



# Vibrational disruption of feeding behaviors of a vector of plant pathogen

Sabina Avosani<sup>1,2</sup>, Alice Berardo<sup>2,3,7</sup>, Nicola M. Pugno<sup>3,4</sup>, Vincenzo Verrastro<sup>5</sup>, Valerio Mazzoni<sup>2</sup>, and Daniele Cornara<sup>5,6,\*</sup>

<sup>1</sup> Department of Civil, Environmental and Mechanical Engineering, University of Trento, Trento, Italy

<sup>2</sup> Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige (TN), Italy

<sup>3</sup> Laboratory of Bio-inspired, Bionic, Nano, Meta Materials & Mechanics, Department of Civil, Environmental and Mechanical Engineering University of Trento, Via Mesiano 77, 38123, Trento, Italy

<sup>4</sup> School of Engineering and Materials Science, Queen Mary University of London, Mile End Road, London E1 4NS, U.K.

<sup>5</sup> International Centre for Advanced Mediterranean Agronomic Studies – Institute of Bari (CIHEAM-Bari). Via Ceglie 9, 70010 Valenzano (BA), Italy

<sup>6</sup> Department of Environmental Science, Policy and Management, University of California, Berkeley (CA), USA

<sup>7</sup> Current address: Department of Civil, Environmental and Architectural Engineering, University of Padua, Via F. Marzolo 9, 35121 Padua, Italy

\* Corresponding author: danielecornara@berkeley.edu

With 6 figures and 2 tables

**Abstract:** Interference with the behaviors associated to host plant recognition, and inter- and intra-specific communication of insect vectors of plant pathogens, could represent a sustainable strategy for reducing or disrupting pathogen transmission. Here, we show that the transmission over a suitable host plant (sunflower) of a vibrational stimulus significantly affects the probing and feeding behavior of the spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae), the main European vector of the fastidious bacterium *Xylella fastidiosa*. Specifically, ca. 30% of the individuals did not even attempt to probe the sunflower plants to which the stimulus was transmitted, while the remaining showed a sex-independent reduction in ingestion of the xylem sap, i.e., *P. spumarius*' main food source, of ca. 67% compared to the control. Even so, the stimulus did not affect the feeding behavior when transmitted to olive plants. The possible reflection of a signal-based vector behavior disturbance on the epidemiology of *X. fastidiosa*, together with future research needs are discussed.

**Keywords:** *Philaenus spumarius*; Aphrophoridae; biotremology; electrical penetration graph (EPG); behavioral disruption

## 1 Introduction

Invasive plant pathogens and pests affect the multifunctionality of agroecosystems in treacherous and often unpredictable ways (Ali et al. 2021; Simberloff et al. 2013). Considering, for example, the case of the vector-borne bacterium *Xylella fastidiosa* ST53 outbreak in olive orchards in Salento (Apulia region, Southern Italy), several authors focused on the significant decrease in table olives and olive oil production as the main consequence of pathogen introduction and spread (Almeida 2016b; Saponari et al. 2019). However, losses in food provisioning are just the tip of an iceberg, with death and removal of infected olive plants recently predicted to potentially prime a cascade of events resulting in the destruc-

tion of the local environment (Ali et al. 2021). Moreover, the current measures aimed at containing *X. fastidiosa* outbreaks by controlling the vector may have major, and overlooked, side effects. In this regard, soil tilling against juveniles and treatments with synthetic pesticides targeting adults have been proposed to control the populations of the meadow spittlebug *Philaenus spumarius* L. (Hemiptera: Aphrophoridae), i.e. the main driver of *X. fastidiosa* secondary spread within Apulian olive orchards (Cornara et al. 2017b, 2019; EFSA et al. 2019). Extensive soil tilling, particularly in dry environments, such as Mediterranean olive orchards, may affect soil quality negatively, augment the risk of desertification, and reduce habitats sheltering beneficial arthropods, i.e., predators, parasitoids, and pollinators (Bodino et al. 2020;

Kairis et al. 2013; Karamaouna et al. 2019; Mesmin et al. 2020; Molinatto et al. 2020). Pesticides, on the other side, are not often compatible with integrated pest management techniques and have been listed among the main drivers of terrestrial biodiversity decline (Desneux et al. 2007; Brühl & Zaller 2019; Chávez-Dulanto et al. 2021; Sánchez-Bayo & Wyckhuys 2019). Such decline of biodiversity might ease the spread of a pathogen as *X. fastidiosa*, which is vectored by generalist insects like *P. spumarius*, as generalists are predicted to occupy the niches left by species affected by the decline (Civitello et al. 2015; Sánchez-Bayo & Wyckhuys 2019). Therefore, tools aimed at tackling an essential factor in pathogen epidemiology as vector abundance, might provoke devastating side effects in the long-term. In addition, even considering just the short-term efficacy of a pesticide-based vector control strategy, chemical control may offer a highly variable reduction in disease risk (Daugherty et al. 2015; Madden et al. 2000). Indeed, even if reducing vector load is hypothesized to decrease the transmission probability (Purcell 1980), pesticides could not prevent feeding behaviors conducive to transmission. It is therefore mandatory to rethink about *X. fastidiosa* control strategies, developing and applying long-term sustainable tools against vectors and safeguarding the ecosystem services. Manipulation of vector behaviors by confounding the cues used by the insect for host-plant recognition, or reducing host plant suitability and residency time, or interfering with insects' communication during crucial steps of their life cycle, could represent an efficient and environmentally-safe strategy for the containment of vector-borne plant pathogens (Fereses & Moreno 2009; Mazzoni et al. 2009; Mokrane et al. 2020).

Biotremology is the science that studies the use of substrate-borne vibrations in animal communication. Many insects groups, including spittlebugs, use vibrational signals for close-range interactions, especially as social and sexual communication, and predator-prey interactions (Avosani et al. 2020; Hill & Wessel 2016; Takanashi et al. 2019; Virant-Doberlet et al. 2019). Characterization and subsequent playback of species-specific vibrational signals on a host plant can therefore be used to disrupt relevant insect behaviors, resulting in the reduction of pest populations (Gordon & Krugner 2019; Mazzoni et al. 2019; Polajnar et al. 2015). For instance, species-specific vibrations transmitted to grapevine plants disrupt the mating behavior of the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Mazzoni et al. 2009, 2019). Aggregation and mating signals have been exploited to develop trapping strategies for invasive pest species such as the brown marmorated stinkbug *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) and the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), respectively (Mankin 2019; Polajnar et al. 2019). However, *P. spumarius* is a highly polyphagous and abundant species, in which mating occurs throughout the season on different host plants, while oviposition happens during the fall on herbaceous

plants after the breakage of the ovarian diapause (Morente et al. 2018; Witsack 1973). Therefore, applying vibrations to disrupt mating as a way to control spittlebug populations appears pointless.

Nevertheless, beside mating, vibrations can be used to affect pest behaviors, such as probing and feeding (Takanashi et al. 2019). Since insect mechano-receptors are usually tuned for specific signal properties (Lakes-Harlan & Strauß 2014; Virant-Doberlet & Cokl 2004), species-specific vibrational signals are more effective in interfering with behaviors than unspecific or broadband noises (Bomford & O'Brian 1990). In particular, the alarm or distress signals emitted by animals can be used as repellent stimuli and are more resistant to habituation, which commonly arise when the stimulus is an unspecific and monotonous noise (Bomford & O'Brian 1990). *Philaenus spumarius* could accordingly be sensitive to signals aimed at repelling conspecifics or expressing stress. In this regard, *P. spumarius* emits distress signals in presence of other individuals, especially after a physical or vibrational interaction, as similarly reported for other insect species (Alexander 1957; Avosani et al. 2020). The female rejection signal is used by *P. spumarius* females to reject courting or approaching males, and likely contains temporal and/or spectral features that can affect crucial behaviors (Avosani et al. 2020). It is therefore possible that the continuous transmission of the rejection signal could disturb the spittlebugs resident on the treated plant and interfere with their activities. (i.e., impair feeding thus possibly decrease the probability of *X. fastidiosa* acquisition (Daugherty & Almeida 2009)).

In this work, we aimed at assessing whether and to which extent a vibrational stimulus based on the intra-specific signal "female-rejection" and transmitted on a suitable host plant, i.e., sunflower (*Helianthus annuus*), could interfere with spittlebug probing and feeding behavior. Here, we present the promising results gathered by coupling real-time probing and feeding behavior observations with recordings of signal transmission and propagation on the tested plants, and discuss the applicability of a vibrational control of spittlebug populations strategy to olive plants.

## 2 Materials and methods

### 2.1 Insects and plants

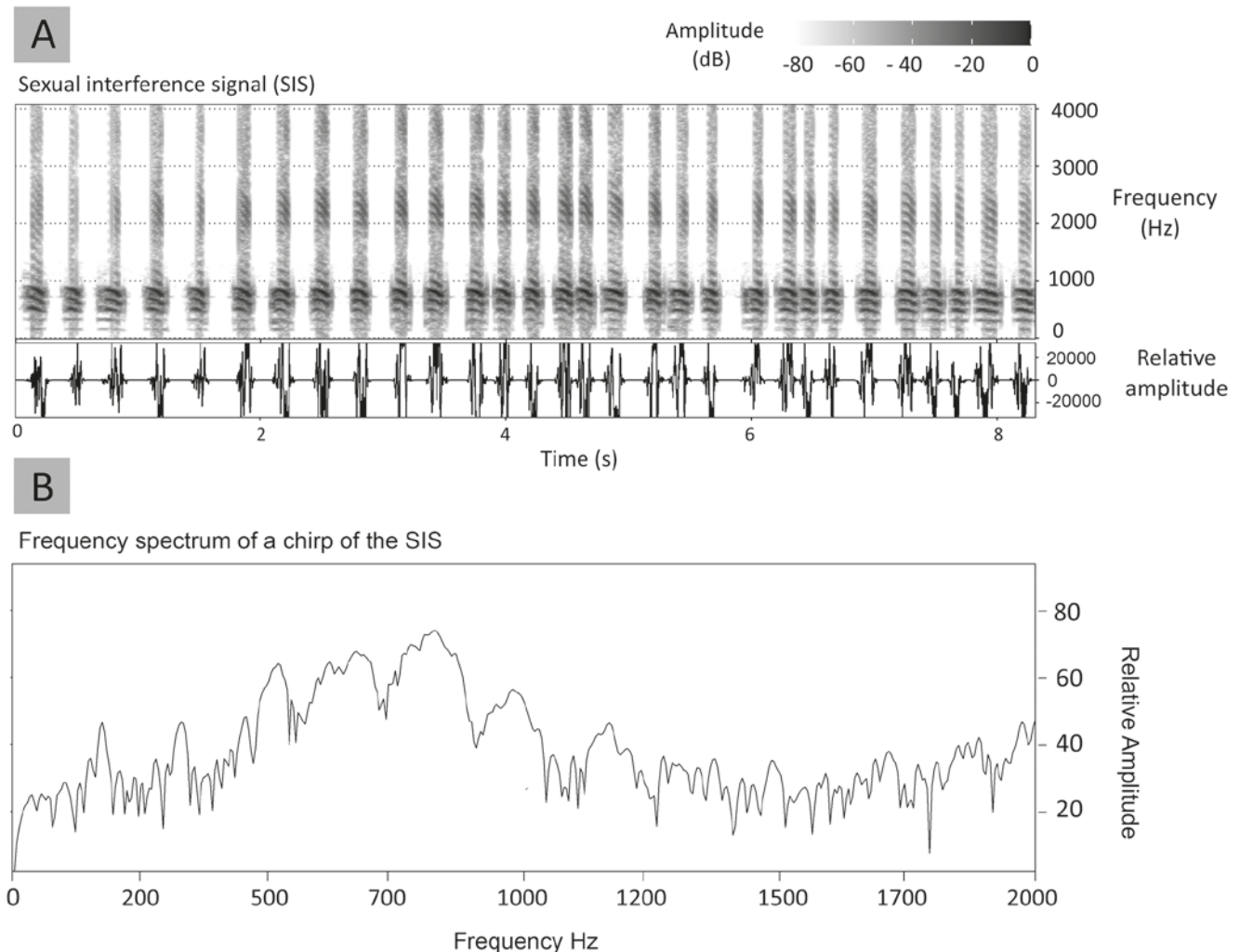
Adults of *P. spumarius* were collected on oak trees (*Quercus ilex*) in Valenzano (Apulia, Southern Italy, *X. fastidiosa*-free area) during June 2020. Collected individuals were caged in insect rearing tents (BugDorm-2120 Insect Rearing Tent, 60×60×60 cm) covered with a nylon net to protect the insects from excessive sunlight and storms and kept on a meadow in the premises of CIHEAM-Bari institute (Apulia, Southern Italy). Plants used for the rearing were sunflower (*Helianthus annuus*) and vetch (*Vicia sativa*), replaced fortnightly, and two-year old *Vitis vinifera* var. Cabernet Sauvignon cuttings;

plants were watered twice per week. For the experiments, we used four week-old sunflower plants (approximately 30 cm tall), grown inside pots ( $5 \times 10 \times 5 \text{ cm}^3$ ) filled with soil and vermiculite (6: 1), and watered twice per week. Olive (*Olea europaea*) plants, var. Ogliarola salentina, were two-year old seedlings pruned in May 2020 and water-fertilized once per week (PLANTAFOL 30 10 10, VALAGRO), in order to obtain fresh shoots of approximately 30 cm by September. All the plants were reared inside a glasshouse under controlled conditions ( $26 \pm 2^\circ\text{C}$ , 60% relative humidity RH).

## 2.2 Vibrational stimuli

A signal designed based on a species-specific (a synthetic interference signal, SIS, Fig. 1) vibrational stimulus was used to investigate if the probing and feeding behavior of *P. spumarius* could be affected by means of vibrations trans-

mitted to a plant. The SIS consisted in a complex signal (Fig. 1) assembled using the audio software Adobe Audition 3.0 (Adobe Systems, Inc., San Jose, CA, USA). The SIS was composed of harmonic elements (the chirps) derived from a female rejection signal (Avosani et al. 2020), which was recorded with a laser vibrometer (VQ-500-D-V, Ometron Ltd., Harpenden, UK) from a reflective sticker glued on a sunflower plant in close proximity to the insect (1 cm). The chirps were amplified (+10 dB boost) in order to increase the amplitude of the frequency bands above 200 Hz (the resonance frequency of the mini shaker). Temporal and spectral features were modified to obtain a stimulus that could both cover the *P. spumarius* signals and be distressing. In this regard, within other insect species (i.e., stinkbugs), distress signals are emitted in response to disturbances and are characterized by high dominant frequencies and fast repetition



**Fig. 1. A)** Spectrogram (above) and oscillogram of the synthetic interference signal (SIS). The signal was designed based on a female rejection signal modified in its temporal and spectral features in order to obtain a signal of 8.3 s of duration and composed by 30 chirps. **B)** Frequency spectrum of a chirp of the SIS (mean duration of 0.18 s and a dominant frequency of 820 Hz).

rates (Lazzari et al. 2006). To create the SIS, the repetition time between chirps was accordingly reduced ( $0.28 \pm 0.06$  s) compared to the original female signal (Avosani et al. 2020), while the chirp dominant frequency was set to 820 Hz. The signal was propagated by using the below-mentioned setup (exciter-amplifier-laptop) to plants of sunflower and olive. The signal was transmitted at two different amplitudes (SIS15 and SIS50, respectively), in that the volume was set from the laptop (default Windows music player), being the volume of the SIS50 three times higher than the SIS15's one. The recorded amplitudes (in  $\mu\text{m/s}$ ) of the two stimuli are reported in results and SM.

Observations of signal propagation and characteristics on both the host plant species were conducted in the biotremology laboratory at Fondazione Edmund Mach (Trentino, Northern Italy), inside a sound insulated chamber maintained at a temperature of  $22 \pm 1$  °C and 65% RH, with two plants (same species) placed on an anti-vibrational table (Astel s.a.s., Ivrea, Italy). Two laser vibrometers were pointed toward the plants on the table and vibrations were simultaneously recorded (VQ-500-D-V, Ometron Ltd., Harpenden, UK and OM-DS VibroGo E 52039, Polytec GmbH, Waldbronn, Germany) by adjusting the laser sensitivity to 5 mm/s/V. The laser was pointed toward small pieces of reflective tape ( $0.5 \times 0.5$  cm<sup>2</sup>) glued to three different points of the plants (two apical leaves and stem). Signals were acquired with a hard drive multichannel LAN-XI data acquisition device (Brüel and Kjær Sound and Vibration A/S) with a sample rate of 8192 Hz. Recordings were then analyzed using Matlab 2020 (1994–2021 The MathWorks, Inc.) to compute the fast Fourier transform (FFT) with window length of 1024 samples, frequency resolution of 8 Hz, 66.7% overlap, and Hann window. The spectra of the recorded signals were then extracted, visualized and compared. Detailed description of signal recordings and analysis are provided in Supplementary Materials (SM1).

### 2.3 Vibration stimuli effect on *Philaenus spumarius* probing and feeding behavior

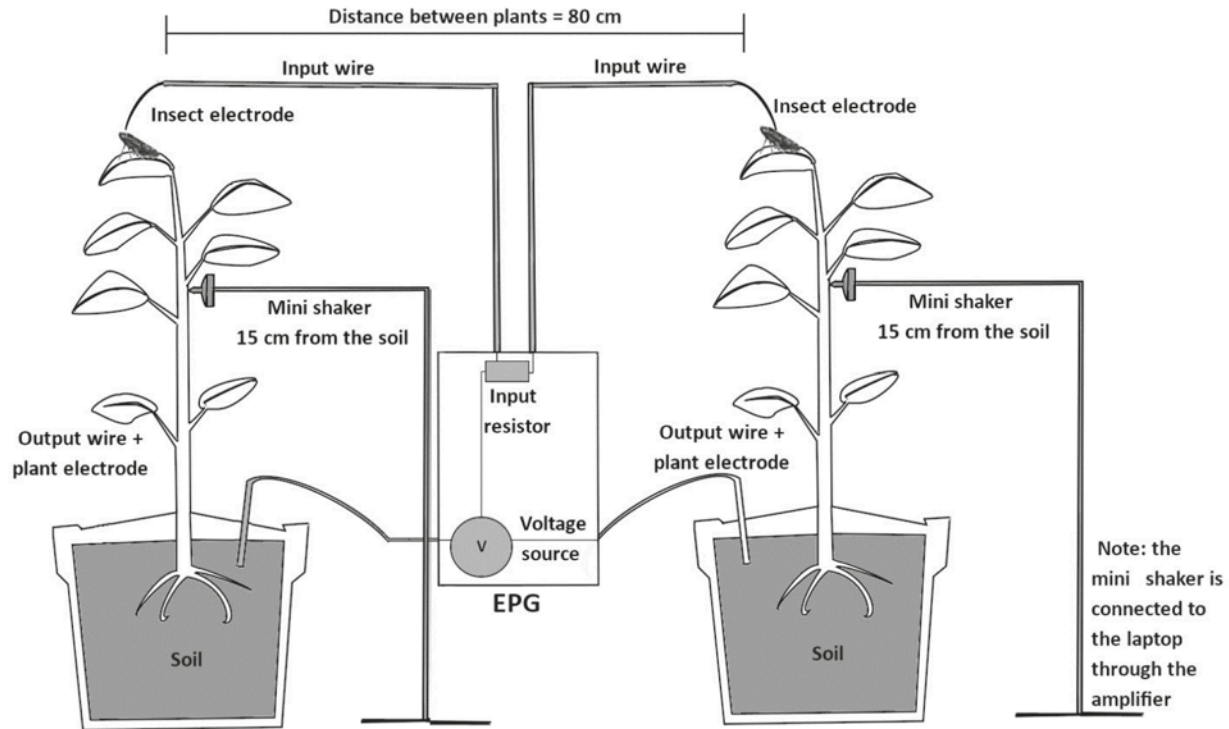
The experiments were conducted in the Electrical Penetration Graph (EPG)-lab at CIHEAM-Bari (Apulia, Southern Italy) at temperature  $25 \pm 1$  °C and RH 65% during August and September 2020. First, we performed EPG-assisted 3h observations of the probing and feeding behavior of *P. spumarius* males and females on sunflower plants treated with SIS at two different volumes (thus two treatments, namely SIS15 and SIS50) and on control. The vibrational stimuli were transmitted by means of an exciter (Visaton BS 76; Visaton GmbH & Co, Germany; also referred to as “mini shaker”) in direct contact with the stem of a sunflower plant by means of a conical rod (5 cm long). The conical rod was perpendicularly pointed on the plant stem halfway between the apical and basal portion, hence ca. 15 cm from the soil. The exciter, kept in position using a clamp, was plugged to an amplifier (Nobsound NS-01G, Nobsound, Shenzhen Cavins Tech

Ltd, China) controlled by a laptop (HPEnvy 15). The plant and the clamp with the exciter were placed inside a Faraday cage hosting the EPG (discussed below). The signals were turned-on 20 min before the insect was placed on the plant and loop-played for the 3h EPG recordings by using the software Windows Music Reader. For the control, we used the same set-up as for treated plants, with the conical rod in contact with the plant, but turning the amplifier off. The experimental design was completely randomized: a single treatment/signal was carried out per time (during each 3h recording a single treatment was performed, with two replicates/plants per time, with the plants at ca. 80 cm from each other) in order to avoid interference among the different signals (Fig. 2). The position (channel of the EPG device used) of each treatment/signal was switched during each recording to avoid position effects. We performed each 3h EPG observation with a single combination insect/plant. Total number of recordings carried out per treatment and sex on sunflower are reported in Table 1.

Second, we conducted a test, namely “Start & Stop”, recording spittlebug males feeding behavior on sunflower plants treated with SIS50 (the stimulus displaying the greatest effect on probing and feeding behavior, discussed in results section). A spittlebug male connected to the EPG-amplifier was permitted to initiate a probe on the sunflower plant connected to a mini shaker (discussed above); the signal (SIS50) was then activated and loop-played for ten minutes once the insect reached a xylem vessel and started xylem ingestion. We therefore analyzed insect probing and feeding behavior during the ten minutes with signal on, and during the successive ten minutes with signal off. A single combination insect/plant was used for each recording. Twenty-six replicates (26 males) were carried out.

Finally, according to the results on sunflower (reported in results section), we tested the effect of SIS50 on *P. spumarius* male feeding behavior on olive plants, following the same design described above for sunflower. A total of 40 replicates, i.e., 20 males for SIS50 and 20 for the control, was carried out.

Spittlebugs were tethered and connected to the EPG amplifiers following the protocol described by Cornara et al. (2018). The insects were offered on either sunflower or olive a 5-cm apical portion of the plant, with access to stem, petiole and leaf. Probing and feeding behavior were recorded for three hours with a Giga 8-DC EPG (EPG-systems, Wageningen, The Netherlands) at 1 Giga Ohm input resistance, assembled inside a Faraday cage, under controlled conditions ( $24 \pm 1$  °C, 40% RH). Output from the EPG at 100x gain was digitalized at a sample rate of 100 Hz per channel and recorded using Stylet+ software (EPG-systems, Wageningen, The Netherlands). For the analysis of the probing and feeding behavior, we followed the waveforms definitions by Cornara et al. (2018) with slight modifications (Markheiser et al., in preparation). Briefly, we considered ten patterns (waveforms representing the different steps/



**Fig. 2.** Experimental setup of the Electrical Penetration Graph (EPG) tests. A specimen of *Philaenus spumarius* connected to a channel of the EPG amplifier by a thin electrode was offered the 5cm apical portion of the host plant, with access to stem, petioles and leaves. The vibrational stimuli were transmitted via a mini shaker, which was in direct contact with the stem of the plant. A single treatment/signal was carried out per time (3h) with two replicates/plants per time (two EPG channel per time, plants at a distance of ca. 80 cm).

**Table 1.** *Philaenus spumarius* tested by EPG (Electrical Penetration Graph) on sunflower.

<b>Female</b>				
Treatment	Tested	Probing individuals <sup>§</sup>	Non-probing individuals <sup>§§</sup>	Percentage non-probing
Control <sup>a</sup>	17	17	0	0
SIS15 <sup>a</sup>	15	14	1	6.66
SIS50 <sup>b</sup>	16	11	5	31.25
<b>Male</b>				
Treatment	Tested	Probing individuals <sup>§</sup>	Non-probing individuals <sup>§§</sup>	Percentage non-probing
Control <sup>a</sup>	18	17	1	5.55
SIS15 <sup>a</sup>	19	19	0	0
SIS50 <sup>b</sup>	14	10	4	28.57

<sup>§</sup> Analysis of probing and feeding behavior was carried out only on probing individuals, excluding insects that did not probe during the 3h EPG

<sup>§§</sup> Insects that performed activities other than probing, as walking, resting, or dubbing plant tissues without inserting the stylets (behavior observed with a  $\times 10$  magnifying lens) into the plant tissues, and were alive and active at the end of the recording.

Different superscript letters indicate significant difference between groups (G-test in a contingency table ( $2 \times 3$ ) followed by a Ryan multiple comparisons for proportions).

behaviors performed by the insect from the insertion of stylets into the plant to their withdrawal): i) np, non-probing; ii) C, pathway; iii) Xc, xylem contact; iv) Xi, xylem ingestion (frequency>0.1Hz); v) LF, low frequency xylem ingestion (frequency<0.1Hz); vi) npN, non-pathway interruption of xylem activity (could be also marked as N); vii) pN, pathway interruption of xylem activity (it is pathway C occurring after the spittlebug initiated xylem-related activities; could be also marked as C and aggregated to pathway C); viii) R, resting; ix) Xe, behavior putatively associated to *X. fastidiosa* inoculation (Cornara et al. 2020); (x) W, stylets withdrawal. Biological meaning of *P. spumarius* EPG waveforms are also reported in Supplementary Materials (SM2 and 3).

Overall, we assessed the differences in probing and feeding behavior among treatments by considering the variations in: i) non-sequential variables (WDI, waveform duration per individual; NWEI, number of waveform events per individual; WDEI, median duration of each waveform event per individual; pWDI, percentage of the total probing time spent in a certain waveform); ii) sequential variables; iii) number of probes (with or without xylem ingestion) performed by the spittlebugs. For a more detailed explanation of sequential and non-sequential variables considered in the present study, and of the waveforms definition and supposed/ascertained biological meaning, please refer to Supplementary Materials (SM2 and 3). Descriptive statistics of the variables recorded in this study (per treatment and sex) are reported in Supplementary Materials (SM4).

We additionally took note of the number of spittlebugs per treatment spending the 3h EPG performing activities other than probing, as walking, resting, or dubbing plant tissues without inserting the stylets (behavior observed with a  $\times 10$  magnifying lens), and alive at the end of the recording; these insects are referred to as “non-probing individuals” in Table 1.

## 2.4 Data analysis

We performed a G-test in a contingency table ( $2 \times 3$ ) followed by a Ryan multiple comparisons for proportions (Ryan 1960) to compare the number of probing individuals (either males or females) between treatments and control. We explored the effect of vibrational signals on spittlebugs probing and feeding behavior (i.e., WDI, NWEI, WDEI, pWDI, probes, and sequential variables) with a linear mixed-effects model (lme; “REML” method). The explanatory variables were treatments (SIS15, SIS50, and Control), sex, and the interaction between these two factors. These variables showed no collinearity. Differences among treatments and control were assessed by Tukey’s test (Tukey’s “honest significant differences” (HSD) method) for pairwise comparison. The data obtained from the EPG were transformed when necessary with  $\ln(x + 1)$  or  $\sqrt{x + 1}$  to reduce heteroscedasticity and improve normal distribution. We accounted for the nested design of the study by including the mini shaker used as random factor. We additionally explored xylem ingestion

duration (Xi WDI) trend during the 3h recording using Generalized Linear Mixed-Effects Models (glmer) with treatment, recording time (hour), their reciprocal interaction, and sex as explanatory variables, and mini shaker and insect identity as random factors (Poisson distribution).

All the analyses were performed in R (R Core Team, 2020). We ran the models using “nlme” and “lme4” packages (Bates et al. 2014; Pinheiro et al. 2020). We checked the models for residual distribution using the “car” package (Fox & Weisberg 2019). There was no evidence of either spatial or temporal autocorrelation of model residuals (analyses performed using the “ncf” and “acf” packages, respectively (Bjornstad 2013)). Graphs were generated using “ggplot2” package (Wickham 2016). Only insects that performed at least a probe, thus insects that inserted stylets into the plant tissues, were considered for statistical analysis.

## 3 Results

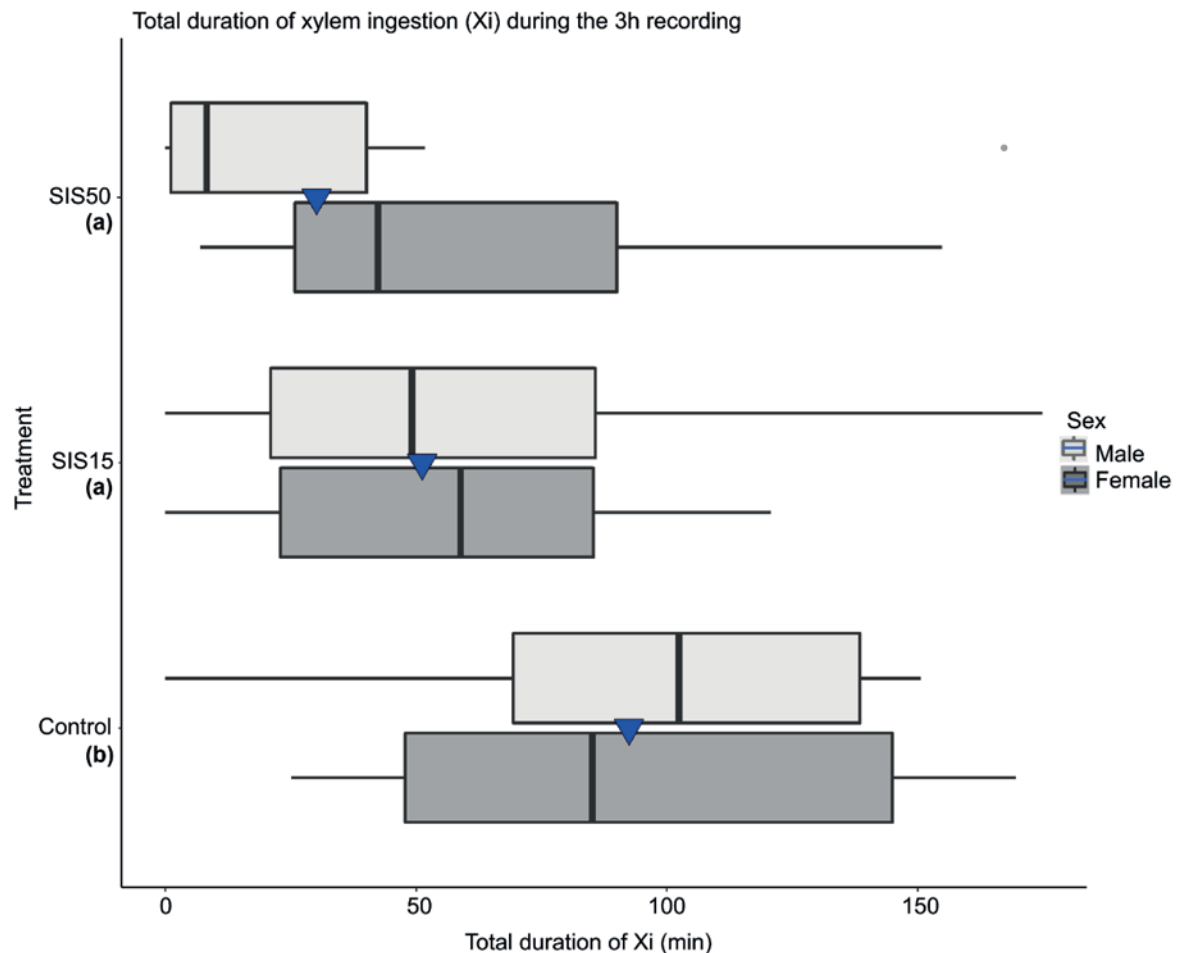
### 3.1 Vibrational stimuli

The greatest number of non-probing individuals during the 3h recordings was observed in the group treated with the SIS50, in that 31.25% of the females and 28.57% of the males performed activities other than probing, such as walking, or resting (Table 1). All these spittlebugs were alive at the end of the EPG. The number of either probing males or probing females was statistically different between groups (males:  $G=7.93$ ,  $df=2$ ,  $p\text{-value}=0.019$ ; females:  $G=8.59$ ,  $df=2$ ,  $p\text{-value}=0.014$ ). In particular, the post-hoc test showed that the treatment with the SIS50 significantly reduced the number of probing individuals compared to either the SIS15 or the control (Table 1).

No significant differences were observed between treatments and control in the total time spent with stylets inserted into the plant tissues (probing time); however, probing in males on plants treated with SIS50 (91.15 min) was considerably shorter than in control males (158.43 min) in terms of median values (See Supplementary Material SM4).

Considering the probes, SIS50 significantly influenced the number of male probes comprising a sustained xylem ingestion (duration of the Xi event longer than 5 min) ( $t=-2.259$ ,  $p=0.026$ ), which resulted reduced compared to control males ( $t=3.241$ ,  $p=0.021$ ).

Regardless of sex, SIS15 and SIS50 had a significant impact on the total duration of xylem ingestion (Xi WDI) (SIS15:  $t=-2.932$ ,  $p=0.004$ ; SIS50:  $t=-3.513$ ,  $p<0.001$ ). Specifically, spittlebugs spent significantly less time in ingesting xylem sap (i.e., their main food source) on plants treated with either SIS15 ( $t=2.932$ ,  $p=0.012$ ) or SIS50 ( $t=3.513$ ,  $p=0.002$ ) compared to control, with no differences between the signals (Fig. 3). However, in terms of median values, the shortest xylem ingestion was observed in the group treated with the SIS50 (Control=92.365 min; SIS15=51.110 min; SIS50=30.080 min), with a reduction



**Fig. 3.** Boxplots representing the total duration of xylem ingestion (Xi WDI) during the 3h recordings on sunflower plants treated with SIS15, SIS50 and control. Time on x-axis is expressed in minutes. The blue triangles indicate the median value of Xi WDI for males and females (pooled data) for each treatment. Letters in bold within brackets indicate statistically significant differences among treatments according to the results from Tukey's test.

of Xi of 67.43%. Similarly, we observed a sex-independent effect of SIS15 ( $t=-4.687$ ,  $p<0.001$ ) and SIS50 ( $t=-3.686$ ,  $p<0.001$ ) on the duration of non-pathway interruptions (npN WDI), which resulted significantly shorter in the treated groups than in the control (SIS15:  $t=4.687$ ,  $p<0.001$ ; SIS50:  $t=3.687$ ,  $p=0.001$ ).

Both SIS15 and SIS50 had a significant effect on the trend of xylem ingestion duration (Xi WDI) during the 3h recording. Specifically, xylem ingestion was significantly shorter on plants treated with the SIS50 starting from the first hour, whilst in the SIS15, xylem ingestion progressively decreased starting from the second and third hour (Table 2; Fig. 4).

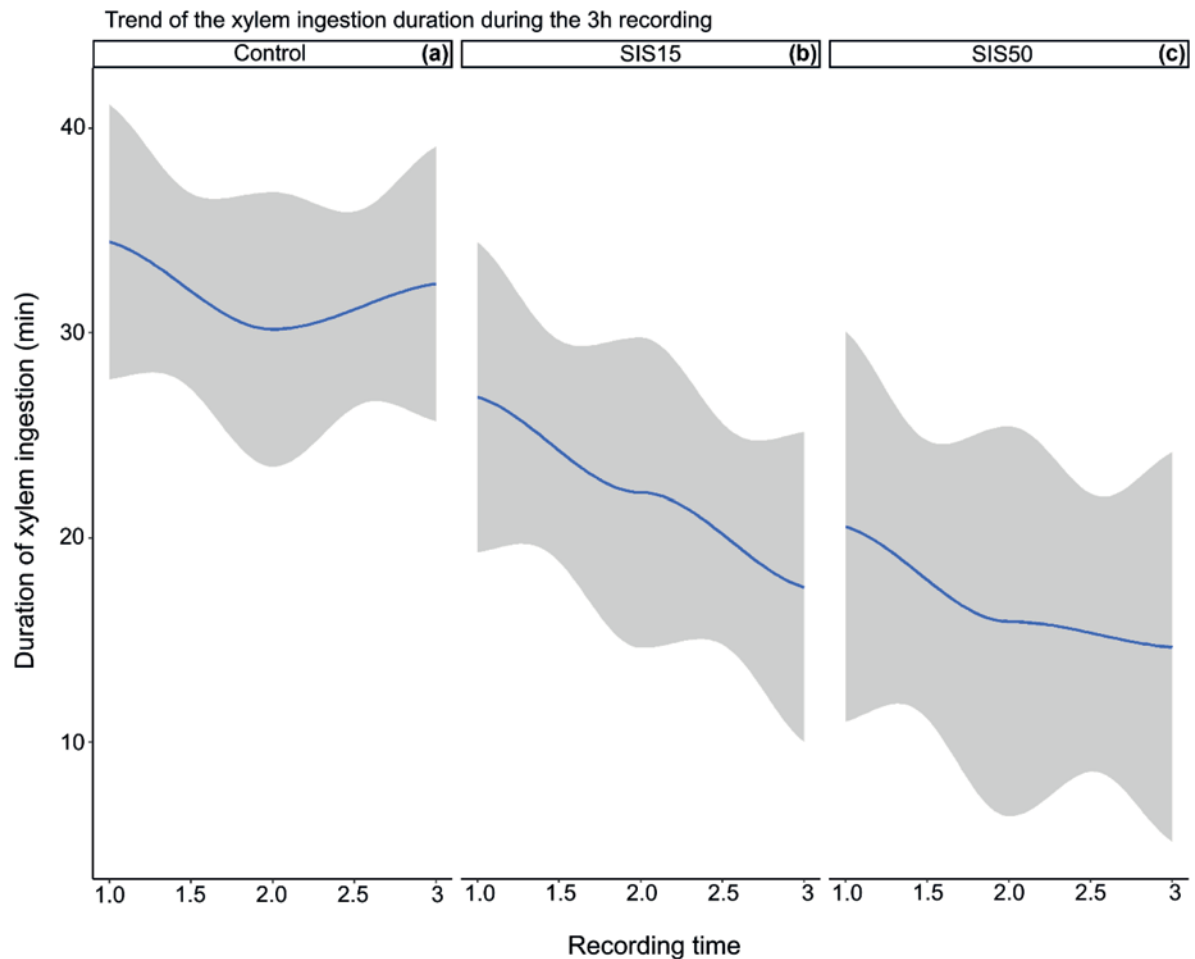
Xylem ingestion and non-pathway interruption durations were reduced on plants treated with either the SIS15 (Xi:  $t=-3.021$ ,  $p=0.003$ ; npN:  $t=-4.752$ ,  $p<0.001$ ) or the SIS50 (Xi:  $t=-3.677$ ,  $p<0.001$ ; npN:  $t=-3.466$ ,  $p<0.001$ ), also when these variables were expressed as percentages of the

total probing time during the 3h EPG recording. The two events were shorter in both groups treated with the SIS15 (Xi:  $t=3.021$ ,  $p=0.009$ ; npN:  $t=-4.75$ ,  $p<0.001$ ) and the SIS50 (Xi:  $t=3.678$ ,  $p=0.001$ ; npN:  $t=3.467$ ,  $p=0.002$ ) than in the control. Considering xylem ingestion, the lowest median value was observed in SIS50, with a reduction of ca. 37% compared to spittlebugs on control plants (Fig. 5).

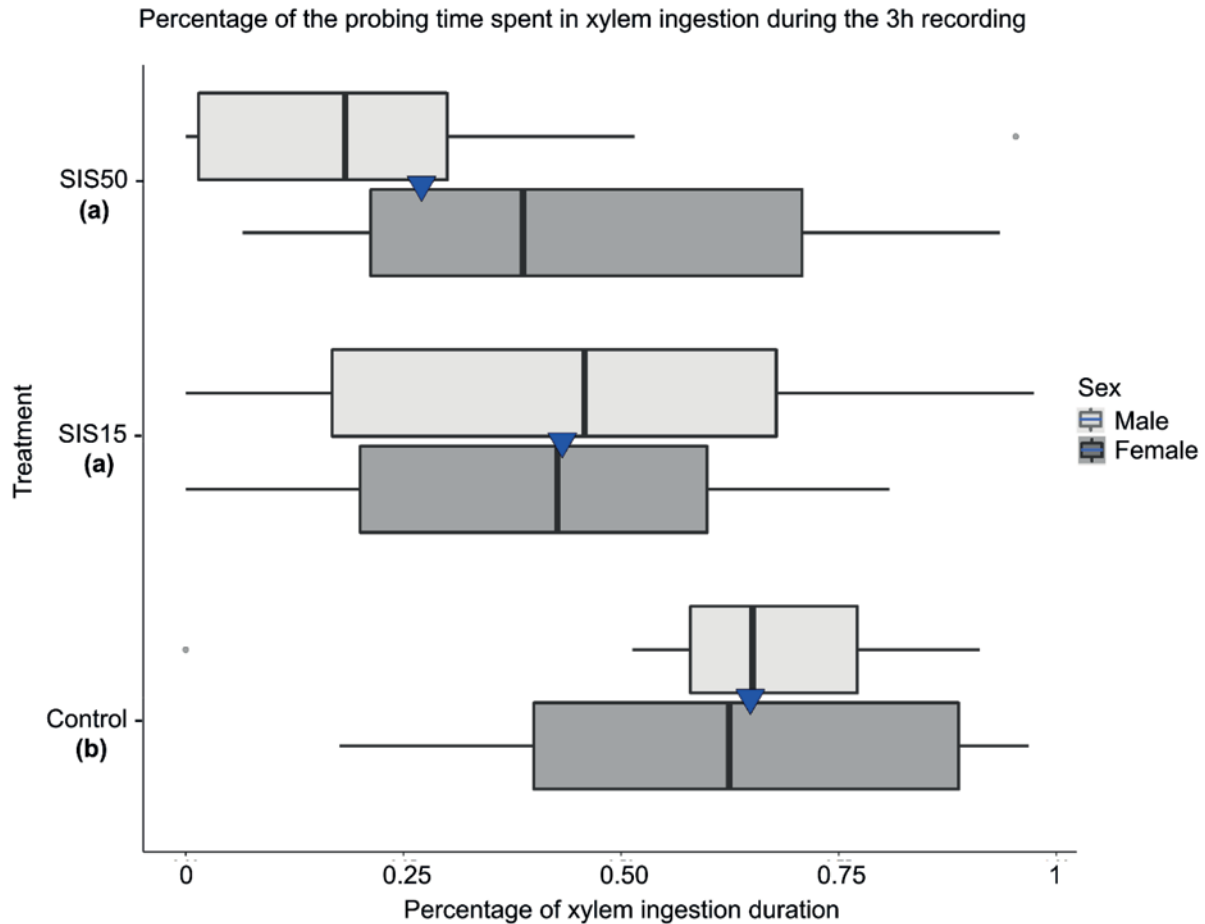
Additionally, the signals impacted the number of xylem ingestions (Xi NWEI; Fig. 6) (SIS15:  $t=-2.886$ ,  $p=0.005$ ; SIS50:  $t=-3.491$ ,  $p<0.001$ ) and of non-pathway interruption events (npN NWEI) (SIS15:  $t=-4.710$ ,  $p<0.001$ ; SIS50:  $t=-3.235$ ,  $p=0.001$ ). Spittlebugs treated with either the SIS15 or the SIS50 performed fewer xylem ingestions (SIS15:  $t=2.887$ ,  $p=0.013$ ; SIS50:  $t=3.491$ ,  $p=0.002$ ) and non-pathway interruptions (SIS15:  $t=4.710$ ,  $p<0.001$ ; SIS50:  $t=3.235$ ,  $p=0.005$ ) compared to control, with no significant difference between treatments.

**Table 2.** Glmer model (A) and Tukey's test (B) results for trend of xylem ingestion duration (Xi WDI) during the 3h EPG recording (confidence level: 0.95).

A)				
	Estimate	Std. error	Z value	p-value
(Intercept)	3.496	0.070	49.611	< 0.001
SIS15	0.007	0.074	0.093	0.926
SIS50	-0.343	0.095	-3.625	< 0.001
Time	-0.030	0.022	-1.374	0.169
Sex	0.019	0.027	0.722	0.470
SIS15: Time	-0.183	0.036	-5.112	< 0.001
SIS50: Time	-0.142	0.046	-3.129	0.002
B)				
Contrast	Estimate	Std. error	Z ratio	p-value
Control,2 – SIS15,2	0.359	0.030	11904	< 0.0001
Control,2 – SIS50,2	0.628	0.038	16678	< 0.0001
SIS15,2 – SIS50,2	0.268	0.041	6625	< 0.0001

**Fig. 4.** Trend of the duration of xylem ingestion (Xi WDI) during the 3h recordings on control sunflower plants and on sunflower plants treated with the SIS15 or the SIS50. Duration of xylem ingestion during the hour is reported on the y-axis (expressed in minutes). The blue lines represent median values, while the confidence intervals (CI 95%) are represented by the gray bands. Letters in bold within brackets indicate statistically significant differences among treatments according to the results from Tukey's test.





**Fig. 5.** Boxplots representing the total duration of xylem ingestion ( $X_i$ ) expressed as percentage of the probing time (pWDI  $X_i$ ) during the 3h recordings on sunflower plants treated with SIS SIS15, SIS50 and control. The blue triangles indicate the median value of  $X_i$  WDI for males and females (pooled data) for each treatment. Letters in bold within brackets indicate statistically significant differences among treatments according to the results from Tukey's test.

No significant differences were observed in the median duration of single waveform events (WDEI).

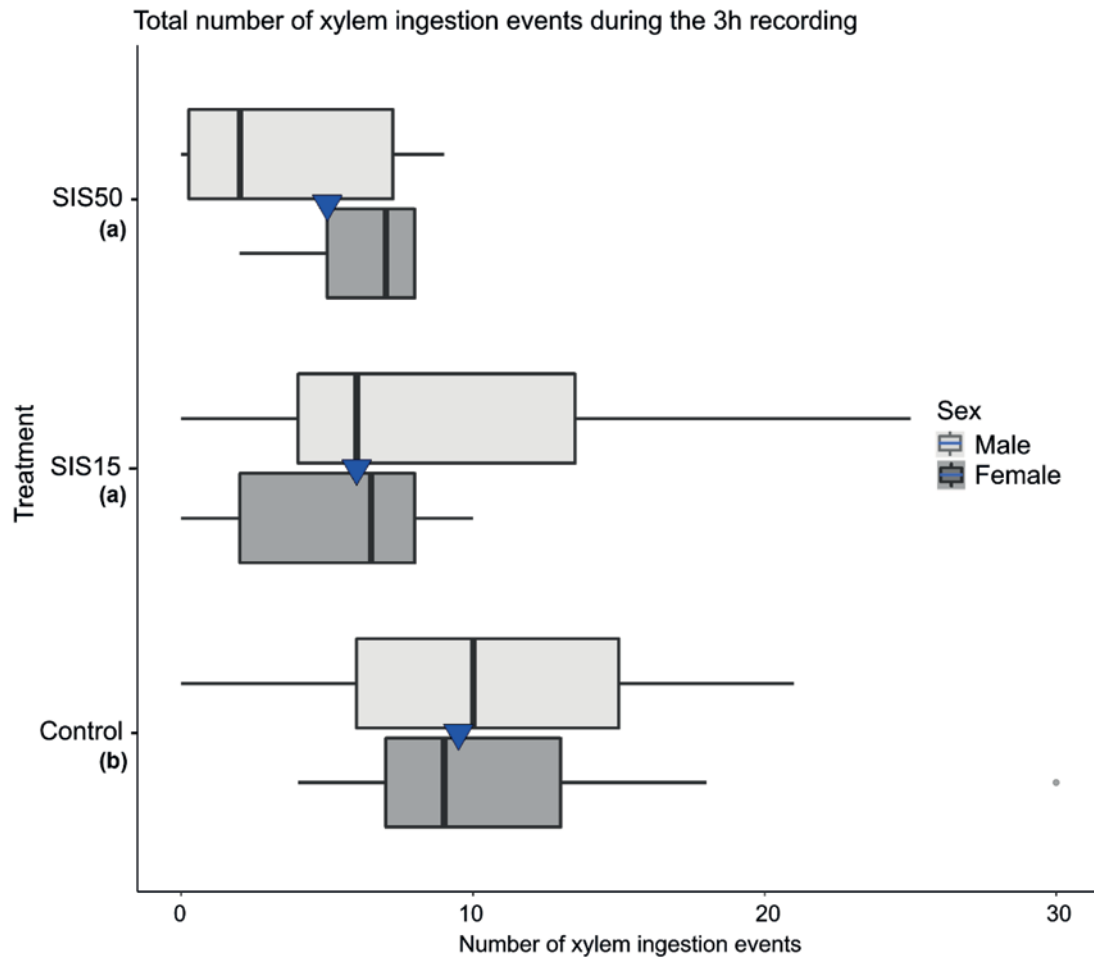
Time required by the insect from the beginning of the recording to perform the first xylem contact (np to 1<sup>st</sup> Xc) was significantly longer on plants treated with SIS50 than in the control group ( $t=-2.469$ ,  $p=0.041$ ). Both SIS15 ( $t=2.806$ ,  $p=0.006$ ) and SIS50 ( $t=3.161$ ,  $p=0.002$ ) affected the time required for the first xylem ingestion (np to 1<sup>st</sup>  $X_i$ ), which was significantly longer for treatments than control (SIS15:  $t=-2.807$ ,  $p=0.017$ ; SIS50:  $t=-3.161$ ,  $p=0.006$ ), with no difference between the two treatments (median values: Control=7.55 min; SIS15=14 min; SIS50=24.40 min). The vibrational signals impacted the time required for sustained xylem ingestion to occur (np to 1<sup>st</sup> sustained  $X_i$ ), namely a xylem ingestion event longer than 5 min (SIS15:  $t=2.225$ ,  $p=0.028$ ; SIS50:  $t=3.508$ ,  $p<0.001$ ). However, according to Tukey's test results, the time required to perform this behavior was significantly longer (more than tripled) in SIS50 (35.80 min) compared to control (11.95 min) ( $t=-3.508$ ,

$p=0.002$ ). No significant differences were observed when considering the time required by the spittlebug to perform the first probe (time to first probe).

In contrast with results obtained on sunflower, no significant differences in probing and feeding behavior of *P. spumarius* on olive plants between control and treatment (SIS50) were observed (Supplementary Materials SM4, Olive section). As expected, considering its low occurrence rate particularly on favorable plants, waveform Xc, putatively associated in *P. spumarius* to *X. fastidiosa* inoculation (Cornara et al. 2020), was not observed in any of the recordings performed either on olive or on sunflower. Additional EPG data are provided in Supplementary Materials (SM4).

### 3.2 "Start & Stop" test on sunflower

Within a short time after its transmission (median: 4 min), the SIS50 elicited the withdrawal of the stylets in 14 out of 26 males tested. Once the stimulus stopped, four of the males that had withdrawn the stylets ceased their feeding activity



**Fig. 6.** Boxplots representing the total number of xylem ingestion events (Xi NWEI) during the 3h recordings on sunflower plants treated with SIS SIS15, SIS50 and control. The blue triangles indicate the median value of Xi WDI for males and females (pooled data) for each treatment. Letters in bold within brackets indicate statistically significant differences among treatments according to the results from Tukey's test.

and jumped off the plant, while the others restarted probing within ca. 3 min (median value). The other twelve tested males continued xylem ingestion during the two-time spans considered, with no evident difference between signal on and off.

### 3.3. Signal propagation and characteristics: sunflower versus olive

The amplitude values of the SISs (SIS15 and SIS50) were lower when recorded from the leaf or the stem of the sunflower. On the other hand, the amplitude of the SIS50 did not differ from stem to leaf on olive plants (see Supplementary Materials SM1). The frequency spectrum of the SISs (Fig. 1) was rather conserved in all its components when transmitted to sunflower, as the main intensity peaks were concentrated on the original frequency bands of the signal. However, the recorded spectrum differed from the original signal in that

most the signal intensity was recorded at 840 Hz, while the spectrum below 400 Hz displayed a very low intensity (Supplementary Materials SM1). The intensity values of the SIS sunflower was 10-fold greater when played at higher amplitudes (SIS50) compared to lower amplitudes (SIS15) (for further information, see Supplementary Materials SM1).

By comparing the amplitude values of the SIS50 on sunflower and olive, a significant decrease in the signal amplitude was recorded (Supplementary Materials SM1, Fig. 3), while the spectrum of the signal (the overall shape) was rather conserved between the two host plants. The signal showed three main frequency peaks in both hosts, namely 536, 680 and 840 Hz. The mean values of the spectra referring to SIS on olive and sunflower plants, the amplitude of the peaks (velocity expressed in  $\mu\text{m/s}$ ) and the dominant frequencies are reported in Supplementary Materials (SM1).

## 4 Discussion

For a vector species, reduced feeding activity may be as important a reflection of potential yield impact as reduced density (Madden et al. 2000). Vector density, time spent on the host plant and transmission efficiency concur in determining *X. fastidiosa* transmission (Daugherty & Almeida 2009; Purcell 1982). In the case of *P. spumarius*' transmission of the fastidious bacterium to olive, the vector density is the main factor underlying the dramatic spread of the pathogen in Apulian olive orchards. In this regard, the relatively low spittlebug efficiency in transmitting *X. fastidiosa* to the host plant is compensated by the high number of individuals residing on olive plants for one to two months from sprouting to summer drought (Bodino et al. 2020; Cornara et al. 2016, 2017). A strategy aimed at reducing vector load and residency time on olive plants could therefore reasonably lead to decrease transmission probability and pathogen spread, at least in the Apulian scenario. Considering the theoretical dynamic of *X. fastidiosa* transmission to olive by the meadow spittlebug, an effective repellent tool should hamper the vector probing and feeding behavior rapidly, within the first minutes of the insect/plant interaction, as bacterial cells inoculation into the host plant is performed few minutes after the insertion of the stylets into the host tissues (Almeida et al. 2005; Cornara et al. 2020). Therefore, the higher the number of probes, and the greater number of vectors probing, the higher the chances for this inoculation behavior to occur (Daugherty & Almeida 2009). On the other hand, bacterium acquisition is associated with xylem sap ingestion from an infected vessel, and to the number of vessels probed by the vector (Almeida 2016a). Tackling transmission is therefore a matter of reducing the chances for a vector to acquire *X. fastidiosa* with the subsequent pathogen spillover. Fewer vector/plant contacts caused by reduced suitability of the host plant and/or reduced permanence of the vector on the substrate, would translate in fewer and shorter xylem contacts, thus in possibly reduced transmission probabilities.

Here, we demonstrate that the transmission over a suitable host plant of a stimulus (SIS) designed based on a vibrational signal used by spittlebugs for intra-specific communication played at high amplitude (SIS50) impeded probing in ca. 30% of the insects tested, causing ceasing of probing activities in around 50% of the tested males upon signal onset. Not only the SIS50 significantly reduced the number of probing spittlebug males and females, but also affected the feeding behavior of those that probed the plant. In this regard, feeding was significantly impaired in probing individuals, with a ca. 67% reduction of the time spent by the individuals in xylem ingestion over the 3h EPG recordings, 37% when just considering the time spent with stylets inserted into the host tissues (probing time). The signal also reduced the number of ingestions performed by the insects, and tripled the time needed for xylem ingestion to occur. This interference is likely the outcome of both: i) a direct

effect of the signal on the spittlebug that ends up diverging from its "normal" behavior because of perceiving a stressing input; ii) an indirect effect, since vibrations could make the plant an unsuitable substrate. Xylem ingestion duration is indeed a clear indicator of host plant suitability, with short duration indicating an unsuitable/barely suitable substrate (Markheiser et al. 2019; Sandanayaka et al. 2013).

In addition, considering the xylem ingestion trend displayed by the spittlebugs on treated plants, SIS50 appears to be a suitable candidate for disrupting *X. fastidiosa* transmission. In fact, spittlebugs on SIS50-treated plants reduced the ingestion of xylem sap from the beginning of the recording, additionally sharply decreasing over time, suggesting a fast repellent action that could be exploited to reduce spittlebugs population on the host plant, thus possibly reduced pathogen transmission chances. However, transmission trials with *X. fastidiosa* on plants treated with SIS50 are urgently needed to confirm our hypotheses.

The SIS15 also impacted the *P. spumarius* feeding behavior, although to a minor extent compared to SIS50. Greater disruption yielded with the latter signal is likely a non-linear response to the ten-fold increase in amplitude recorded with high compared to low volume. In the leafhopper *Scaphoideus titanus*, the inhibition of a crucial behavior such as male calling was achieved by using a noise transmitted at relatively high amplitudes. Although the noise reduced the searching behavior when perceived by the leafhopper male at amplitudes above 2.5  $\mu\text{m/s}$ , the complete disruption of the male-female communication occurred when the noise amplitude exceeded 15  $\mu\text{m/s}$  (Polajnar et al. 2016). Considering that *S. titanus* signals reached amplitudes of 50  $\mu\text{m/s}$  when recorded from the leaf where the insect is placed, mating disruption was achieved when the noise amplitude was 1/3 the amplitude of the natural emitted signal. These outcomes suggest that a disturbance stimulus can be efficient even if the signal reaches the insects at very low amplitudes (Eriksson et al. 2011). In our study, the main SIS50 frequency peaks could reach *P. spumarius* at amplitudes ranging from a minimum of 6  $\mu\text{m/s}$  to a maximum of 150  $\mu\text{m/s}$  on sunflower, thus resulting in a strong behavioral response, such as the immediate alteration of the feeding activity. The inefficacy of SIS50 in affecting the spittlebug probing and feeding behavior on olive is likely related to the dramatic decrease in signal amplitude on the woody plant compared to sunflower, particularly in correspondence of the three main frequency peaks recorded in both hosts, namely 536, 680 and 840 Hz. However, the lack of behavioral alteration on "vibrationally-treated" olives could not be ascribed just to signal amplitude in correspondence of these three peaks, given the values of this parameter are rather similar between SIS15 on sunflower and SIS50 on olive (SM1 Table 1). Low frequency components may have played a role, given that small insects such as *S. titanus* perceive and respond to low frequencies (220–250 Hz, for instance) also when the amplitude values range between 1 and 0.1  $\mu\text{m/s}$  (Eriksson et al. 2011, 2012). Of par-

ticular interest is therefore the spectrum of the SIS below 400 Hz. In fact, the fundamental frequency of *P. spumarius* vibrational signals ranges between 150 and 200 Hz (Avosani et al. 2020) and insect mechanoreceptors such as the femoral-chordotonal organ are responsive to low and medium frequency vibrations (200-900 Hz) in an amplitude-dependent fashion (Stein & Sauer 1999). In olive, the spectrum below 400 Hz is drastically reduced, whilst it is conserved on sunflower although displaying a reduced amplitude compared to the original signal (Fig. 1, Supplementary Materials SM1, Fig. 3). The lowest velocity threshold values perceived by insects are in the range between 1 and 10  $\mu\text{m/s}$ , which corresponds to the amplitudes measured in the frequency components below 400 Hz when the SIS was played on sunflower at low amplitude (SIS15). The long exposure to the SIS low-frequency components likely resulted in an accumulation of stress for the insect and in a progressive reduction of the feeding activity, while no behavioral effects were observed in olive due to the absence of these components and the overall lower amplitude values. Accordingly, increasing the amplitude of the frequency peaks within the 0-400 Hz range could lead to a greater impact in terms of spittlebugs feeding disruption. Overall, we hypothesize that the SIS frequency components we recorded on the tested plants elicited the behavioral responses (i.e., the feeding impairment) on sunflower when reached the insect at high amplitudes, although further research is indeed needed to characterize the impact of each of the signal features on spittlebugs behavior. Lastly, considering that plants are frequency and amplitude filters with different resonance properties depending on the species (Mazzoni et al. 2014; Michelsen et al. 1982; Polajnar et al. 2012), studies should be addressed at effectively transmit a conserved signal to olive plants, maximizing the feeding (and potentially pathogen transmission) disruption effect.

Beside the signal features and its behavioral role, the phenology and life cycle of the insect must be considered while designing “behavioral” control methods. In *P. spumarius*, the SIS50 affected the feeding behavior in August-September (the period the experiment was carried out), when females in nature emit mating signals, whereas rejection signals are mainly produced in spring (when females are sexually immature) (Avosani et al. 2020). The SIS50 had accordingly an apparently (although non-statistically significant) more marked effect on males than on females, being males during the period the tests were performed responsive to female mating signals (Avosani et al. 2020), which could have been masked by SIS in September. If the temporal and spectral features of SIS50 could both mask the female signals and cause distress, they could lead *P. spumarius* males and females to find a more suitable host for feeding and mating. Different levels of disturbance (i.e., soil tilling and vibrational stimuli) could accordingly affect adult populations by eliciting mass movements to other hosts and influence the oviposition suitability of certain sites, thus impacting the following popula-

tions (Bodino et al. 2019). A strategy aimed at repelling the spittlebug from olive plants is very promising, as this insect is highly polyphagous and can thrive on hosts alternative to olive (Weaver & King 1954; Bodino et al. 2019). In this regard, to estimate the efficiency of a similar strategy, it is crucial to assess the landscape composition of olive groves, considering the surrounding habitats, which can provide alternative hosts for spittlebug adults.

Beside the landscape composition, other factors such as the phenology and mating status could influence the efficacy of the vibrational disturbance. Further research should therefore analyze the effect of SIS, together with other species-specific signals composing the vast repertoire composing *P. spumarius* communication (for example the male-male signal (Avosani et al. 2020), throughout the season and on mated and unmated individuals, with a particular emphasis on spring and summer period, when *X. fastidiosa* transmission to olive occurs (Cornara et al. 2017).

To conclude, this work demonstrates, for the first time, that the playback of an *ad-hoc* designed signal based on species-specific vibrations can be exploited to impair probing and feeding behaviors of *P. spumarius*, when these stimuli are transmitted to an herbaceous host such as sunflower. This strategy is potentially applicable to all those vectors of plant pathogens using vibrations for short-range communication. Although our results raise numerous further experimental questions (i.e., what are the signal features responsible for the feeding impairment, how the SIS could be transmitted to olive trees, if the SIS could reduce *X. fastidiosa* acquisition and/or inoculation), they also could pave the way for sustainable strategies aimed at cohabiting with the fastidious bacterium, by mitigating the ecosystem impact either of the pathogen itself or of the suggested or currently applied integrated pest management approaches.

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