcanzando un 50% de éxito de detección 82 h después de alimentarse;

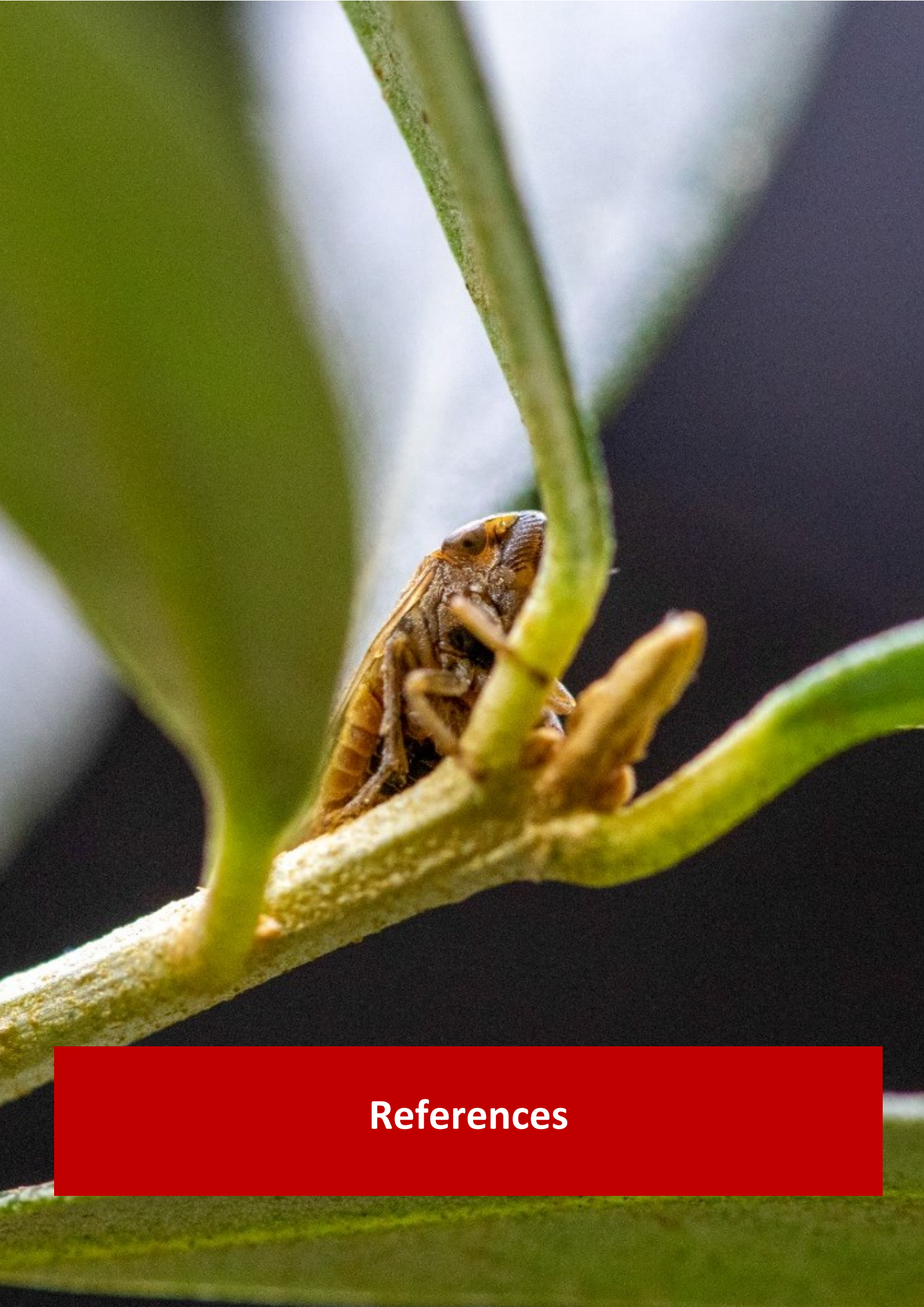
Los resultados presentados en esta tesis son solo los primeros pasos para encontrar e implementar medidas sostenibles para reducir la propagación de *X. fastidiosa*.

Un mayor número de investigaciones sobre cómo el paisaje, las prácticas agrícolas y la composición de la cubierta vegetal afecta a la comunidad Cicadomorpha, centrándose esencialmente en los vectores y potenciales vectores de *X. fastidiosa*, es esencial para comprender mejor qué contribuye a las variaciones en la abundancia y diversidad de estos insectos.

También se necesitan más estudios centrados en *Cicadella viridis*, el insecto que se alimenta del xilema, y que es el más abundante en los viñedos muestreados. Esto es necesario para comprender mejor su papel en la transmisión y diseminación de *X. fastidiosa* en agroecosistemas distintos de los viñedos.

Son necesarios estudios complementarios sobre preferencia alimentaria de los vectores y también de seguimiento de la abundancia estacional de *P. spumarius* para profundizar en el conocimiento de sus patrones de comportamiento y aumentar la protección del olivo frente a la bacteria. Así como el estudio del perfil de volátiles de los diferentes cultivares de olivo para entender en qué medida estos volátiles impulsan la respuesta olfativa de *P. spumarius*.

Y, por último, también sería esencial realizar más estudios para evaluar los cebadores diseñados en la presente tesis y utilizarlos en otros insectos con el objetivo de encontrar otros depredadores potenciales.



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