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Host finding and probing behavior by *Philaenus spumarius* on olive varieties with a different degree of susceptibility to *Xylella fastidiosa*

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Abstract

Abundance on and access time to the host plant are the pivotal factors in *Xylella fastidiosa* transmission to olive by the meadow spittlebug *Philaenus spumarius*. Therefore, olive varieties suitable for the vectors, i.e., plants providing all the necessary cues to the insect for their location, settling and acceptance, and devoid of antixenotic defenses, could be more susceptible to infection than varieties non- or less suitable for the vector. Here we evaluated whether a bacterium-susceptible olive variety, Ogliarola Salentina, could be a more suitable host for *P. spumarius* than the two resistant varieties Leccino and FS-17. We carried out: (i) an evaluation of between-hosts and within-host preference; (ii) an insect survival analysis; (iii) an Electrical Penetration Graph (EPG)-assisted analysis of the probing behavior; (iv) light microscopy of the tissues the spittlebugs had access to; (v) an analysis of the xylem sap primary metabolites. In choice tests, the insect exhibited a significant preference for Ogliarola Salentina. In addition, spittlebugs displayed longer xylem sap ingestion bouts on the bacterium-susceptible variety compared to resistant genotypes, possibly because of differences in the xylem sap chemical profile rather than xylem anatomy. Spittlebugs preference for Ogliarola over both Leccino and FS-17 could be a relevant and so far overlooked component of the low disease prevalence in these two olive varieties reported in Southern Italian olive orchards. Overall, our data point toward the importance of incorporating studies on vector-plant interaction and host traits of resistance to the vector in research on genotypes resistant to *X. fastidiosa*.

Keywords Spittlebugs · Vector-plant interaction · Xylem sap · Primary metabolites · Resistant genotypes

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Introduction

The xylem limited vector-borne bacterium *Xylella fastidiosa* is a major phytopathogen native to the Americas and causal agent of devasting diseases worldwide affecting grapevine, citrus, olive, and stone fruits among other crops. From the Pierce's disease of grapevine in California to the citrus variegated chlorosis in Brazil, this bacterium causes large economic losses, dramatic transformations of the landscape, and, in some cases, social turmoil (Almeida 2016; Schneider et al. 2020; Kahn et al. 2023).

In south-eastern Italy (Apulia region), the Olive Quick Decline Syndrome (OQDS) caused by *X. fastidiosa* subspecies *pauca* (ST53 group) is wiping out the olive oil production and depriving the local population of thousands of centenarian olive trees, as well as of their landscape and cultural heritage (Cornara et al. 2018; Saponari et al. 2019; Vanhove et al. 2019; Morelli et al. 2021). The only epidemiologically relevant vector of *X. fastidiosa* described so

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far in all the European outbreaks is the meadow spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae), responsible for the secondary olive-to-olive transmission of the bacterium (Cornara et al. 2018).

In Apulia the spread of *X. fastidiosa* has been facilitated by the planting of extensive monoculture of olive varieties susceptible to the bacterium, as Ogliarola Salentina and Cellina di Nardò, in addition to favorable environmental conditions for both the bacterium and the insect vectors (Saponari et al. 2019; Morelli et al. 2021).

However, not all the olive cultivars are equally susceptible to *X. fastidiosa*: the varieties Leccino and FS-17 (also known as "Favolosa") exhibit lower *X. fastidiosa* prevalence under field conditions, together with mild or no OQDS symptoms, and bacterial populations ten to 100 times lower compared to the susceptible varieties (Saponari et al. 2017; Pavan et al. 2021). Several research projects aimed at understanding the genetic basis of this resistance are currently ongoing. Giampetruzzi et al. (2016) reported an up-regulation of genes encoding receptor-like kinases (RLK) and receptor-like proteins in Leccino in response to *X. fastidiosa* infection, while Ogliarola plants react to the bacterium with the expression of genes related to the abscisic acid (ABA) metabolism, possibly aimed at maintaining cell wall structural integrity. Montilon et al. (2022) observed that in Leccino plants infected with the bacterium, occluded xylem vessels are mainly filled with callose-like granules. Additionally, Leccino's pit membranes have a compact and not degraded structure that does not allow bacterial movement.

The search for sources of resistance to *X. fastidiosa* within the olive germoplasm is of paramount importance for restoring the agricultural productivity in infected areas, reducing the reliance on long-term unsustainable control practices as ground cover removal and pesticide applications targeting the insect vectors (Avosani et al. 2021; Pavan et al. 2021). However, current research is overlooking a fundamental component in the epidemiology of any vector-borne plant pathogen, i.e., insect vector interaction with the host plant (Fereres and Moreno 2009; Daugherty et al. 2010). The host plant localization, landing, acceptance, and settlement by insects are driven by the right set of stimuli, which include biotic and abiotic factors, as well as learning (Dethier 1982; Avosani et al. 2023). Structural and chemical characteristics of the host plant are assessed by the insect through a sequence of behavioral steps stimulated by specific cues (Backus et al. 1985). If the plant cues match the insect requirements, the plant will be located and accepted as suitable host. Plants resistant to phytophagous insects bear structural and/or chemical traits that impair location, settling and acceptance (Mitchell et al. 2016). Therefore, resistant plants are expected to host a lower number of insects (if any) for a shorter time span compared to susceptible/suitable hosts.

Vector abundance and time spent on host plants, together with vector infectivity and transmission efficiency, are key factors for the transmission of *X. fastidiosa* (Purcell 1981; Daugherty and Almeida 2009). Specifically, in the case of transmission of *X. fastidiosa* to olive by *P. spumarius*, abundance and time of access to the plant have been estimated to be the most important factors in the bacterium epidemiology in southern Italy, given the relatively low efficiency of the olive-to-olive transmission by this vector (6–11%) compared for example to the grapevine-to-grapevine transmission by the blue-green sharpshooter *Graphocephala atropunctata* (Hemiptera: Cicadellidae: Cicadellinae) (up to 92%) (Hill and Purcell 1995; Cornara et al. 2017; Bodino et al. 2023).

Therefore, plants suitable to vectors, i.e., plants providing all the necessary cues to the insect for their location, settling and acceptance, and devoid of antixenotic defenses, could be epidemiologically more relevant, serving as both sources (for pathogen acquisition) and recipient (exposed to inoculation) of the bacterium, than plants showing structural and/or chemical traits not, or less, attractive for the vector.

In the present work we aimed at characterizing *P. spumarius* interaction with an olive variety susceptible to *X. fastidiosa*, i.e., Ogliarola Salentina, and the resistant varieties Leccino and FS-17. Our goal was to understand whether *P. spumarius* exhibits a preference for the *X. fastidiosa*-susceptible variety, and to infer structural and chemical characteristics of the plants underlying host selection and acceptance by the insect vector. Our hypothesis is that the two varieties resistant to *X. fastidiosa* are also less attractive and suitable for *P. spumarius*.

To test this hypothesis, we carried out: (i) an evaluation of between-hosts and within-host preference; (ii) a survival analysis; (iii) an Electrical Penetration Graph (EPG)-assisted analysis of the probing behavior; (iv) light microscopy of the tissues the spittlebugs had access to in the three olive varieties tested; (v) an analysis of xylem sap primary metabolites.

Materials and methods

Insects and plants rearing

A colony of *P. spumarius* was started from field-collected (non-infected area in Apulia, Italy) third to fourth instar nymphs. The juveniles were reared on grapevine (*Vitis vinifera* cv Cabernet Sauvignon), vetch (*Vicia sativa*), sunflower (*Helianthus annuus*), fava bean (*Vicia faba*), and *Sonchus* sp. until adulthood inside Bugdorm cages (Bugdorm.com, MegaView Science Co., Taichung 407,008, Taiwan) (approx. 500 nymphs per cage) kept in a greenhouse shaded with a shading net at 24–18 °C (day-night), photoperiod 16:8 L:D (light: darkness), 60% RH (relative humidity). One- to two-month-old adults reared on grapevine and sunflower (same

conditions as for the nymphs) water-fertilized daily (sunflower was replaced bi-weekly) were used for all the tests.

Olive plants were one year old self-rooted cuttings grown in perlite and peat moss (1:1) inside 1 L pots. Plants were maintained inside an insect-proof greenhouse, at a temperature of 22 ± 1 °C and 50% RH (no photoperiod control), water-fertilized twice a week with Plantafol 20:20:20 (NPK, Valagro SpA, 66,041 Atessa (CH), Italy) 1 g/L, and trimmed to 60 cm (single green shot and a wooden basal part, both of approx. 30 cm) one-week before the experiment onset.

Evaluation of between-hosts and within-host preference and survival analysis

We conducted an experiment aimed at evaluating within-host preference and survival under no-choice conditions. An olive plant (actively sprouting single green shoot, ca. 30 cm height) was caged within a plastic and mesh cage, keeping the wooden part and the pot outside the cage (10 replicates per variety, i.e., Ogliarola (Og), Leccino (Le), and FS-17) (Online resource 1, Fig. 1a); five *P. spumarius* individuals of same sex were introduced inside the cage (five cages with males, and five with females, thus 50 spittlebugs per variety). We conducted daily surveys (at 12 am) for 38 days taking note of alive/dead individuals, and of the position of individuals either on the cage or the plant. Each shoot was divided in three portions, basal, median, and apical, of approx.10 cm each (herein also referred to as "strata"). Inside each strata, we further evaluated the tissue the spittlebug settled on, i.e., stem, petiole, leaf (abaxial or adaxial).

For the assessment of between-host preference (preference among FS17, Ogliarola (Og) and Leccino (Le)), *P. spumarius* females were caged singly inside a BugDorm (BugDrom 2F-400, W75 x D75 x H115 cm) containing three olive plants (free-choice), one plant per variety, at the same phenological stage (one green single shoot (GSS) and wooden basal part (MS), both of approx. 30 cm. GSS and MS were each hypothetically divided in three portions, apical, median, and basal, of ca. 10 cm each) (Online resource 1, Fig. 1b). We recorded the position of each individual at 1–3-6–9-12–24-48–72 h post-exposure, thus the variety, the strata, and the tissue (petiole, stem, leaf abaxial or adaxial) selected. Fifteen biological replicates (fifteen cages with three plants each) were carried out, with the position of the plants inside the cage switched for each cage to avoid positional effects.

The choice and no-choice tests were carried out in June, i.e., the period when newly emerged adult spittlebugs move from the ground cover to olive plants in Southern Italy.

Data analyzes was performed with R version 4.0.3 (R Core Team 2020). Data exploration was carried out following the protocol described in Zuur et al. (2010) and model assumptions were verified following the protocol of Zuur

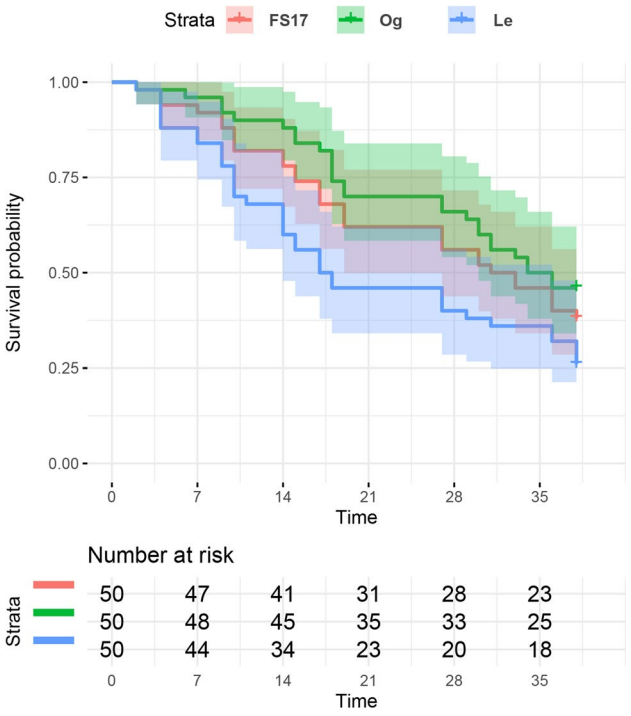

Fig. 1 Kaplan–Meier plot describing the survival curves for *Philaenus spumarius* at three olive varieties (FS17, Og, Le). The curves represent the estimated survival probability at each time point. *Y*-axis shows the probability of being alive at each time point, while *X*-axis shows the time-points. The shaded areas around the lines represent the 95% CI. Panel down shows the corresponding number of insects at risk at each time-point. The term "strata" refers to the three olive varieties tested: FS-17 (FS17, red); Ogliarola (Og, green); Leccino (Le, blue)

and Ieno (2016). For the first trial (no-choice), we first tested the preference of *P. spumarius* among the different portions/strata (apical, middle, base) and the cage. Model assumptions revealed the data suffered from overdispersion, therefore we adopted a mixed model for zero-inflated data using Package "glmmDMB" (Bolker et al. 2012). The number of individuals per plant strata was used as response variable, while the strata and the sex were used as categorical factors and cages as replicates. Visual inspection of Q-Q plots and residuals plotted against fitted values revealed no obvious deviation from the canonical assumptions of normally distributed and homogenous model residuals. Akaike information criterion (AIC) was used to select the best fitting model (Pinheiro and Bates 2000). Given that the insects stayed mainly on the apical part of the plant, we performed GLMM (family = poisson) for within-host preference of *P. spumarius* (plant tissue: leaf adaxial, leaf abaxial, petiole, stem) in the apical part chosen by the individuals in each of the three different varieties (FS17, Og and Le) and by sex along days, using cages as replicates. In order to evaluate spittlebugs survival on the three varieties, we considered the Overall Survival (OS) from the date of first observation (day

1) to the date of death (event). Alive insects were censored at the date of last follow-up (day 38). OS was described using Kaplan–Meier curves (Kaplan and Meier 1958) in the three categories of the variable "variety". We calculated the median survival time and its associated non-parametric confidence interval and compared between the three categories of "variety" using Cox's proportional hazards (PH) model, considering "Leccino variety" as model baseline. We adjusted Cox PH model for sex and considered a two-sided 5% significance level. For analyzes we used R packages "survival" (Therneau and Lumley 2015) and "survminer" (Kassambara et al. 2017).

For the second trial, host preference and within-shot preference along the observation time (72 h) were evaluated by PERMANOVA (permutations = 999, method = "euclidean") (Anderson 2017), Package Vegan (Oksanen et al. 2019). Pairwise. Wilcox. test with Bonferroni correction was used for multiple comparisons.

EPG-assisted analysis of the probing behavior

One to two months old adult spittlebugs were collected in 2 ml aerated plastic tubes, then anesthetized placing the tube at − 20 °C for 2 min, and tethered (18 µm gold wire glued on the pronotum with Ted Pella, no. 16062; Pelco® Conductive Silver Paint (Ted Pella, Redding, CA, USA)). Wired spittlebugs were caged inside aerated Petri-dishes (where they were able to move and get accustomed to the wire), then starved for one hour. After the starvation period, the wire-electrode connection was strengthened with a drop of conductive silver paint, before plugging the electrode with the insect into the EPG probe. The recordings were performed under controlled conditions at 23 ± 1 °C, 50% RH, and artificial light of 1,000 ± 200 lx, during the period May–July. Three rounds of 4 h recordings with both males and females on the olive apical part (the insect had access to stem, petiole and leaf; see results from the within-host preference trial) were carried out per day (8–12am; 12am–4 pm; 4–8 pm) in the period June-July, always during sunny days, taking note of insects' age, sex, and time of the day during which the recording was carried out (EPG round). A total of 32 recordings for Ogliarola (17 males and 15 females), 36 for Leccino (18 males and 18 females), and 31 for FS-17 (14 males and 17 females) were considered valid (clear signal and absence of aberrant electrical patterns) and analyzed.

An eight-channel DC-EPG amplifier (model Giga-8d, EPG-Systems, Wageningen, Netherlands) was used for the EPG-recordings. Data were acquired with 'Stylet + d' software (EPGSystems, Wageningen, Netherlands). EPG waveforms were manually marked in 'Stylet + a' software (EPG Systems, Wageningen, Netherlands) and then analyzed by "XylFeed" software (Markheiser et al. 2023). Briefly, following the protocol described by Markheiser et al. (2023), we considered ten patterns of waveforms representing the different behavioral steps 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 > 0.1 Hz); (v) LF, low frequency xylem ingestion (frequency < 0.1 Hz); (vi) N, non-pathway interruption of xylem activity; (vii) R, resting; (viii) Xe, behavior putatively associated with *X. fastidiosa* inoculation (Cornara et al. 2020, 2022); (ix) W, stylets withdrawal; (x) Esc, escaping of the individual from the host plant.

Probing behavioral differences among the olive varieties ((i) Sequential variables (np to first Xc; np to first Xi; np to first LF; first C to first Xc; first C to first Xi; first C to first LF; time to first probe; time to first probe with Xi and time to first probe with LF); (ii) Non-sequential variables (WDI, waveform duration per individual; NWEI, number of waveform events per individual; WDEImd, median duration of each waveform event per individual)) were investigated by Mixed-Effects Linear Models (LME, restricted maximum likelihood method), using variety, sex, and the interaction between them as explanatory variable, and the EPG round as random factor. The interaction term was removed from the model in case of non-significance. Differences in the number of probes (successful (with xylem ingestion), unsuccessful (without xylem ingestion), initial (without xylem ingestion, but with xylem contact) and total (successful + initial + unsuccessful)) were evaluated through Generalized Linear Mixed Models (GLMM) with negative binomial distribution (link-function: log). The explanatory variables were species, sex, and the interaction among them; EPG round was included as random factor. The interaction term was removed from the model in case of non-significance.

LMEs were used to assess variety-related differences in NWEI and WDI hourly trends during the 4 h recording. The explanatory variables were the variety, the time of the recording (expressed in hours), and the interaction between these factors, while sex and EPG round were included as a random factors. For LME, the data obtained from the EPG were transformed, when necessary, with ln $(x+1)$ or $\sqrt{(x)}$ to reduce heteroscedasticity and improve normal distribution. In case of a statistically significant effect ($P < 0.05$) of model variables, pairwise comparison were conducted by Tukey's HSD (honest significant differences) test using "emmeans" package (Lenth 2021). The Ogliarola variety was set as the model intercept.

Models were run using the "glmmTMB" package, while residual distribution was checked using the "DHARMa" package. There was no evidence of either spatial or temporal autocorrelation of model residuals according to analyzes performed using the "ncf" and "acf" packages (Bjornstad, 2022), respectively. Explanatory variables showed no collinearity. A summary of the raw dataset was prepared using Past 4.03 software

(PAST—Download (lo4d.com)). Plots were generated using "ggplot2" package (Wickham et al. 2016).

Microscopy on the tissues the spittlebugs had access to in the three olive varieties tested

Two 2 cm apical olive samples per variety (each sample containing stem, leaf midrib and petiole) were randomly selected among those where valid EPG recordings with xylem ingestion (waveform Xi) were produced (thus olive plants where the spittlebug accessed the xylem vessels and ingested xylem sap). At the end of the 4 h EPG, the portion was cut with pruning shears and stored in 2% glutaraldehyde in 0.05 M potassium phosphate 0.1 M sodium cacodylate buffer pH 7.2 at − 4 °C until being processed (one month after sampling). Each sample and portion (stem, petiole and midrib) was observed by light microscopy in order to collect data on: (1) diameter of the xylem vessels (µm); (2) depth of the xylem vessels (distance from the border/epicuticle in µm); (3) xylem vessels density (number/mm^2). Ten transversal cross Sects. 2 mm thick per portion per each sample were sectioned with a microtome and scrutinized with a Zeiss Axioskop 40 (Carl Zeiss Microscopy, Thornwood, NY, United States NIKON Microscope Eclipse 80i, Nikon Inc., Melville, NY, U.S.A.), at a resolution 10X magnification for the diameter, and 4X for the depth and the density. The images were captured with a digital camera QIMAGING QICAM Imaging software NIS-Elements by Nikon (Nikon Corporation, Tokyo 108–6290, Japan), and analyzed by SW Image J (Schneider et al. 2012).

Differences among the three varieties in xylem vessels diameter (µm), depth (distance from the epidermis; µm), and aggregation (number of vessels per mm^2) were analyzed by LME, using the variety, the portion (stem, leaf midrib and petiole), and their interaction as explanatory variables, and the sample as random factor. Data were transformed, when necessary, with ln $(x+1)$ or $\sqrt{(x)}$ to reduce heteroscedasticity and improve normal distribution. In case of a statistically significant effect ($P<0.05$) of model variables, pairwise comparison were conducted by Tukey's HSD (honest significant differences) test using "emmeans" package (Lenth 2021). The Ogliarola variety was set as the model intercept. Models were run using the "glmmTMB" package, while residual distribution was checked using the "DHARMa" package. There was no evidence of either spatial or temporal autocorrelation of model residuals according to analyzes performed using the "ncf" and "acf" packages (Bjornstad, 2022), respectively. Explanatory variables showed no collinearity.

Analysis of xylem sap primary metabolites by high performance liquid chromatography coupled to triple quadrupole mass spectrometer (HPLC/MS–MS)

The xylem sap from four olive plants per variety, randomly selected among those where valid EPG recordings with xylem ingestion were produced (two males and two females), was extracted with a Scholander pressure chamber. After inserting the shoot in the chamber, 2 cm of the main stem was debarked and disinfested with a sterile paper moistened in ethanol to avoid contamination of the xylem sap from bark and phloem. Ethanol was left to evaporate before sap extraction. The pressure was increased gradually until xylem sap drops were observed, but to a maximum of 25 bars of pressure to limit external contamination derived from cell rupture. Xylem sap was collected in 2 mL sterile microcentrifuge tubes placed on ice. An average of 1.5 mL of xylem sap was collected per plant; samples were stored at − 80 °C until the chemical analysis (Anguita-Maeso et al. 2021). The xylem sap from each plant was collected immediately after the end of the 4 h EPG round.

Xylem sap were filtered at 0.2 µm using regenerated cellulose syringe filters and diluted 1:10 by volume with LC/MS grade water. Three microliters of diluted xylem sap were injected in the UHPLC Ultimate 3000 system (Dionex Thermo Fisher Scientific, MA, US) equipped with LPG-3400RS pump, WPS-3000 autosampler, TCC-3000 column oven, and a Photodiode Array Detector PDA 3000. Chromatographic separation was obtained by the column Zorbax Eclipse C18, 10 cm of length, 2.1 mm of internal diameter, 1.8 µm of particles size (Agilent, CA, US) using a binary gradient with formic acid 0.1% in water (solvent A), methanol/acetonitrile/formic acid (50/50/0.1 vol) (solvent B). All reagents were LC/MS grade from Sigma Aldrich (MO, US). The solvent B gradient program was 5% initial, isocratic for 1 min, increased to 28% in 4 min, then to 55% in 20 min, and finally to 90% in 2 min, isocratic for 3 min, equilibration to the initial conditions for 5 min. The column compartment was set at constant temperature of 30 °C, and the mobile phase flow rate at 0.25 mL/min. The identification of compounds was performed by using a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer equipped with a HESI interface. The MS and HESI conditions were: capillary temperature 330 °C; source heater temperature 280 °C; nebulizer gas N_2; sheath gas flow 35 psi; auxiliary gas flow 10 arbitrary units; capillary voltage − 2.8 kV. Data were acquired in negative ionization mode using a data-dependent method. The data-dependent settings were: full scan from 100 to 850 m/z, activation level 500 counts, isolation width 1 Da, default charge state 2, CID energy 20, collision gas pressure 1.5 mTorr of Argon bip. All data were acquired and processed using Xcalibur v 3.0 (Thermo

Fischer Scientific). The identification of primary metabolites (amino acids, organic acids, sugars) was achieved by comparing $[M-H]^-$ and MS/MS fragmentation patterns with literature data or MZ cloud database.

To compare the quantity of each identified compound among olive varieties samples, the molecular mass signal was extracted from the Total Ions Chromatogram, and the area of the relative peak was recorded. All obtained values were standardized using Fisher *z*-transformation method. Data grouped for *cv* were tested for normal distribution (Shapiro–Wilk test) and analyzed by ANOVA and post hoc Tukey test, using SPSS 22 (Software SPSS—Italia IBM, NY, US).

Results

Evaluation of between-hosts and within-host tissue preference and survival analysis

For the no-choice experiment, Zero-inflated mixed models showed a significant preference of *P. spumarius* for the apical stratum independently of sex and olive variety (Online resource 1, Table 1 and 2). Considering within-host preference for the apical stratum, GLMM showed that most insects preferred the stem tissue (Online resource 1, Table 3), independently of sex and time. However, the interaction tissue * variety * time showed a significant selection trend, with the number of individuals settling on the leaf (either abaxial or adaxial) decreasing over time, oppositely to the trend observed for the stem (Online resource 1, Table 3). For the survival analysis under no-choice conditions, a total of 150 individuals were considered, with 95 deaths taking place. When looking at the total deaths (Online resource 1, Table 4), values did not differ among varieties; however, the median overall survival for Leccino was the half value of that observed in the other two varieties. A significant difference (adjusting for sex) was observed only between Ogliarola and Leccino (HR = 0.5227, 95% CI (0.3178–0.8597); $p = 0.0106$), with a lower risk of death events over time in Ogliarola. Kaplan–Meier plot (Fig. 1) shows the survival curves for *P. spumarius* on the three olive varieties (FS17, Ogliarola, Leccino) highlighting a lower survival of individuals on Leccino variety.

For the second experiment (choice test), results of PERMANOVA showed a significant preference of *P. spumarius* for the variety Ogliarola compared to FS17 ($p = 0.024$) and Leccino ($p = 0.0005$). There was no significant difference between FS17 and Leccino ($p = 0.714$). Spittlebugs made their choice within the first 24 h of plant access, with no significant host switching thereafter (Online resource 1, Fig. 2, 4 and 5). The results of preference of portion (strata) of the plant by time showed a preference for the GSS portion ($p = 0.012$) independently of variety ($p = 0.114$), time ($p = 0.997$) or the interaction between them ($p = 0.999$).

EPG-assisted analysis of the probing behavior

The total duration of the xylem contact pattern (Xc WDI $z = 2.499$, $p = 0.0124$), and of the single xylem contact events (Xc WDEI $z = 2.917$, $p = 0.00354$) were shorter in *P. spumarius* males compared to females, independently of the variety. Xylem ingestion was overall significantly shorter in Leccino compared to Ogliarola either considering only Xi WDI ($z = 2.80$, $p = 0.00514$), or the sum of high frequency (Xi) and low frequency (LF) xylem ingestion (Xi + LF WDI) ($z = 2.326$, $p = 0.020$), with no difference between Leccino and FS-17. The single xylem ingestion bouts (Xi

Table 1 Cultivars comparative data on carbohydrates, organic acids and amino acids expessed in z-score (Fisher distribution). The mean values, obtained analyzing the three pools of sap samples, are reported. Only significant values of ANOVA were reported together with the Tukey post hoc results for pairwise comparisons (significant differences indicated with letters). Negative or positive values means that the compound concentration in the relative samples is higher or lower than the general mean computed considering all samples

	Favolosa		Leccino		Ogliarola		Sign
Carbohydrates							
Mannitol	1.0	a	−0.1	b	−0.9	b	0.024
Hexose	0.5		−0.3		−0.2		
Organic acids							
Citric	0.7		0.1		−0.7		
Malic	0.7	a	0.5	a	−1.3	b	0.001
Isocitric	0.8	a	0.2	a	−1.0	b	0.030
Succinic	0.5		0.2		−0.7		
Amino acids							
Choline	0.7		−0.5		−0.2		
Arginine	−0.2		−0.7		0.9		
Glutamine	0.7		−0.1		−0.6		
Tyrosine	0.0		0.6		−0.6		
Leucine_Isoleucine	0.4		−0.2		−0.2		
Phenylalanine	0.5		−0.1		−0.4		

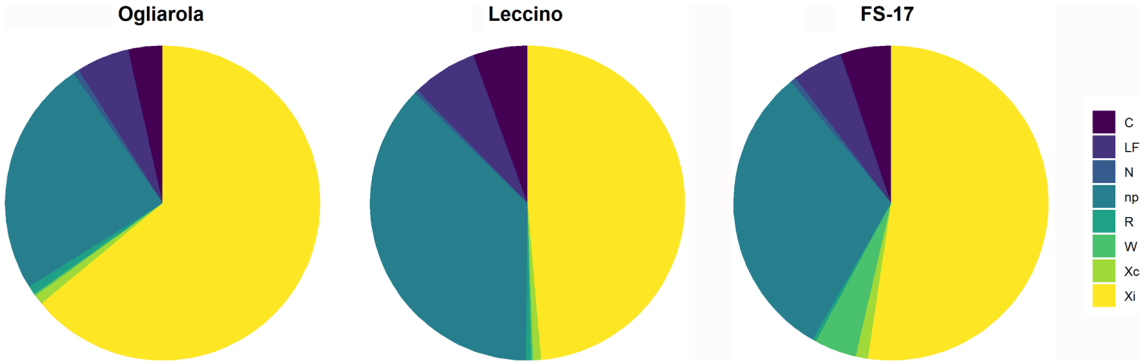

Fig. 2 Duration of each probing behavioral pattern/waveform (WDI) during the 4 h access to the three olive varieties. EPG WAVEFORMS = np: non-probing; C: pathway (stylets outside xylem); Xc: xylem contact (tasting of the xylem sap, and testing the mechanical strength of the sealing); Xi (xylem ingestion); LF (low-frequency xylem ingestion, i.e., peaks with a frequency < 0.1 Hz); N (non-pathway interruption; termed *X* according to Backus nomenclature); R (resting); Unk (unknown behavior); W (stylets withdrawal from plant tissues). No escape (Esc) events were observed

WDEI) were significantly longer in Ogliarola compared to both FS-17 ($z = 2.179$, $p = 0.0293$) and Leccino ($z = 2.020$, $p = 0.0434$), with no difference between Leccino and FS-17 (Fig. 2 and 3). The total duration of xylem-sap ingestion (Xi WDI) showed a similarly decreasing trend over the 4 h recordings in all the varieties (Fig. 3).

Spittlebugs on FS-17 exhibited significantly more pathway events (C NWEI) ($z = 2.21$, $p = 0.0273$) and more stylets

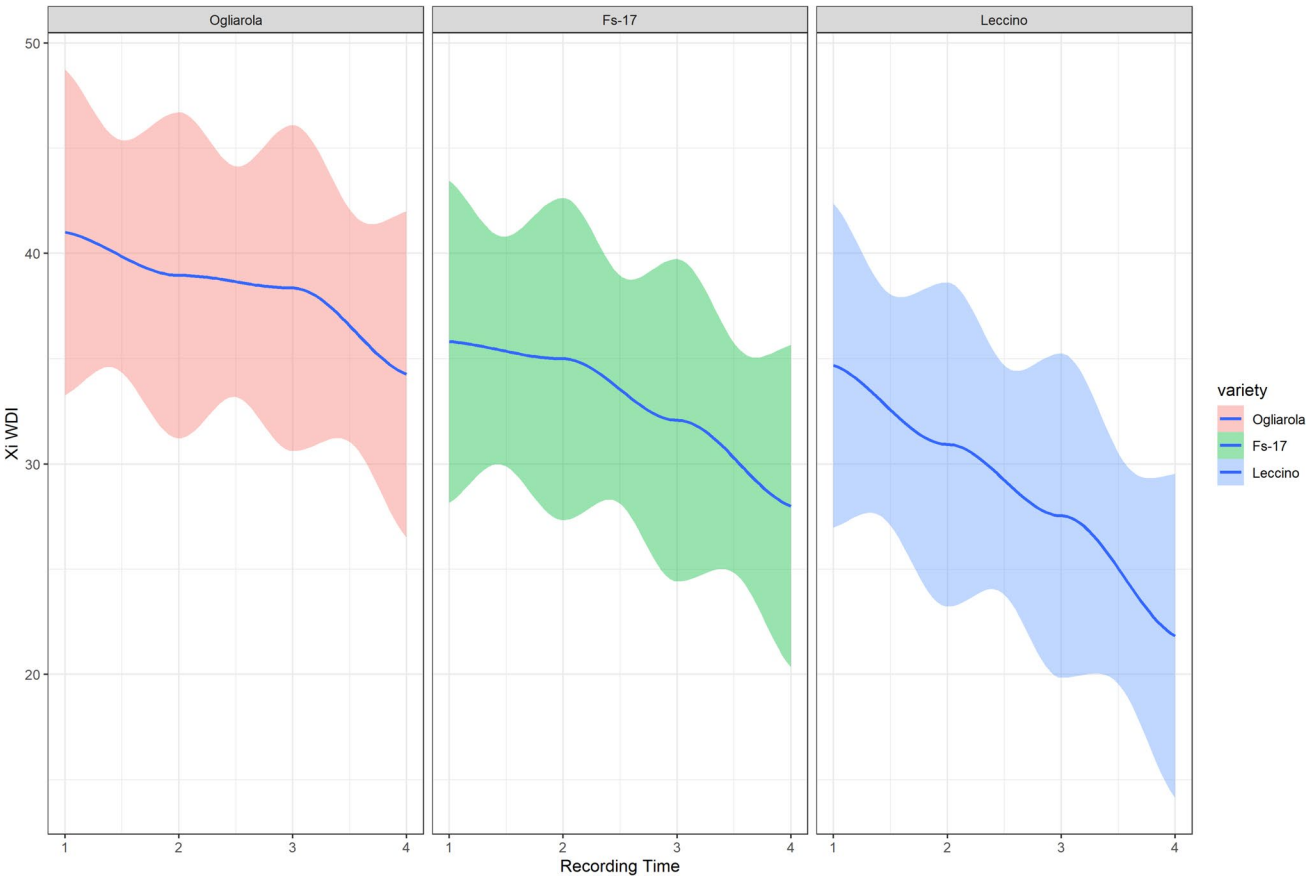

Fig. 3 Xylem ingestion trends in the three olive varieties during the 4 h EPG. *x* axis: duration of the xylem ingestion expressed in minutes. *y* axis: hour of the recording

withdrawal events (W NWEI) ($z = 1.96$, $p = 0.0499$) than Ogliarola; the differences between the two bacterium-resistant varieties were non statistically significant. There was no difference in the sequential variables among the varieties. The pattern leading to *X. fastidiosa* inoculation (waveform Xe) was present only in two recordings, one female and one male spittlebug both on Leccino. No escape (Esc) events were observed. A summary of all the EPG variables considered is reported in Online resource 2.

Microscopy on the tissues the spittlebugs had access to in the three olive varieties tested

Hundreds of xylem vessels were scrutinized for each of the two samples (comprising stem, leaf, and petiole) produced per variety (Fig. 4, 5). FS17 had significantly larger xylem vessels than both Ogliarola and Leccino in all the three portions analyzed, namely leaf midrib (Ogliarola: $t = 26.804$; $p < 0.001$; Leccino: $t = 24.442$, $p < 0.001$), petiole (Ogliarola: $t = 21.414$, $p < 0.001$; Leccino: $t = 25.706$, $p < 0.001$), and stem (Ogliarola: $t = 45.430$; $p < 0.001$; Leccino: $t = 40.983$, $p < 0.001$). On the other hand, petiole vessels in Leccino were significantly narrower than in Ogliarola ($t = 6.204$, $p < 0.001$).

Stem xylem vessels were deeper (more distant from the epidermis) in FS17 than Ogliarola ($t = 26.591$, $p < 0.001$) and Leccino ($t = 25.564$, $p < 0.001$), with no significant differences between Ogliarola and Leccino. On the other hand, Leccino vessels were shallower than FS17 in the leaf midrib ($t = 5.606$, $p < 0.001$), and shallower than both Ogliarola ($t = 13.613$, $p < 0.001$) and FS17 ($t = 15.279$, $p < 0.001$) for the petiole.

Xylem vessels were more aggregated (higher number of vessels per mm^2) in Ogliarola stem compared to FS17 stem ($t = 6.262$, $p < 0.001$), and in Ogliarola midrib compared to FS17 midrib ($t = 3.163$, $p = 0.0475$). Considering the petiole, FS17 had significantly less aggregated vessels than both Ogliarola ($t = 6.653$, $p < 0.001$) and Leccino ($t = 4.068$, $p = 0.002$). The differences in vessels aggregation between Leccino and Ogliarola were non statistically significant.

The total counting of the number of vessels and areas (mm^2) scrutinized per variety and per portion, the summary statistics, and the graphics generated by visreg, are reported in Online resource 3.

Analysis of the xylem sap primary metabolites of the olives the insects had access to

We observed statistically significant differences among the varieties in the concentration of mannitol, malic acid, and isocitric acid (Table 1). Mannitol concentration was significantly higher in FS-17 than both Ogliarola and Leccino. The xylem sap of the two *X. fastidiosa*-resistant varieties contained a greater concentration of malic and isocitric acids than the susceptible Ogliarola. Regarding the other two organic acids identified, i.e., citric and succinic acids, even though the variety-related difference was not statistically significant, the concentration showed the same trend FS-17 > Leccino > Ogliarola.

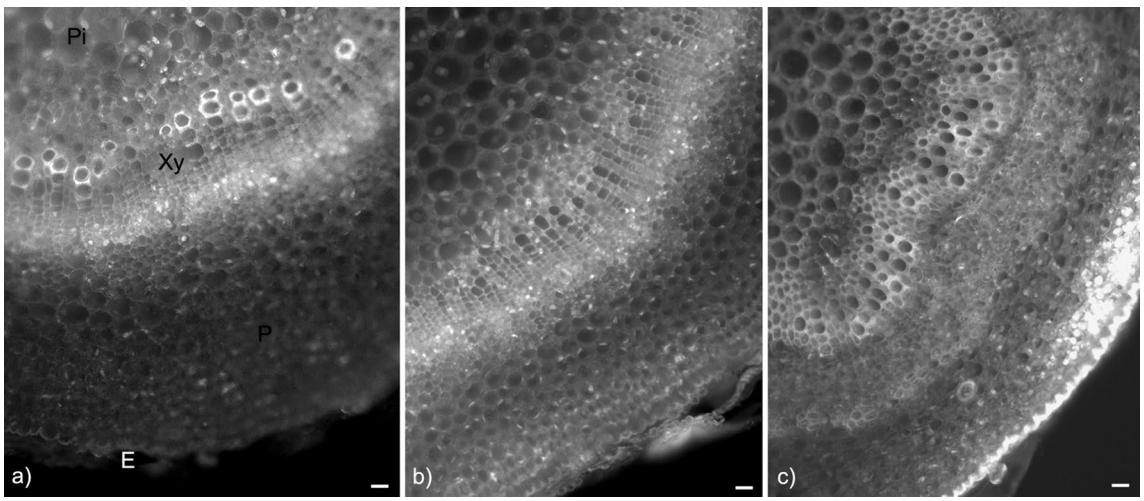

Fig.4 Micrographs of the stem cross sections of FS17 **a**, Leccino **b** and Ogliarola **c** cultivars. Pi: pith; Xy: xylem vessels; P: parenchima; E: epicuticle. Scale bars: 50 mm

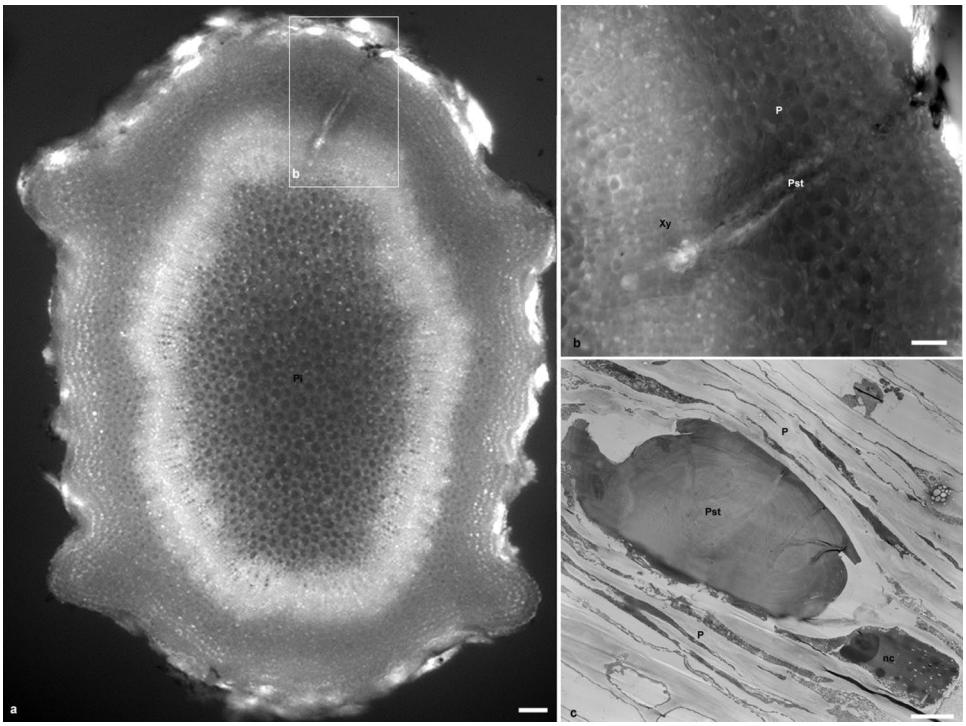

Fig. 5 Micrographs of the stylets trace (Pst) of *Philaenus spumarius* in the stem of olive plants var. Leccino observed under optical (**a**) and electron (**c**) microscopy. White boxed area in **a** is enlarged in (**b**). **b** Close-up of distal part of the salivary sheath as it passes the parenchymal cells (P) and terminates in a large, mature xylem cell (Xy). The spittlebug stylets penetrated intercellularly between parenchyma cells (**c**). The stylets penetration in Leccino's stem and related cells compression led possibly to the nearby necrosis (nc); alternatively, the necrosis was associated with a hypersensitivity response of the variety. The portion was fixed in 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH7.2) for 2 h and post-fixed at 4 °C in 1% osmium tetroxide in the same buffer for 2 h. Overnight bulk staining in 0.5% aqueous uranyl acetate, dehydration in graded ethanol dilutions, and embedding in TAAB Spur resin followed. Thin sections, obtained by the ultramicrotome REICHERT SUPERNOVA (Leica Reichert Division–Austria) on a MICROSTAR diamond knife (MICRO ENGINEERING Huntsville USA), were stained with lead citrate before observations with a FEI Morgagni 282D Transmission Electron Microscope (ThermoFisher, Waltham, MA, U.S.A.) at 80 kV accelerating voltage. Samples were analyzed by 20 sections of 80 nm in thickness. Pi, pith. Scale bars, a 1 mm, b and c 500 nm

Discussion

The abundance and time spittlebugs spend on olive plants, rather than transmission efficiency, seem to be the main factors underlying the dramatic spread of *X. fastidiosa* in Southern Italian olive orchards, as well as in all the bacterium outbreaks around Europe (Cornara et al. 2017, 2019; Bodino et al. 2023). If during host plant recognition and acceptance the spittlebug senses traits indicating plant unsuitability, it would abandon it and search for more suitable hosts. Thus, the lower the abundance and the shorter the time on a plant, the lower the number of probes and the chances of engaging with behavioral activities conducive to bacterium acquisition and inoculation by the vector (Daugherty and Almeida 2009; Cornara et al. 2020).

In the present study we observed that Leccino and FS-17 varieties are less suitable hosts for the meadow spittlebug *P. spumarius* when compared to the bacterium-susceptible variety Ogliarola Salentina. When given a choice, the meadow spittlebug significantly prefers Ogliarola over Leccino and FS-17 varieties, also engaging in longer xylem ingestion on the former compared to the latter.

In choice trials, spittlebug females significantly preferred Ogliarola over Leccino and FS-17, with the final choice made within the first 24 h, and no significant host-switch occurring thereafter. Cascone et al. (2022) reported *P. spumarius* females are strongly repelled by Volatile Organic Compounds (VOCs) produced by FS-17, while no response (neither attraction nor repellence) to Leccino was observed. Orientation toward a host and landing are driven by the right stimulus or set of stimuli, including biotic and abiotic factors and learning (Diether 1982). In xylem-sap feeders, the main stimuli driving host finding and landing are visual and olfactory cues (Avosani et al. 2023). According to Patt and Setamou (2014), volatiles mainly act by increasing xylem feeders' responsiveness to visual quality of the foliage, being the latter, the pivotal stimulus permitting the insects to detect a suitable host. The preference for the Ogliarola variety

observed in our choice trial could therefore have been the result of the perception by the spittlebug of more attractive visual (shape, size, color, light polarization (Prokopy and Owens 1983)) and chemical (VOCs) stimuli in the bacterium-susceptible compared to the bacterium-resistant varieties (Leccino and FS-17). However, the determination of the main stimuli driving host finding in spittlebugs deserve further dedicated investigations.

Our results also show that Ogliarola is not just more attractive when a choice is given to the insect, but also a more suitable host than the other varieties tested. The duration of the xylem ingestion is the main indicator of host suitability for xylem-feeders (Markheiser et al. 2020). *Philaenus spumarius* on Ogliarola displayed indeed significantly longer duration of the xylem ingestion phase compared to Leccino, longer single xylem ingestion bouts than both Leccino and FS-17, and the lowest absolute risk of death events during the 38-day long survival trial among the varieties tested. No other probing behavioral variable tested, either sequential or non-sequential, significantly differed among the varieties, apart from the higher number of pathways (EPG waveform C) and stylets withdrawal (EPG waveform W) observed in FS-17. Overall, our data suggest that, upon landing on the plant, the processes of host recognition through labial dabbing and sensing of chemical cues on the plant surface, and the successive stylets insertion and penetration searching for xylem vessels, do not differ among the varieties. The absence of statistically significant differences in time required to contact xylem vessels and engage with xylem ingestion, indicate no major differences among the varieties in either biochemical composition and structure of the leaf cuticle, epidermis, and mesophyll impacting the probing behavior, or in chemical cues triggering the initiation of xylem ingestion.

As for probing and feeding (Sogawa 1976), also the onset and the maintenance of ingestion depend possibly on different sets of positive sensory inputs stimulating the central nervous system. Amino acids, particularly glutamine and asparagine, have long been considered the main drivers of host plant acceptance in xylem-sap feeders (Horsefield 1977; Brodbeck et al. 1990; Andersen et al. 1992; Thompson 1994, 2004). The xylem-sap amino acids repertoire did not show significant differences among the olive varieties, and indeed the spittlebugs exhibited no variety-dependent differences in behaviors associated with xylem contact or initiation of the sap ingestion. The only primary xylem sap metabolites (among those tested) that significantly diverged between Ogliarola and both Leccino and FS-17 were organic acids, and specifically malic acid and isocitric acid, whose titer was significantly higher in the resistant ones compared to the susceptible. Therefore, while amino acids could be the main chemical cue triggering the ingestion onset upon xylem contact and trial ingestion, the right balance of organic acids, alone or in combination with secondary metabolites not tested here, could be crucial for the maintenance of xylem sap ingestion. Our observation on the correlation between organic acids and insect feeding is consistent with data reported Kim et al. (1976) and Yoshihara (1980) on phloem feeders, with high concentrations of organic acids (oxalic and trans-aconitic) in rice plants phloem shortening the duration of ingestion bouts in the planthopper *Nilaparvata lugens* Stal (Hemiptera: Delphacidae). The higher suitability for *P. spumarius* of the bacterium-susceptible Ogliarola observed in our EPG experiment was also further confirmed by our survival experiment, where a greater risk of death events (statistically significant in the case of Leccino), probably associated with a reduced uptake of xylem sap, was observed in the bacterium-resistant varieties.

The presence within the xylem-sap bacterium-resistant varieties of feeding deterrent compounds, either organic acids or other primary and/or secondary matabolites that we did not characterize, is also reflected by the greater number of pathways and stylets-withdrawal events in FS-17, and of xylem egestion (Xe) events in Leccino. The Xe-waveform has been recently described as the pattern leading to *X. fastidiosa* inoculation into the host plant (Cornara et al. 2020, 2022). The behavior is possibly associated with the discharging of the content of the precibarium and the cibarium out of the food canal to free the precibarial sensilla from obstructions (Cornara et al. 2023). Toxicants and feeding deterrents might also trigger this behavior, as hypothesized by Bextine et al. (2004) observing greater *X. fastidiosa* transmission rate in plants treated with systemic insecticides.

Our data are consistent with previous research on insects probing behavior on pathogen-susceptible versus pathogen-resistant genotypes. Kleina et al. (2020) observed reduced settling and feeding by the xylem-sap feeder *Bucephalogonia xanthophis* (Hemiptera: Cicadellidae) and *Sibovia segata* (Hemiptera: Cicadellidae) on plum genotypes resistant to *X. fastidiosa*. Similarly, Ripamonti et al. (2022) reported feeding (sap ingestion) by the phloem-feeder *Scaphoideus titanus* (Hemiptera: Cicadellidae) as the only significantly divergent probing behavioral pattern between the grapevine variety Barbera (susceptible to the Flavescence Doree phytoplasma), and the varieties Moscato and Brachetto (tolerant to the phytoplasma).

The xylem anatomy in olive plants the spittlebugs were given access to seems not relevant in determining insect acceptance of the host and settling preference, at least for the three olive varieties selected in the present study. Microscopy studies conducted so far point toward xylem vessels narrowness, i.e., the higher proportion of relatively narrow vessels, as the underlying cause of the resistance to *X. fastidiosa* in the Leccino variety. FS-17 resistance seems instead not associated with xylem vessel diameter, given diameter values are similar to those measured in susceptible varieties

(Sabella et al. 2019; Walker et al. 2023). However, previous analysis of the xylem anatomy of olive plants with a different degree of susceptibility to the fastidious bacterium were conducted without focusing on the plant tissues spittlebugs prefer to alight on, as the apical part of the stem, leaf petioles and midribs. Considering instead the insect vector-plant interaction and specifically the plant tissues where the insect vectors prefer to feed, our data indicate resistance to spittlebugs does not depend on xylem vessels depth, aggregation, and diameter in the varieties tested. Indeed, the greater depth and lower aggregation of xylem vessels in FS-17 compared to Ogliarola has no or marginal incidence on the probing activity and the chances to find a xylem vessel. Similarly, differences in xylem vessels diameter seem not to correlate with the duration of xylem ingestion bouts, with Leccino and Ogliarola exhibiting similar diameter values but different sap ingestion duration.

The present study represents a first step toward disentangling spittlebug-host plant interactions in light of incorporating plant resistance to spittlebugs in the research on *X. fastidiosa* resistant genotypes. We observed significant differences in *P. spumarius* host finding and probing behaviors between an olive variety susceptible to *X. fastidiosa*, Ogliarola Salentina, and the two bacterium-resistant genotypes identified so far, namely Leccino and FS-17. When given a choice, spittlebugs tend to prefer Ogliarola over the two *X. fastidiosa*-resistant varieties. Additionally, Leccino and FS-17 are less suitable hosts for the spittlebug compared to Ogliarola, possibly because of differences in xylem sap metabolome among the varieties tested.

Vector preference for bacterium-susceptible over bacterium-resistant varieties could be a relevant and so far overlooked component of the bacterium epidemiology in Southern Italian olive orchards. Overall, our data point toward the importance of incorporating studies on vector-plant interaction and host traits of resistance to the vector in research on genotypes resistant to *X. fastidiosa*.

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Author contributions Conceptualization: DC; RA, AF. Data curation: DC, VZC, ZH, AT, ADS. Formal analysis: DC, VZC, ZH. Funding acquisition: DC; RA, AF. Investigation: DC, VZC, ZH, SA, CL, AT, ADS. Methodology: DC, AF. Project administration: DC, RA, AF. Supervision: DC, AF. Writing original draft: DC. Writing, review & editing: All the co-authors.

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Data availability Raw data are reported in Online resources 1, 2 and 3. EPG recordings are available on Zenodo (the Zenodo link will be added upon paper acceptance).

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no competing interest.

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