






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

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Funding information

Fundação para a Ciência e a Tecnologia, Grant/Award Number: LA/P/0007/2020, 2020.07051.BD and UIDB/00690/2020; Horizon 2020 Framework Programme, Grant/Award Number: Grant Agreement 727987

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 behaviour 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 towards 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' behavioural 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

behaviour, cis-3-hexen-1-ol, cis-3-hexenyl acetate, vectors, videotracking, *Xylella fastidiosa*

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 (Cavaliere et al., 2019). On the other hand, little is known about the ability of *C. viridis* to transmit the bacteria. Nonetheless, Bodino et al. (2019) 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 behaviour (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 behaviour 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 behaviour towards a food source or a suitable oviposition site, or repulsive behaviours against predators, or toxic substances (Depetris-Chauvin et al., 2015). Volatile Organic Compounds (hereafter VOCs), emitted by plants, provide important cues driving the behaviour 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 behaviour 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 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).

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 behaviour.

Movement is also fundamental to animal behaviour, 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 and 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 1 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/s² (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 and a constant acceleration of 152 m/s² (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 min after the first probe (Cornara et al., 2020). 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.

2 | MATERIALS AND METHODS

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).

2.1.1 | 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°C for 5 min. Then, a fibre 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.

2.1.2 | GC-MS conditions

Chromatographic analysis was performed using a Shimadzu GC-2010 Plus. The volatile compounds were eluted from the fibre by thermal desorption for 1 min in the injection port of the chromatography system (220°C). The fibre 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 as follows: 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.

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 (41°47'53.2"N, 6°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 and 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 hr (L:D) photoperiod. After a week in these conditions, the insects were used in the olfactory and movement parameter assays.

2.3 | Multiple-choice olfactory response assays

A four-arm olfactometer (Figure S1) was used to test the behavioural 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 3.1.). 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 odour source container. Each odour 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 cm³/min. Each volatile compound was applied (10 µg) separately to strips of filter paper (4 mm × 25 mm) placed in the odour 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 odours 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 hr. After starvation, one insect each time was released into the central hole of the arena, and its behaviour was recorded for 20 min 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 cm² around each air entry. Choice was codified through two behavioural 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.

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 min 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.

2.5 | Data analysis

2.5.1 | 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 *post hoc* 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).

2.5.2 | Multiple-choice olfactory response assays

For each species, the behaviour was analysed with the Noldus Observer XT 11.5 software (Noldus et al., 2001). Generalized Estimating Equations ($\alpha = 0.05$) 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 *post hoc* Tukey's tests ($\alpha = 0.05$) 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'.

2.5.3 | 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 ($\alpha = 0.05$) in PAST.

3 | RESULTS

3.1 | Volatile characterization

In total, 83 compounds were identified from the three plant species (Table S1). 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-3-hexenyl 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-3-hexenyl 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-3-hexenyl 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 (Figure S2 and Table S1). 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, $42.07 \pm 10.38 \mu\text{g/g}$, $29.87 \pm 4.83 \mu\text{g/g}$ and $1.80 \pm 0.64 \mu\text{g/g}$, for cis-3-hexen-1-ol; and, $28.29 \pm 6.22 \mu\text{g/g}$, $4.87 \pm 1.93 \mu\text{g/g}$ and $31.77 \pm 7.11 \mu\text{g/g}$, for cis-3-hexenyl acetate. Therefore, these two compounds were selected for the olfactory assay due to their frequency and quantity.

3.2 | Multiple-choice olfactory response assays

At the lowest VOCs concentration ($5 \mu\text{g}/\mu\text{l}$), the females of *P. spumarius* were significantly attracted by the two volatile compounds (Table 1 and Figure 1). 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 2), whereas males of *P. spumarius* showed 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 $10 \mu\text{g}/\mu\text{l}$ (Table 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 1). Regarding the total length of stay in each area, it was not significantly influenced by treatment or sex for the highest concentrations (20 and $30 \mu\text{g}/\mu\text{l}$) (Table 2). At these concentrations (20 and $30 \mu\text{g}/\mu\text{l}$), no significant differences were detected in the choice of *P. spumarius*.

In the case of *Cicadella viridis*, neither the frequency of visits nor the total length of stay significantly differed between treatments for any concentration (Table 1 and Table 2). However, the frequency of visits to each treatment varied significantly between sex for all the concentrations (Table 1).

3.3 | Estimation of movement parameters

Females of *P. spumarius* were able to walk a total distance of $2.42 \pm 0.20 \text{ m}$ in 10 min, with a mean velocity of $0.43 \pm 0.04 \text{ cm/s}$. Both movement parameters (total distance and mean velocity) were significantly higher compared with the males ($p < .01$) (Figure 3a1 and a2). Regarding the total time of moving, there were no significant differences between males and females ($p = .81$) (Figure 3a3). Females of *C. viridis* walked with mean velocities significantly higher ($p < .01$) than the males (Figure 3b1 and b2). In 10 min, females of *C. viridis* walked $3.13 \pm 0.33 \text{ m}$ with a mean velocity of $0.63 \pm 0.07 \text{ cm/s}$. Females also were moving significantly more time than males (Figure 3b3).

TABLE 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	<i>Philaenus spumarius</i>			<i>Cicadella viridis</i>		
			df	χ^2	p	df	χ^2	p
5 $\mu\text{g}/\mu\text{l}$	Choice	Frequency	3	40.50	<.01	3	3.98	.263
	Sex		1	5.90	.015	1	10.63	<.01
	Choice:Sex		3	28.10	<.01	3	3.68	.298
10 $\mu\text{g}/\mu\text{l}$	Choice	Frequency	3	9.32	.524	3	6.42	.093
	Sex		1	0.41	.524	1	29.86	<.01
	Choice:Sex		3	9.07	.028	3	1.10	.777
20 $\mu\text{g}/\mu\text{l}$	Choice	Frequency	3	3.33	.343	3	4.79	.188
	Sex		1	12.96	<.01	1	6.80	<.01
	Choice:Sex		3	3.98	.263	3	1.74	.628
30 $\mu\text{g}/\mu\text{l}$	Choice	Frequency	3	0.51	.920	3	3.45	.327
	Sex		1	29.98	<.01	1	10.22	<.01
	Choice:Sex		3	1.26	.740	3	11.12	.011

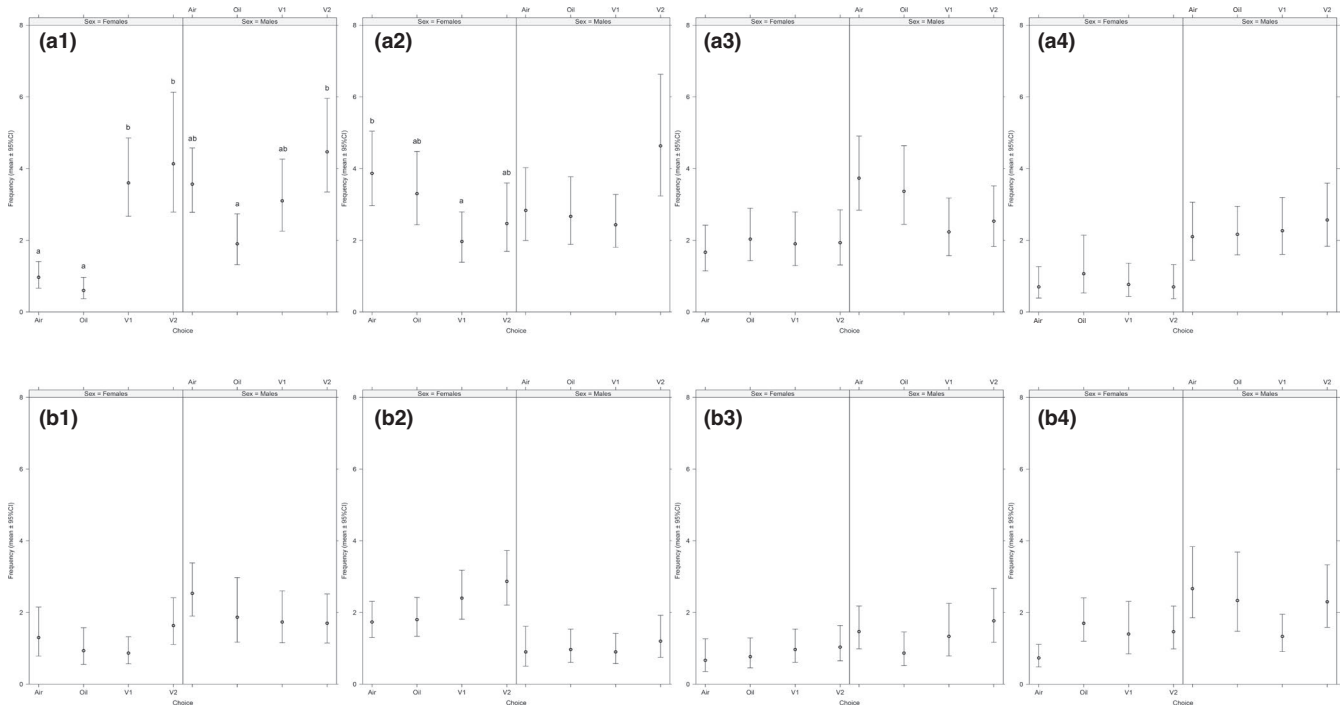


FIGURE 1 Number of visits (mean \pm 95%CI) of females and males of *Philaenus spumarius* (a) and *Cicadella viridis* (b) to cis-3-hexenyl acetate (V1) and cis-3-hexen-1-ol (V2), Air and Oil in a 4-choice olfactometer at different concentrations of volatiles (5, 10, 20 and 30 $\mu\text{g}/\mu\text{l}$)

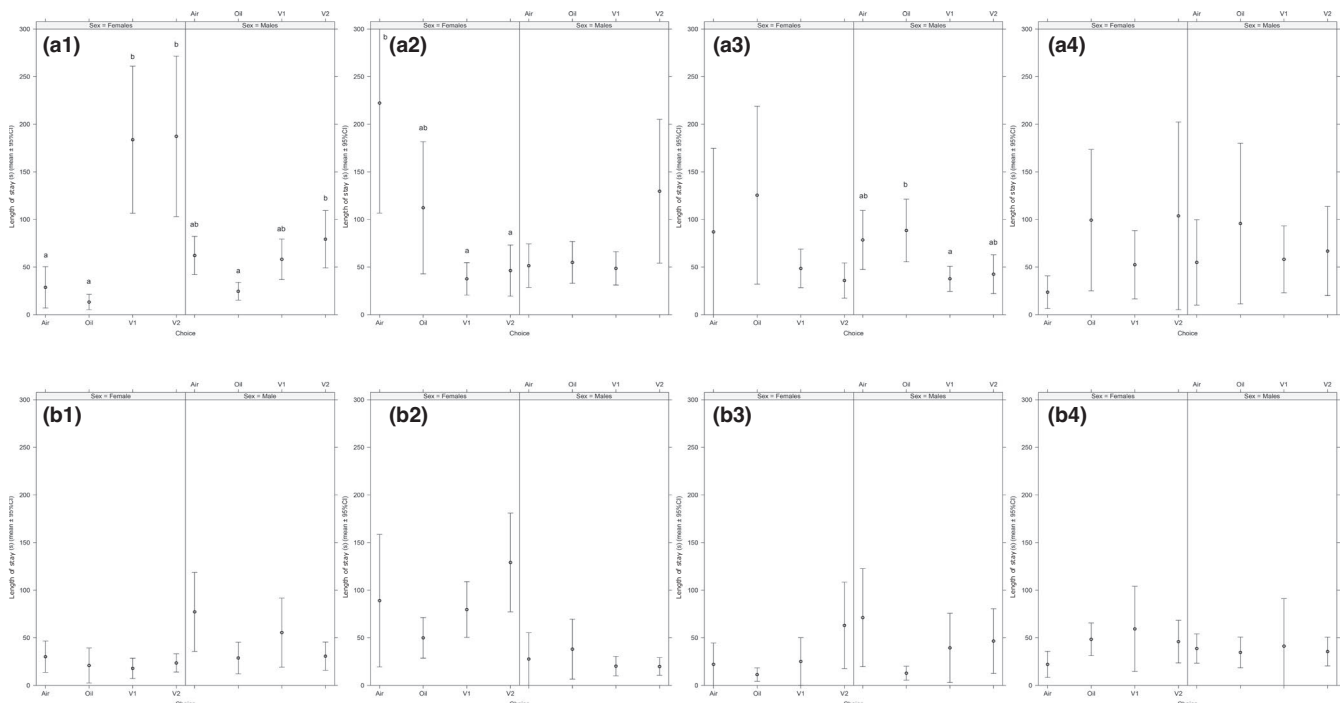


FIGURE 2 Total length of stay (mean \pm 95%CI) of females and males of *Philaenus spumarius* (a) and *Cicadella viridis* (b) to cis-3-hexenyl acetate (V1) and cis-3-hexen-1-ol (V2), Air and Oil in a 4-choice olfactometer at different concentrations of volatiles (5, 10, 20 and 30 $\mu\text{g}/\mu\text{l}$)

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 analysed. 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*.

TABLE 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	<i>Philaenus spumarius</i>			<i>Cicadella viridis</i>		
			df	χ^2	p	df	χ^2	p
5 $\mu\text{g}/\mu\text{l}$	Choice	Length of stay	3	50.30	<.01	3	5.59	.1332
	Sex		1	8.30	<.01	1	8.70	<.01
	Choice:Sex		3	20.40	<.01	3	4.41	.215
10 $\mu\text{g}/\mu\text{l}$	Choice	Length of stay	3	14.81	<.01	3	3.05	.383
	Sex		1	2.44	.1181	1	20.47	<.01
	Choice:Sex		3	15.41	<.01	3	9.18	.027
20 $\mu\text{g}/\mu\text{l}$	Choice	Length of stay	3	9.35	.025	3	15.93	<.01
	Sex		1	0.48	.486	1	1.06	.304
	Choice:Sex		3	1.34	.72	3	3.42	.332
30 $\mu\text{g}/\mu\text{l}$	Choice	Length of stay	3	5.03	.170	3	3.00	.390
	Sex		1	0.00	.970	1	0.41	.520
	Choice:Sex		3	1.54	.670	3	4.71	.190

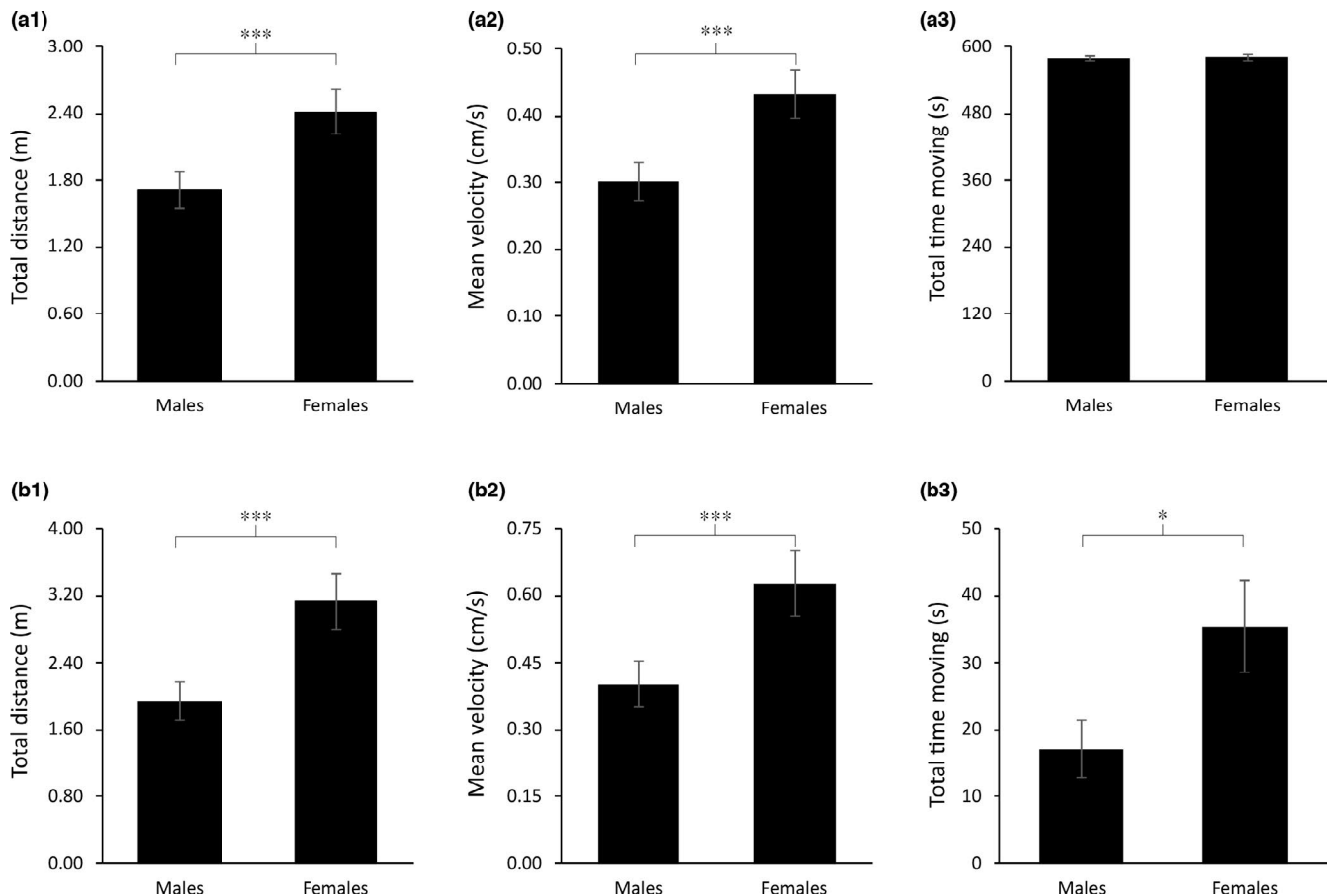


FIGURE 3 Movement parameters (walk) obtained for males and females of (a) *Philaenus spumarius* and (b) *Cicadella viridis*. (1) Distance walked (mean \pm SE) in 10 min; (2) Velocity (mean \pm SE); (3) Total time walking (mean \pm SE). Significant differences are showed by asterisks (***) $p < .001$; (*) $p = .02$

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., 2017).

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 (Ameje 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 $\mu\text{g}/\mu\text{l}$ 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) behavioural 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 behaviour 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., 2021; 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* (Burrows, 2007; Cornara et al., 2018) 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 behaviour. 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 travelling 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 travelled and flight duration.

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

ACKNOWLEDGEMENTS

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020) unit and to the Associate Laboratory SusTEC (LA/P/0007/2020); and to the research Project XF-ACTORS 'Xylella fastidiosa Active Containment Through a multidisciplinary-Oriented Research Strategy' (Grant Agreement 727987). Isabel Rodrigues acknowledges the Ph.D. grant (2020.07051.BD) provided by FCT. Nuno Rodrigues thanks to National funding by FCT – Foundation for Science and Technology, P.I., through the institutional scientific employment programme – contract.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

Conceptualization: JAP, JBM and NR; Methodology: IR, JBM, NR and JAP; Statistical analysis: JBM and IR; Writing – Original draft preparation, review & editing: all authors contributed equally. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Data supporting the information shown in the results have been uploaded to Zenodo (<https://zenodo.org/>). Rodrigues et al. (2021): Olfactory responses sampling data set [Data set]. Zenodo. DOI: <https://doi.org/10.5281/zenodo.5761817>

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How to cite this article: Rodrigues, I., Benhadi-Marín, J., Rodrigues, N., Baptista, P., & Pereira, J. A. (2022). Olfactory responses to volatile organic compounds and movement parameters of *Philaenus spumarius* and *Cicadella viridis*. *Journal of Applied Entomology*, 00, 1–12. <https://doi.org/10.1111/jen.12992>