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Elucidating Overlooked Aspects of the Tripartite Interaction *Philaenus spumarius*-*Xylella fastidiosa*-Host Plant for a Sustainable Bacterium Control

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To my mom and dad, the quiet strength behind every step I take

To my husband, the light that made the path brighter

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Summary

The introduction and spread of *Xylella fastidiosa*, a xylem-limited bacterium native to the Americas, in Southern Italian olive groves has caused economic, ecological and social turmoil. The meadow spittlebug, *Philaenus spumarius*, is the main European vector of *X. fastidiosa* subsp. *pauca* (ST53) to olive. Conventional control strategies, *i.e.,* quarantines, uprooting, ground-cover management, and pesticides application, have so far failed to contain the bacterium spread and may pose serious risks to agroecosystems and human health. Sustainable and effective alternatives are therefore imperative to interfere with vector-host plant interactions, and limit successive transmission. However, developing such strategies requires a detailed understanding of which are the pivotal cues underlying host location and acceptance. This PhD thesis focused on the drivers of host plant acceptance by spittlebugs, with the goal of elucidating what are the plant traits underlying host acceptance and permanence by the insect.

The dissertation opens with a conceptual synthesis of behavioral manipulation as a multimodal approach aimed at interfering with insect-plant interaction through the manipulation of the cues underlying insect's interaction with the surroundings, including the plant. The review highlights the knowledge gaps on how spittlebugs locate and accept host plants, noting that host location is primarily driven by visual cues supported by olfaction, whereas host acceptance depends on the plant's structural and, crucially, chemical traits. These gaps underscore the need to identify what are the plant traits driving these behaviors (Chapter **I**). Therefore, the following research investigates what influences host plant acceptance by *P. spumarius*, *i.e.,* a pivotal step for the transmission and spread of *X. fastidiosa,* firstly through assessing its varying preference among olive varieties with different susceptibility to the bacterium. Using choice tests, survival analysis, Electropenetrography (EPG), microscopy, and xylem primary-metabolite profiling, the study compares the susceptible variety Ogliarola Salentina to the tolerant genotypes, Leccino and FS-17. Spittlebugs showed a significant preference for Ogliarola, exhibiting longer xylem ingestion periods. These behavioral differences were most plausibly explained by differences in xylem sap chemistry rather than vascular anatomy, indicating that chemical cues play a dominant role in spittlebugs host acceptance (Chapter **II**).

Building on these findings, the chemical determinants of host acceptability were investigated by linking spittlebug probing behavior with the xylem sap metabolite composition, including both primary and secondary metabolites, of olive varieties differing in susceptibility to *X. fastidiosa*. The analysis revealed that host acceptance is not driven by single compounds but

rather by the overall metabolic profile of the xylem sap. In particular, varieties characterized by higher concentrations of organic acids were the least suitable for sustained feeding, suggesting that these compounds may act collectively as deterrents. These results represent a crucial step for *X. fastidiosa* management spread through the selection of germplasm resistant to the vector or via agronomic practices that manipulate xylem chemistry to reduce plant suitability (Chapter **III**). Previous comparisons between the least and most preferred olive varieties by *P. spumarius* (*i.e.,* Leccino and Ogliarola) suggested that resistance traits to spittlebugs may exist within the olive germplasm. To further explore this evidence, host plant suitability to spittlebugs was evaluated across fourteen olive varieties widespread in Apulia region, Southern Italy. The results indicate that some genotypes, such as Leccino, Pendolino and Arbequina, possess characteristics that reduce vector acceptance and permanence on the host, largely due to an unsuitable xylem chemical profile. These traits could play a significant role in limiting *X. fastidiosa* transmission through reduced vector activity (Chapter **IV**).

The final part of the work provides further evidence that xylem sap chemistry is a key determinant of host plant acceptance by spittlebugs. Combining spittlebug probing behavior analysis with untargeted metabolomics under three water regimes, revealed that water status modulates spittlebug-plant interaction and plant acceptance in a variety-dependent manner: while in the drought-resistant Ogliarola the water regime had no effect on plant-insect interaction, in the drought-sensitive Leccino spittlebugs exhibited a significant preference for severely stressed plants. The xylem sap chemical profile of the latter, characterized by a lower expression of organic acids, resulted more suitable for spittlebugs as evidenced by longer feeding phases. These results strengthen the hypothesis that variations in xylem sap composition, rather than water status alone, drive host suitability to the vectors. Moreover, they suggest that managing agronomic inputs, such as irrigation, could offer a means to modulate vector-plant interactions and potentially reduce transmission risk (Chapter **V**).

Overall, this dissertation demonstrates that the xylem sap chemical profile is a pivotal factor underlying host plant acceptance by spittlebugs. Variations in xylem composition influence vector probing and feeding behavior, determining plant suitability and, consequently, the potential for *X. fastidiosa* transmission. A less favorable chemical profile would reduce vector activity and permanence on the host plant, ultimately limiting opportunities for bacterium acquisition and inoculation. These findings provide a mechanistic understanding of vector-plant interactions and highlight the potential of selecting or managing olive varieties with unfavorable xylem profiles to hinder disease epidemiology.

Sintesi

L'introduzione e la diffusione di *Xylella fastidiosa*, un batterio limitato allo xilema e originario delle Americhe, negli oliveti dell'Italia meridionale ha provocato gravi conseguenze economiche, ecologiche e sociali. Il principale vettore europeo di *X. fastidiosa* subsp. *pauca* (ST53) nell'olivo è la sputacchina dei prati, *Philaenus spumarius*. Le strategie di contenimento convenzionali — quarantene, eradicazioni, gestione della copertura erbosa e applicazioni di pesticidi — non sono finora riuscite a limitare la diffusione del batterio e possono comportare rischi significativi per gli agroecosistemi e la salute umana. Diventa quindi imprescindibile lo sviluppo di alternative sostenibili ed efficaci che interferiscano con le interazioni tra vettore e pianta ospite, riducendo così le trasmissioni successive. Tuttavia, la messa a punto di tali strategie richiede una comprensione approfondita dei segnali chiave che determinano la localizzazione e l'accettazione dell'ospite.

Questa tesi di dottorato si concentra sui fattori che influenzano l'accettazione della pianta ospite da parte delle sputacchine, con l'obiettivo di chiarire quali tratti vegetali determinino l'accettazione e la permanenza dell'insetto sulla pianta.

La dissertazione si apre con una sintesi concettuale sulla manipolazione comportamentale come approccio multimodale volto a interferire con le interazioni insetto-pianta attraverso la modulazione dei segnali che guidano l'interazione dell'insetto con l'ambiente circostante, pianta inclusa. La rassegna evidenzia le lacune conoscitive riguardo ai meccanismi con cui le sputacchine localizzano e accettano le piante ospiti, sottolineando che la localizzazione è principalmente guidata da segnali visivi supportati dall'olfatto, mentre l'accettazione dipende dalle caratteristiche strutturali e, soprattutto, chimiche della pianta. Tali lacune confermano la necessità di identificare i tratti vegetali che determinano questi comportamenti (Capitolo I).

La ricerca presentata successivamente indaga quindi i fattori che influenzano l'accettazione dell'ospite da parte di *P. spumarius* — un passaggio cruciale per la trasmissione e diffusione di *X. fastidiosa* — valutando innanzitutto le preferenze dell'insetto tra varietà di olivo con diversa suscettibilità al batterio. Attraverso test di scelta, analisi di sopravvivenza, Elettropenetrografia (EPG), microscopia e profilazione dei metaboliti primari dello xilema, lo studio confronta la varietà suscettibile Ogliarola Salentina con i genotipi tolleranti Leccino e FS-17. Le sputacchine hanno mostrato una preferenza significativa per Ogliarola, con periodi di ingestione xilematica più lunghi. Queste differenze comportamentali sono risultate

verosimilmente associate a differenze nella chimica della linfa xilematica piuttosto che nell'anatomia vascolare, indicando un ruolo dominante dei segnali chimici nell'accettazione dell'ospite da parte delle sputacchine (Capitolo II).

Partendo da tali risultati, sono stati approfonditi i determinanti chimici dell'accettabilità dell'ospite collegando il comportamento di sondaggio delle sputacchine con la composizione metabolica della linfa xilematica, includendo metaboliti primari e secondari, in varietà di olivo con diversa suscettibilità a *X. fastidiosa*. L'analisi ha evidenziato che l'accettazione dell'ospite non è mediata da singoli composti, bensì dall'intero profilo metabolico dello xilema. In particolare, le varietà con concentrazioni più elevate di acidi organici sono risultate meno idonee al nutrimento prolungato, suggerendo un ruolo deterrente di tali composti. Questi risultati rappresentano un passo cruciale nella gestione di *X. fastidiosa* attraverso la selezione di germoplasma resistente al vettore o mediante pratiche agronomiche in grado di modulare la chimica dello xilema per ridurre l'idoneità della pianta (Capitolo III).

Confronti precedenti tra le varietà di olivo meno e più gradite a *P. spumarius* (Leccino e Ogliarola, rispettivamente) indicavano la possibile presenza di tratti di resistenza alle sputacchine all'interno del germoplasma olivicolo. Per approfondire questa evidenza, è stata valutata l'idoneità di quattordici varietà diffuse in Puglia nei confronti delle sputacchine. I risultati indicano che alcuni genotipi, come Leccino, Pendolino e Arbequina, possiedono caratteristiche che riducono l'accettazione e la permanenza del vettore sull'ospite, principalmente a causa di un profilo chimico xilematico sfavorevole. Questi tratti potrebbero avere un ruolo rilevante nel limitare la trasmissione di *X. fastidiosa* attraverso la riduzione dell'attività del vettore (Capitolo IV).

L'ultima parte del lavoro fornisce ulteriori evidenze che la chimica dello xilema è un fattore chiave nell'accettazione dell'ospite da parte delle sputacchine. Combinando l'analisi del comportamento di sondaggio con la metabolomica untargeted sotto tre regimi idrici, è emerso che lo stato idrico modula l'interazione pianta-insetto in maniera dipendente dalla varietà. Mentre nella varietà tollerante alla siccità Ogliarola lo stress idrico non ha influenzato l'interazione, nella varietà sensibile Leccino le sputacchine hanno mostrato una preferenza per le piante gravemente stressate. Il profilo chimico dello xilema in queste ultime, caratterizzato da minori concentrazioni di acidi organici, è risultato più favorevole all'alimentazione, come evidenziato da fasi di ingestione più prolungate. Questi risultati rafforzano l'ipotesi che le variazioni nella composizione dello xilema, più che lo stato idrico in sé, determinino l'idoneità

dell'ospite per il vettore. Inoltre, suggeriscono che la gestione degli input agronomici, come l'irrigazione, possa offrire uno strumento per modulare le interazioni vettore-pianta e ridurre il rischio di trasmissione (Capitolo V).

Nel complesso, questa tesi dimostra che il profilo chimico della linfa xilematica rappresenta un fattore determinante per l'accettazione della pianta ospite da parte delle sputacchine. Le variazioni nella composizione xilematica influenzano il comportamento di sondaggio e alimentazione del vettore, determinando l'idoneità dell'ospite e, di conseguenza, il potenziale di trasmissione di *X. fastidiosa*. Un profilo chimico meno favorevole riduce l'attività e la permanenza del vettore sulla pianta, limitando le possibilità di acquisizione e inoculazione del batterio. Questi risultati forniscono una comprensione meccanicistica delle interazioni vettore-pianta e mettono in evidenza il potenziale della selezione o gestione di varietà di olivo con profili xilematici sfavorevoli al vettore come strategia per ostacolare l'epidemiologia della malattia.

INTRODUCTION

1. Introduction

1.1. The meadow spittlebug: *Philaenus spumarius*

Commonly known for its unique color polymorphism and the spittle produced by its juvenile stages, the meadow spittlebug *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) has gained attention in the last decade because of its role in *Xylella fastidiosa* subsp. *pauca* (ST53) epidemiology in southern Italian olive orchards (Cornara *et al.,* 2017). This ubiquitous, common and very abundant insect is a highly polyphagous xylem feeder and has one of the widest host plant ranges among herbivore insects, encompassing grasses, forbs, shrubs and trees (EFSA, 2023). *Philaenus spumarius* is a univoltine specie, *i.e.,* has an annual life cycle: eggs overwinter on straw and debris, development occurs through five nymphal instars and takes from 35 to 100 days, depending on weather and geographical location (Weaver & King, 1954; Cornara *et al.,* 2018). Nymphs start to emerge in spring and feed mainly on dicotyledons, producing foamy masses that protect them from solar radiation (Chen *et al.,* 2018). Adults start to emerge in late April–May (southern Italian conditions) and tend to migrate from herbaceous ground cover to perennial hosts such as olive trees and other woody plants in late spring/early summer. Adult spittlebugs move back to herbaceous cover in late summer/early autumn, where females lay eggs that will hatch the following spring (Cornara *et al.,* 2018; Morente *et al.,* 2018; Bodino *et al.,* 2019).

Both juveniles and adults feed on xylem fluid, overcoming the relatively high fluid tensions (Horsfield, 1977; Malone *et al.,* 1999). Beside being highly dilute and nutritionally poor, xylem sap is maintained under negative pressure, which varies with plant water status and environmental conditions (Redak *et al.,* 2004). To overcome xylem tension and successfully extract the sap, spittlebugs, as well as other xylem feeders, rely on a large muscular cibarial pump supported by a specialized filter chamber in their gut (Cheung & Marshall, 1973; Backus, 1988; Malone *et al.,* 1999). Research studies demonstrated that ingestion is significantly impaired when xylem water potentials fall below approximately -1.5 to -2.0 MPa (Andersen *et al.,* 1989, 1992; Malone *et al.,* 1999; Bergman *et al.,* 2021). Additionally, it was previously hypothesized that nitrogen-fixing legumes, together with other plants rich in xylem amino acids, served as preferred hosts, particularly during the nymphal stages (Horsfield, 1977; Thompson, 1994, 2004).

1.2. *Xylella fastidiosa*: A devastating xylem-limited pathogen

Although direct damage by adult spittlebugs is generally considered limited, yield losses have been reported in some crops such as strawberries and alfalfa (Weaver & King, 1954). Nevertheless, their role as vectors of the bacterium *X. fastidiosa* makes them a serious threat to agriculture and landscape (Cornara *et al.,* 2018). *Xylella fastidiosa* is a gram-negative xylem-limited bacterium, colonizer of both plants and insects and can infect more than 650 plants species (Hopkins, 1989; Newman *et al.,* 2003; Chatterjee *et al.,* 2008). It is the causal agent of several devastating plant diseases across the world, *e.g.,* Pierce's Disease on grapevines, Citrus Variegated Chlorosis, and Leaf Scorch Disease on Almonds (Hill & Purcell, 1995; Purcell, 1997; Hopkins & Purcell, 2002; Almeida, 2016; Sicard *et al.,* 2018). *Xylella fastidiosa* is native to the Americas, but global trade of plant materials has led to the current worldwide expansion of the bacterium (Almeida & Nunney, 2015; Landa *et al.,* 2020). In Europe, the first detected outbreak of *X. fastidiosa* was reported in 2013 on olive trees in Apulia region, Southern Italy. The strain ST53, belonging to the subspecies *pauca*, was demonstrated to be the causal agent of the Olive Quick Decline Syndrome (Saponari *et al.,* 2017), leading to a massive dieback of susceptible olive trees.

Xylella fastidiosa is transmitted in a peculiar mode that could be defined as propagative persistent but non-circulative, involving xylem-sap-feeding insect vectors, such as sharpshooters (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Aphrophoridae). In American agroecosystems, xylem-sap feeder communities are dominated by sharpshooters, whereas in Europe, spittlebugs are the most prevalent (Cornara *et al.,* 2018; Sicard *et al.,* 2018; Esteves *et al.,* 2019). Spittlebugs are highly adaptable, polyphagous, and abundant insects capable of inhabiting a broad range of terrestrial environments (Bodino *et al.,* 2021). These characteristics make them effective vectors of *X. fastidiosa* (Cornara *et al.,* 2017).

The transmission process of *X. fastidiosa* is intimately linked to the feeding behavior of these vectors and their interactions with host plants. During feeding on an infected plant, bacterial cells are ingested and retained in the insect's foregut. When the insect subsequently feeds on a healthy plant, the bacterium can be inoculated into a new host after stylets penetration and contact with xylem vessels (Cornara *et al.,* 2017).

The probability of successful *X. fastidiosa* transmission can be expressed as the product of three components: vector infectivity, transmission efficiency, and vector activity, defined by the abundance of vectors on host plants and the duration they spend on it (Purcell, 1981;

Daugherty & Almeida, 2009). In olives systems, spittlebugs exhibit relatively low transmission efficiency (6-11%) compared with the high efficiency reported for sharpshooter transmission to grapevines (up to 92%) (Hill & Purcell, 1995; Cornara *et al.,* 2017; Bodino *et al.,* 2023). However, this limitation is compensated by their high population densities and prolonged residence in olive canopies throughout the season, which increase the number of vector-plant contacts over time. Consequently, vector activity, reflecting both abundance and the time spent on individual hosts, emerges as a critical determinant of *X. fastidiosa* spread in olive landscapes. Therefore, better results in limiting pathogen spread could be achieved by understanding these dynamics and developing strategies that reduce vector-plant interaction and interfere with host plant location and acceptance, thus curbing vector activity.

1.3. Management of *Xylella fastidiosa* and its vectors

In the absence of a cure for *X. fastidiosa*, management in European olive orchards focuses on removing sources of infection, monitoring propagative material, and controlling vector populations. The latter consists of intensive tillage to reduce juvenile spittlebug populations, and the application of insecticides targeting adults (Lago *et al.,* 2022). However, these measures are often ineffective and unsustainable: repeated tillage can degrade soil fertility, accelerate erosion, and reduce habitats for beneficial arthropods (Kairis *et al.,* 2013; Ali *et al.,* 2021), while insecticide use poses risks to biodiversity, ecosystem services, and human health (Biondi *et al.,* 2012; Chagnon *et al.,* 2015; Kim *et al.,* 2017; Labadessa *et al.,* 2024). Overall, these interventions have yielded inconsistent results in limiting the spread of the bacterium (Bragard *et al.,* 2013; Daugherty *et al.,* 2015; Lago *et al.,* 2022). Therefore, the containment of *X. fastidiosa* requires innovative management strategies that are both environmentally and economically sustainable. In particular, control approaches that disrupt the interactions between vectors and host plants and aim to reduce vector-host contacts, hold considerable promise. Such strategies target the transmission cycle, interfering with host location, acceptance, and feeding behaviors.

Host location in spittlebugs has been recently demonstrated to be mainly mediated by visual cues, as these insects possess potential visual capacities and sensitivity to polarized light, with olfactory stimuli likely playing a complementary role (Lazar *et al.,* 2025). Once a potential host is located, spittlebugs rely on contact chemosensory cues to assess plant suitability (Backus & McLean, 1985; Backus, 1988). Host acceptance occurs when the sensory stimuli

perceived during probing exceeds a behavioral threshold that triggers stylet insertion and sustains xylem ingestion. Acceptance is, indeed, influenced by the balance between feeding stimulants and deterrents, the insect's physiological condition (*e.g.,* nutritional state), and previous feeding experiences (learning), all of which shape the decision whether a plant becomes a feeding and, consequently, a transmission host (Dethier, 1982; Chapman, 2003; Markheiser *et al.,* 2020; Tinelli *et al.,* 2025).

Thus, a plant resistant or less susceptible to the vector, *i.e.,* displaying anatomical and/or chemical traits reducing palatability for the insect, could theoretically be a poor source/recipient for the pathogen, given the expected low vector activity. Specifically, shortening the insect-plant interaction, by deterring or delaying probing and feeding activities fundamental for pathogen transmission, could in turn limit the ability of the vector to acquire pathogens from infected plants (source) and subsequently inoculate them to recipient ones (Daugherty & Almeida, 2009; Le Roux *et al.,* 2010). Therefore, determining the cues underlying spittlebugs behavior, *i.e.,* host location and acceptance, is of paramount importance for the development of effective and sustainable disease management strategies (Fig. 1) (Chatterjee *et al.,* 2008; Retchless *et al.,* 2014; Sicard *et al.,* 2018).

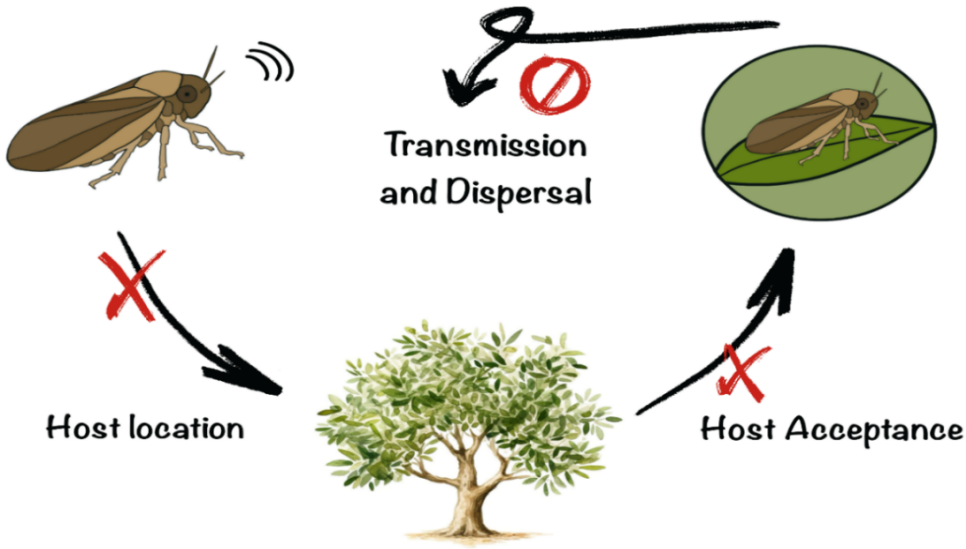


Figure 1. Conceptual diagram illustrating how disrupting host location and acceptance behaviors in vectors may limit pathogen transmission and dispersal (Source: original).

1.4. Spittlebugs' host plant acceptance

Upon landing, the structural and chemical characteristics of the plants serve as important cues in the process of recognizing suitable host. Although the underlying mechanisms remain poorly

understood in spittlebugs, both xylem anatomy and sap composition are likely to influence how vectors identify, settle on, and feed from potential hosts. Structural traits, including vessel diameter, depth and aggregation, may affect the insect's ability to insert the stylets and the rapid finding of a xylem vessel, along with the rapid onset of a continuous xylem ingestion, while the chemical profile of the sap, such as its nutrient content and specialized metabolites, can shape feeding behavior and host preference.

- **Plant anatomy:**

Plant anatomy could represent the first line of defense against herbivores settling. Before reaching the vascular system, insects must first overcome external plant barriers that can influence host selection and acceptance. The physical characteristics of the plant surface, such as the presence of trichomes and epidermal texture, can affect both settling and feeding. Indeed, glandular trichomes have been shown to act as mechanical barriers that hinder feeding and mobility of *P. spumarius* nymphs (Hofman & McEvoy, 1985).

Beyond these external defenses, internal anatomical traits, particularly those related to the xylem, play a crucial role in the subsequent stages of the plant-vector-bacterium interaction. The structure of the xylem vessels not only determines the accessibility and suitability of the feeding site for xylem feeders but also shapes the colonization and movement of *X. fastidiosa*. The structural characteristics of xylem vessels, particularly vessel diameter, length, density, and pit membrane porosity, has been demonstrated to be associated with differential host susceptibility and disease severity. For instance, narrower vessels and higher vessel density in the bacterium tolerant variety, Leccino, were potentially associated with the reduced bacterial colonization and slower systemic spread, leading to milder symptoms and improved hydraulic resilience (Petit *et al.,* 2021; Walker *et al.,* 2023; Sabella *et al.,* 2024). This anatomical resistance is thought to constrain bacterial movement, limit biofilm expansion, and enhance host compartmentalization through tyloses and gel occlusions (Rapicavoli *et al.,* 2017; Schneider *et al.,* 2020). In contrast, susceptible cultivars, *i.e.,* Ogliarola and Cellina di Nardò, were characterized by wider xylem vessels that facilitate longitudinal spread of *X. fastidiosa* and accelerate vessel dysfunction via embolism and biofilm accumulation (Brodersen & McElrone, 2013; Sabella *et al.,* 2019, 2024).

While most of the studies have focused primarily on plant-pathogen interactions, an important aspect has been largely overlooked: the concurrent influence of xylem vessels structure on

vector behavior and transmission dynamics. The efficiency with which xylem feeders acquire and transmit *X. fastidiosa* depends on their ability to access and sustain feeding from host plants. Therefore, xylem anatomy not only mediates pathogen movement within the vascular system but may also influence host settlement and acceptance by the vector. This dual relevance underscores the need to extend anatomical investigations beyond pathogen resistance, integrating plant structural traits into a broader tripartite framework encompassing host, pathogen, and vector interactions.

- **Plant chemistry:**

Insects depend on multiple sensory modalities, such as vision, olfaction, and taste, to detect, evaluate, and select suitable food sources. Among these, taste plays a crucial role once the insect has landed on a potential host, determining whether this plant will be accepted or rejected as "food source" (Jaenike, 1990; Chapman, 2003). Plants chemical profile is, therefore, a critical factor shaping host acceptance. Once the insect has access to the plant, it initiates probing looking for gustatory cues driving ingestion and final acceptance. These chemical cues play a central role in determining vector behavior and feeding preference (Andersen *et al.,* 1989, 1992; Brodbeck *et al.,* 1990; Rogers & Newland, 2003; Andersen *et al.,* 2014; Ripamonti *et al.,* 2022). When the chemical composition of xylem sap matches the nutritional requirements of the insect, it stimulates feeding initiation and supports sustained ingestion, leading vectors to remain longer on the host and feed for extended periods. This prolonged feeding and increased activity substantially increases the likelihood of both pathogen acquisition and inoculation. Conversely, unfavorable chemical profiles may discourage feeding and reduce vector permanence on the plant, decreasing the probability of successful transmission events (Purcell, 1981; Daugherty & Almeida, 2009; Le Roux *et al.,* 2010; Mitchell *et al.,* 2016). Xylem-feeding insects assess the quality of xylem sap through specialized precibarial gustatory sensilla, enabling insects to avoid unpalatable food sources or toxic substances and allowing it to modulate the initiation and duration of feeding based on its nutritional needs (Backus & McLean, 1985; Backus, 1988; Rogers & Newland, 2003). Chemical stimuli can thus be classified as either phagostimulants, *i.e.,* favorable compounds that promote feeding, or phagodeterrents/antifeedants, which inhibit or reduce feeding. The relative balance between these opposing chemical signals determines host acceptance and suitability for xylem-feeding insects (Dethier, 1980; Chapman, 2003). Beyond chemical cues, xylem feeders must also overcome the negative pressure of xylem sap under which xylem sap is maintained, a condition that varies with plant water status and environmental factors (Redak

et al., 2004). Spittlebugs, as well as other xylem feeders, rely on a powerful muscular cibarial pump to generate the suction forces required to extract sap from xylem vessels under tension (Cheung & Marshall, 1973; Backus, 1988; Malone *et al.,* 1999). However, its efficiency is often determined by xylem water potential levels, with thresholds ranging approximately between -1.5 and -2.0 MPa (Andersen *et al.,* 1989, 1992; Malone *et al.,* 1999; Bergman *et al.,* 2021).

Several research studies have investigated the role of plant chemical traits in determining the feeding preference and host acceptance in xylem-feeding insects, such as sharpshooters and spittlebugs. Early studies proposed that nitrogen availability, particularly in the form of amides such as glutamine and asparagine, acts as a key phagostimulant, stimulating ingestion and promoting sustained feeding (Horsfield, 1977; Andersen *et al.,* 1992, 2005; Brodbeck *et al.,* 1990, 1999; Thompson, 1994, 2004). Specifically, Brodbeck *et al.* (1990) demonstrated that preferred host plants by the glassy-winged sharpshooter *Homalodisca vitripennis* Say (Hemiptera: Cicadellidae), typically contain higher concentrations of amino acids (glutamine and asparagine), which influence host selection and acceptance, triggering the onset of continuous xylem ingestion. Moreover, work by Andersen *et al.* (2005) confirmed the central role of glutamine in host acceptance, reporting a strong positive correlation between *H. vitripennis* abundance and glutamine concentrations in host plants. In contrast, these authors observed a negative correlation between insect abundance and the amino acid proline, suggesting an inhibitory or deterrent effect on feeding. Organic acids, although present in variable concentrations across hosts, showed weaker correlations with leafhopper abundance compared with amino acid profiles (Andersen *et al.,* 2005). Conversely, plants with lower amino acid or higher inorganic ion concentrations tend to be less suitable, leading to reduced feeding or frequent host-switching behavior (Andersen *et al.,* 1989; Brodbeck *et al.,* 1990, 1999).

Despite the extensive evidence linking amino acids to host preference in xylem feeders, knowledge of how other chemical traits shape host acceptance remains comparatively limited. Research on organic acids, secondary metabolites, and other xylem compounds has lagged far behind the detailed work conducted on amino acids. While the chemical drivers of sharpshooters' host acceptance have been extensively investigated, the chemical cues underlying host suitability for the meadow spittlebug remain poorly characterized. A deep understanding of the sensory perception and chemical stimuli mediating vector feeding behavior and activity would pave the way for managing *X. fastidiosa* vectors through the

selection/breeding of germplasms resistant to the vector and thereby less prone to serve as source/recipient of the bacterium. Additionally, as the chemical profile of xylem sap varies significantly with environmental conditions, fertilization, abiotic stresses and season of the year (Tromp, 1979; Ferguson *et al.,* 1983; Andersen *et al.,* 1989, 1992, 1995; Brodbeck *et al.,* 1990, 1999; Redak *et al.,* 2004), there is also potential for managing xylem feeders by manipulating exogenous factors (*e.g*., water and fertilizers), and rendering host plants unacceptable food sources.

1.5. Objectives of the thesis

The overall objective of this dissertation was to characterize insect-plant interactions that determine host plant acceptance by *P. spumarius* on olive, with a particular focus on the plant traits that underline host preference and permanence by the insect. The work begins with a synthesis of current knowledge and key knowledge gaps regarding: i) the mechanism by which spittlebugs locate and accept their host plants; and ii) the potential use of behavioral manipulation strategies to interfere with and disrupt vector-host plant interactions (**Chapter I**). To address these gaps, host plant acceptance by *P. spumarius* was investigated by assessing its preference among olive varieties with different susceptibility to *X. fastidiosa,* focusing on the structural and chemical characteristics of the plants (**Chapter II**). This was followed by an in-depth investigation of the role of xylem sap chemistry in mediating spittlebug-olive interaction, identifying metabolites affecting olive plant's suitability for *P. spumarius*, *i.e.,* the compounds mediating plant acceptance and settling (**Chapter III**). Consequently, host suitability was evaluated across a broader set of olive varieties widespread in Apulia Region, by developing a plant suitability index integrating multiple EPG variables reflecting the likelihood of each olive genotype to serve as favorable host for spittlebugs (**Chapter IV**). Finally, the influence of water status on vector-plant interactions was examined in olive varieties differing in drought response and spittlebug suitability, along with associated shifts in xylem sap chemistry that modulate host plant acceptability (**Chapter V**).

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CHAPTER I

Intruding into a conversation: how behavioral manipulation could support management of *Xylella fastidiosa* and its insect vectors

2. Intruding into a conversation: how behavioral manipulation could support management of *Xylella fastidiosa* and its insect vectors.

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REVIEW

Intruding into a conversation: how behavioral manipulation could support management of *Xylella fastidiosa* and its insect vectors

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Abstract
Behavioral manipulation (BM) is a multimodal control approach based on the interference with the stimuli mediating insect perception and interaction with the surroundings. BM could represent a win–win strategy for the management of vector-borne plant pathogens as the bacterium *Xylella fastidiosa*, since it could reduce the number of vectors alighting on host plants and, consequently, the chances for transmission to occur. In this review, we summarized current knowledge and highlighted gaps in information on (i) how insect vectors of *X. fastidiosa* in general, and more specifically the meadow spittlebug *Philaenus spumarius*, locate and accept the host plant; and (ii) how behavioral manipulation techniques could be applied to disrupt the vector–host plant interaction. Finally, we discussed how diverse BM strategies could be combined with other integrated pest management tools to protect olive groves from inoculation with the fastidious bacterium.

Keywords Olive · Spittlebugs · Sharpshooters · Transmission · Vector-borne plant pathogens

Introduction

Native to the Americas, the gram-negative, xylem-limited bacterium *Xylella fastidiosa* is a colonizer of both plants and insects and can infect more than 650 plant species (Almeida 2016; Sicard et al. 2018; Morelli et al. 2021). *Xylella fastidiosa* is characterized by various phylogenetic clades, each with a limited host range, and only particular bacterial genotypes cause specific plant diseases. The current main grouping comprises the subspecies *fastidiosa, multiplex,* and *pauca* (Morelli et al. 2021). The bacterium is transmitted only by xylem-sap feeding insects, such as sharpshooters (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Aphrophoridae). The inoculation of

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bacterial cells may occur few minutes upon stylets insertion into the host plant tissues and contact with a xylem vessel (Cornara et al. 2020, 2022).

Invasions of new areas and continents largely depend on trade of plant material, and diverse introductions recently resulted in the establishment of *X. fastidiosa* in Europe (Martelli 2016; Strona et al. 2017). The region Apulia, in south-eastern Italy, was the first area of the Old World where a *X. fastidiosa* outbreak was reported; the strain ST53, belonging to the subspecies *pauca*, likely introduced around 2008, was demonstrated to be the causal agent of the olive quick decline syndrome (Saponari et al. 2017; Vanhove et al. 2019). The Italian outbreak could be thought as a "perfect storm" in which the combination of several factors resulted in an unprecedented spread of the fastidious bacterium and the loss of millions of olive trees, and, as a consequence, in economic and social turmoil. Firstly, *X. fastidiosa* was accidentally introduced into an environment (Apulia) where a native, competent, and polyphagous vector, the meadow spittlebug *Philaenus spumarius* (L.) (Hemiptera Aphrophoridae), is very abundant (Cornara et al. 2018a). Secondly, olive trees, hosts of both the spittlebug and the strain ST53, cover the area, forming an almost continuous forest interspersed with Mediterranean maquis sheltering the vectors during hot and dry summers (Cornara et al. 2018a; Bodino et al. 2019). Thirdly, pest management efforts were belated because symptoms of the disease can show one-to-two years after infection and due to the difficulties in uprooting infected trees, which represent a cultural heritage for the local residents (Almeida et al. 2019; Saponari et al. 2019). Currently, the main control strategies against *P. spumarius* in olive orchards consist in tilling, which is aimed at reducing the herbaceous cover hosting the spittlebug juveniles, and in mandatory pesticide applications (Saponari et al. 2017, 2019; Lago et al. 2021, 2022). Notwithstanding these efforts, *X. fastidiosa* infections continue to expand, with a yearly rate of movement of the invasion front of about 10 km, leaving devastation in its wake (Saponari et al. 2019). As a fact, the bacterium is causing immeasurable environmental losses and is rapidly accelerating the desertification of the region, due to tree removal and drawbacks of vector control measures, which pose a risk to biodiversity (Sánchez-Bayo and Wyckhuys 2019; Ali et al. 2021). New strategies that do not threaten the environment and that should be compatible with other integrated pest management techniques are urgently needed for the long-term management of the bacterium and its vector.

As mentioned above, *X. fastidiosa* can be theoretically transmitted by any xylem feeder, but the relevance of a vector in the bacterium epidemiology depends on its ecological attributes (e.g., vector density, distribution and dispersal), among which host selection plays a pivotal role. By understanding the stimuli underlying the behaviors leading to host plant location and acceptance by the insect vector, it would be possible to develop behavioral control strategies aimed at disrupting one or more aspects of these processes (Foster and Harris 1997; Mazzoni and Anfora 2021). Behavioral manipulation could represent a win–win strategy in the fight against *X. fastidiosa* because it could decrease both the vector-borne spread of the bacterium and reliance on pesticides (Mazzoni and Anfora 2021).

In this review, we summarize current knowledge and highlight information gaps on i) how insect vectors of *X. fastidiosa* in general, and more specifically the meadow spittlebug *Philaenus spumarius*, locate and accept the host plant; and ii) how behavioral manipulation techniques could be applied to disrupt the vector–host plant interaction.

Introducing behavioral manipulation

Behavioral manipulation (BM) methods for pest control are based on techniques aimed at interfering with crucial behaviors of target pests, in order to reduce their impact on crop production (Foster and Harris 1997; Cowles 2004; Gut et al. 2004; Mazzoni and Anfora 2021). BM may affect both intra- and interspecific communication; typical examples of it are semiochemical-based techniques such as mating disruption, which is largely adopted to control a wide range of crop pests (Benelli et al. 2019; Cardé 2021; Nieri et al. 2022). Usually, BM strategies do not eradicate the pest, but tend to reduce the density of its population, decreasing direct damages to crops and/or the transmission of vector-borne pathogens (Hooks and Fereres 2006). Even if BM does not offer a quick or definitive suppression of pests compared to insecticides, it provides long-term and area-wide control, often lasting throughout the lifecycle of the crop–pest interaction (Foster and Harris 1997; Cowles 2004). In addition, limitations of BM are counterbalanced by its sustainability and compatibility with other pest management strategies, such as biological control (Foster and Harris 1997; Cowles 2004; Pecenka et al. 2021).

Alteration of behaviors related to plant–insect interaction (i.e., host finding and feeding) may seem more advantageous than mating disruption, which reduces local populations but does not guarantee plant protection, especially considering potential immigration of pests from outside the crop (Foster and Harris 1997). In the case of vector-borne plant pathogens, BM might reduce the transmission rate by lowering the contacts between the pest and the target crop (Miller and Strickler 1984; Todd and Nault 1990; Cowles 2004; Almeida et al. 2005; Hooks and Fereres 2006; Hu, Mou and Tsai 2020).

In this context, to control a vector-borne bacterium as *X. fastidiosa,* the prerequisite for an efficient BM strategy is knowledge regarding the exogenous and endogenous factors

governing the vector's host plant selection, which can be divided into host finding (thus, orientation and landing) and host acceptance (e.g., probing, feeding and oviposition) (Schoonhoven 1968; Visser 1983, 1986). Intraspecific communication, particularly mating behavior, will be also treated within the present review, since it is crucial to better understand the insect behavioral ecology once landed on the host plant. In this perspective, studying how the pest selects its hosts and communicates can provide insights regarding how to interfere with its landing, settling and permanency on the plant (i.e., by reducing mate and host finding, by impairing feeding by means of vibrational stimuli and/or by genome editing (Mazzoni and Anfora 2021; Salvagnin et al. 2018)).

Host finding

The search for a new host is generally triggered by changes in environmental factors or by modifications of the insect's physiology such as food deprivation or egg maturation. On the other hand, landing is driven by the right stimulus or set of stimuli, which may include abiotic and biotic factors such as host plant-mediated and non-host cues (i.e., wind, rain, the presence of hetero- and conspecifics, etc.) as well as learning (Miller and Strickler 1984; Visser 1986; Bernays 1996; Finch and Collier 2000; Cowles 2004; Hodkinson 2009; Döring 2014). In the last decade, an impressive number of works regarding host finding and acceptance in phytophagous insects has been published. However, information regarding how these insects perceive and evaluate their environment is rather fragmented (Prokopy and Owens 1978, 1983; Todd and Nault 1990; Stenberg and Ericson 2007; Döring 2014). The majority of published works suggests that two main sensory modalities govern this process: vision and olfaction (Prokopy and Owens 1983; Miller and Strickler 1984; Dicke 2000; Beyaert and Hilker 2013; Ben-Yakir et al. 2020). All *X. fastidiosa* competent vectors presumably use semiophysicals (namely, visual and vibrational cues) and semiochemicals (odors) for host finding (Krugner et al. 2019; Nieri et al. 2022). In particular, visual cues are likely critical in host detection, while volatiles and xylem-sap metabolites, are key components of host acceptance.

Vision

Among herbivorous insects (especially auchenorrhynchans), vision is probably the sensory modality driving host localization at long distances. For instance, in the leafhopper *Empoasca vitis*, the suppression of opsin genes within the eyes was sufficient to hinder tropism toward hosts, especially at relatively long distances from the plant (Zhang et al. 2018). Phytophagous insects elaborate visual cues in function of i) their distance from the plant, and ii) their position in vicinity of the potential host (i.e., nearby or inside the canopy). Thereby, distant plants are presumably perceived as indistinct shapes against the horizon, while, as the insect approaches, the visual quality of the foliage is used to identify hosts and triggers landing. Once inside the crop or the canopy, visual cues such as shape, size, color, brightness and polarization, guide the insect toward the preferred plant and/or structure (Prokopy and Owens 1983). For instance, Moericke (1955) and Kennedy and colleagues (1961) described how insects, in this case aphids, respond to ultraviolet and human-visible light, which govern attack flights or trivial flights involving landing on green plants or long migratory flights, respectively.

Color is a key factor driving host finding and landing in herbivorous insects. Color is composed of different spectral characteristics: hue is the dominant wavelength, brightness the total amount of light reflected, and saturation the spectral purity of the reflected light. Insects may utilize any of these cues in different combinations, and many have true color vision, which is defined as intensity-independent discrimination of objects, based on their spectral properties (Van der Kooi et al. 2021). Yet, the way in which insects perceive colors differs fundamentally from humans (Chittka and Döring 2007). To the best of our knowledge, a systematic understanding of behavioral responses to colors in spittlebugs and related *taxa* is missing, together with data on spectral reflectance of traps used in behavioral experiments. Nonetheless, mechanisms discovered in other hemipteran insects such as aphids (Döring 2014; Döring and Kirchner 2022) may help to guide the interpretation of experiments with spittlebugs or closely related species. This approach may also be used to compare behavioral differences between species, which may be remarkable. For example, green is attractive to most leafhoppers (i.e., *Empoasca* spp.), which prefer bright colors corresponding to young leaves (Zhang et al. 2018; Bian et al. 2020), while whiteflies such as *Bemisia tabaci* prefer yellow (Blackmer and Byrne 1993) and thrips (e.g., *Frankliniella occidentalis*) are extremely sensitive to blue (Blackmer and Byrne 1993; Zhang et al. 2018; Ren et al. 2020). Differences occur even between sexes, as in the leafhopper *Scaphoideus titanus,* in which males are mostly attracted from yellow, while females seem to prefer red, probably because this color recalls the grapevine bark, thus oviposition sites (Mazzoni et al. 2011). This example suggests the presence of another spectral channel (and consequently, receptors), maximally sensitive in the orange or red range. Similar preferences have been observed in some Australian hemipterans as well (Farnier et al. 2014).

Multichromatic vision do not likely play an important role in the discrimination of different plant colors (Prokopy and Owens 1983), considering that many plants share similar spectral characteristics (Farnier et al. 2014). Light, scattered from or transmitted through leaves, is most intense around

500–580 nm, in the green-yellow part of the spectrum, resulting in green-yellow objects being extremely attractive for phytophagous insects seeking for vegetation (Prokopy and Owens 1983). Given that information is currently scarce on behavioral or physiological responses of spittlebugs to this color range, we may only speculate what colored stimuli are used by these insects. For many herbivorous insects, yellow colors indicate young leaves rich in nitrogen, which is a limiting factor in many plants (Mooney and Gulmon 1982). It must be said, however, that yellow leaf color is not necessarily an indicator of high quality (Döring and Kirchner 2022). Future research should therefore assess whether specific spectral properties of leaves of these plants are related to food quality and are consequently relevant for xylem feeders. In this regard, research on behavioral and electrophysiological responses to colors is needed, as it would facilitate the development of new trapping devices for the control of spittlebug and sharpshooter vectors in the field.

In the case of *P. spumarius*, the sole study that investigated the effectiveness of differently colored sticky traps dates back to 1967 and demonstrated that yellow is the most attractive color compared to the others tested, which included white, blue, green, orange, red and pink (it must be noted that these were the colors as perceived by human observers). A low number of *P. spumarius* was recorded on white, blue and pink traps, whereas intermediate numbers were found on green, orange and red traps (Wilson and Shade 1967). However, the yellow sticky traps currently employed in the field do not efficiently trap spittlebugs, especially in olive groves, suggesting that there are other attractive cues (different wavelengths, saturation, shape or factors other than color) involved (Morente et al. 2018). For instance, insects can detect the linear polarization of light, which represents the oscillation of incident photons in a common plane. Light, reflected from the shiny, waxy cuticle of leaves, becomes linearly polarized (Wehner 2001). The observer perceives this light together with the diffuse, unpolarized light, scattered from leaves or reflected through leaves. The ratio between the polarized and unpolarized light depends on the chemical and physical properties of the cuticle and the leaf parenchyma, and it could therefore be used by insects to detect or evaluate potential hosts. For instance, polarized reflections are exploited by butterflies to evaluate and identify host plants (Kelber et al. 2001), in that they choose oviposition sites based on the degree of polarization of reflected light (Blake et al. 2019). Aquatic bugs have photoreceptors optimized for detecting water bodies using polarization vision as well (Schwindt 1984a, 1984b), proving that hemipterans possess a object-directed polarization vision that could be used to select targets.

The life cycle of *P. spumarius* potentially influences the way insects perceive and process visual stimuli, considering that the transition to adulthood means passing from a sedentary, semiaquatic lifestyle to a dry and free-living one (Halkka et al. 1977; Keskinen and Meyer-Rochow 2004; Cornara et al. 2018a). In this univoltine species, eggs are laid on straw very close to the soil, while nymphs crawl on plants in the surrounding ground cover after hatching. Nymphs likely do not seek specific host plants given that they may thrive on hundreds of herbaceous species, while the production of a watery foam is essential for their survival as it avoids desiccation. The life of the nymph is consequently rather sedentary until adulthood. On the other hand, the adult spittlebug emerges from the foam covered by an exoskeleton protective against sunlight and provided with wings and legs capable of impressive jumps. They are hence ready to explore the outside world and find shelters if the herbaceous cover dries out in summer (Yurtsever 2000; Cornara et al. 2018a; Bodino et al. 2020). In this perspective, the eyes of *P. spumarius* are expected to undergo significant changes during adult molting giving that adults walk, jump and fly to find hosts. They need appropriate visual acuity, achieved during metamorphosis by an increase in size and number of facets within the compound eye. The cornea thickens and the rhabdoms become thinner, making the adult eyes tougher and improving vision compared to juveniles (Keskinen and Meyer-Rochow 2004). Nothing is known, however, about how *P. spumarius* eyes respond to specific visual requirements, and what stimuli, attractive or repellant, might be involved in orientation and behavioral changes.

If visual cues are pivotal, repellant stimuli could be used to reduce host finding by the spittlebug and integrated with attractive stimuli to develop push-and-pull strategies. Some plant species evolve resistant traits that help reduce detection by pests, even when this implies a lower reflection of optimum light wavelengths. For example, plants can reduce aphid landing by reflecting wavelengths other than the yellow or green—in the UV spectrum—or by producing waxless and glossy leaves (Eigenbrode and Espelie 1995; Stapleton and Summers 2002; Moharramipour et al. 1997; Smith and Chuang 2014).

Visual background appearance is another driving force guiding insects toward vegetated ground surfaces that may be used for vector management (Döring and Röhrig 2016). The marked contrast in the light spectrum between plants and soil is attractive to insects, especially when substantial amounts of bare soil surround a plant or a crop (Prokopy and Owens 1978, 1983; A'Brook 1968). A comparable situation occurs in the olive orchards ravaged by *X. fastidiosa* in southern Italy, where the herbaceous cover dries out in summer, leaving olive trees and a few evergreen shrubs surrounded by bare red soil (Fig. 1) (Cornara et al. 2018a; Bodino et al. 2019; Saponari et al. 2019). As noted above, current control techniques aimed at reducing vector populations involve removal of the ground cover in olive orchards.



Fig. 1 Visual cues may govern the movement of the spittlebug *Philaenus spumarius* toward olive plants, considering contrast of colors between the red bare soil and the green olive canopy. The spittlebug may move from the drying herbaceous vegetation or from surrounding olive/wild trees

Even if this tillage decreases the number of nymphs developing on the understory, it could potentially increase the risk of attracting adult spittlebugs from outside the orchard. In this regard, even if the dispersal of *P. spumarius* seems limited in olive orchards, it is influenced by several factors, such as agroecosystem structure and landscape composition (Santoiemma et al. 2019; Bodino et al. 2021b). As reported by Lago et al. (2022), interplant movements could occur during night or dusk, suggesting that also the time of the day should be considered. Provided that visual stimuli are available for *P. spumarius*, imagine a spittlebug adult searching for a host in proximity of a tilled plot: would not be the leafy and green canopy of an olive tree easy to spot against the red bare soil? Even though this is only a hypothesis, it would be of paramount importance to investigate the role of color background in host localization by this insect, especially considering that similar information could provide novel insights regarding the relation between ground cover removal and spittlebug abundance on olive canopies.

Finally, it has been noted that xylem feeders discriminate against plants showing symptoms of severe water stress and/or of bacterial infection (Marucci et al. 2005; Mizell et al. 2008; Rashed et al. 2011; Daugherty et al. 2011; Krugner et al. 2014). In particular, visual stimuli influence host selection by sharpshooter vectors of *X. fastidiosa* in the Americas, as these insects discriminate against infected grapevines, which are subjected to water stress and presumably represent a low-quality food source (Daugherty et al. 2011, 2017; Del Cid et al. 2018). *Philaenus spumarius* may show a similar preference, which would affect pathogen acquisition and, consequently, the spread of the olive disease caused by the fastidious bacterium. As pointed out by Daugherty and colleagues (2011), when investigating whether alternate hosts in the field represent pathogen reservoirs, it is important to study how vectors respond to infection in these plant species. In fact, *X. fastidiosa* infection level and symptom severity differ among different host species, and trends in quality over time may influence host phenotype and, as a consequence, vector behaviors (Hill & Purcell 1995; Purcell, 1997; Daugherty et al. 2011, 2017). Considering that *X. fastidiosa*, *H. vitripennis* and *P. spumarius* have broad host ranges (Krugner et al. 2019; Almeida et al. 2005; Cornara et al. 2018a, b), it would be crucial to unveil how infection and symptom expression impact the acceptance of different host plants by vectors. Similar information would, in our opinion, allow to better characterize *X. fastidiosa* epidemiology and develop strategies aimed at reducing vectors landing on host plants.

Olfaction

Plants emit volatile organic compounds (VOCs) that can be detected by phytophagous insects during flight and landing via olfactory receptors on their antennae, or with other sensilla on tarsi and mouthparts (Visser 1986; Andersson et al. 2015). Depending on the species, either host-specific volatiles or specific ratios of ubiquitous plant odors (such as green-leaf volatiles) are used by insects to find preferred hosts (Bruce et al. 2005; Riffell et al. 2009; Bruce and Pickett 2011). Extensive research has proven that odors are crucial components in long- and short-range localization of food sources within insect *taxa* such as Lepidoptera and Diptera, as well among some hemipterans, such as stinkbugs (Pentatomidae). Electrophysiological and behavioral tests on sternorrhynchans such as aphids (Aphidoidea), whiteflies (Aleyrodoidea) and psyllids (Psylloidea) demonstrated not only that odors trigger physiological responses, but also that they influence the orienting behavior of these insects, even if solely at short distances from the plant (Bruce et al. 2005; Coutinho-Abreu et al. 2014).

In contrast, auchenorrhynchans (cicadas, spittlebugs, leaf-, plant- and treehoppers) have small antennae, and most studies suggest that olfaction has little role in mate and host finding, although exceptions have been reported (e.g., Mazzoni et al. 2009; Ranieri et al. 2016; Zhang et al. 2018). For instance, the cixid planthopper *Hyalesthes obsoletus* has sensible sensilla that are finely tuned to perceive preferred hosts (Riolo et al. 2012). In many leafhopper and planthopper species, chemical stimuli such as plant volatiles are similarly associated to host detection, though in most cases odors just increase responsiveness to visual stimuli (Obata et al. 1981; Todd et al. 1990; Cook and Denno 1994; Fereres and Moreno 2009; Mazzoni et al. 2009; Rossi Stacconi et al. 2014; Darshanee et al. 2017; Grange et al. 2017). Enhanced responses to visual cues attributable to plant volatiles have also been observed in the sharpshooter *Homalodisca vitripennis*, a *X. fastidiosa* vector in North America (Patt and Sétamou 2007; Krugner et al. 2019). Among these and other xylem feeders, a variety of cues are presumably used to track the physiological state of host plants and consequently assess whether they can provide adequate nutrients (Mizell and French 1987; Andersen et al. 1992; Brodbeck et al. 1999; Redak et al. 2004).

The evidence produced so far on the relevance of olfactory cues in long- and short-range host plant location by *P. spumarius* is fragmentary and inconclusive. Morphological studies showed that the antennae of *P. spumarius* bear a lower number of antennal sensory structures than leafhoppers and planthoppers (Ranieri et al. 2016). The morphology of *P. spumarius* sensilla suggests that their main function is thermo-/hygroreception, notable considering that humidity plays a pivotal role in the life cycle of spittlebugs (Weaver and King 1954; Tichy and Loftus 1996; Ranieri et al. 2016; Cornara et al. 2018a). At any rate, the general organization of some basiconic and coeloconic sensilla is consistent with an olfactory function (Ranieri et al. 2016), and electroantennographic (EAG) studies confirmed that the antennae of both *P. spumarius* males and females respond to a wide range of VOCs belonging to different chemical classes (Germinara et al. 2017). Recent experiments also proved that spittlebugs are attracted and/or repelled by different aromatic plants, and that the response depends on the sex of the insect and on the distance from the odor source (Ganassi et al. 2020). Cascone and colleagues (2022) observed a negative correlation between the amount of limonene, 3-octanone, camphor, geraniol, and (-)-myrtenol and attractiveness toward *P. spumarius* females. Among EAG-active host plant volatiles, spittlebug females were apparently attracted only by (+)-camphor, cis-3-hexenyl-acetate and cis-3-hexen-1-ol (Anastasaki et al. 2021; Rodrigues et al. 2022). With regard to olive, females were attracted by the blend of VOCs emitted by varieties susceptible to *X. fastidiosa* (Ogliarola, Rotondella and Frantoio) and repelled by a resistant one (FS-17), while males were totally unresponsive (Cascone et al. 2022).

Besides the evidence produced so far, literature on spittlebug species others than *P. spumarius* suggest that there is room for repellent compounds to be found and exploited. The Neotropical spittlebug pest *Mahanarva spectabilis*, for instance, likely used olfactory cues to distinguish among sugarcane cultivars and pasture grasses, both under greenhouse and field conditions. While attractive cultivars were most susceptible to nymphs and adults, volatiles emitted by a resistant cultivar had a repellent effect, suggesting that host suitability may be discriminated through odor cues (Auad and De Resende 2018; Silva et al. 2019). By identifying odors that are less attractive for pest insects, it might be possible to develop resistant varieties emitting repellent volatiles, or to identify plant volatiles that could be used to develop repellent tools. Novel techniques in the field of genome editing may also be employed to protect plants, considering that the plant susceptibility to pest attacks may be reduced by modifying its emission of kairomones, as suggested by Salvagnin and colleagues (2018).

Host acceptance

Despite decades of effort, knowledge on the biochemical and biophysical factors driving the acceptance of host plants by xylem feeders is still scattered. Once on a plant, the insect proceeds through a sequence of brief stereotypical behaviors aimed at discriminating whether the plant could be an acceptable food source or oviposition substrate. Each behavioral step is composed of overlapping fixed action patterns,

and behavioral responses proceeding to completion without sensory feedback are triggered by a specific stimulus or set of stimuli (Backus 1985). Host acceptance therefore depends on a threshold of positive sensory inputs to the central nervous system that triggers stylets insertion (probing) and tasting/testing ingestion and maintains ingestion and swallowing. The factors determining host plant acceptance include i) intensity of olfactory and gustatory feeding stimulants, ii) intensity of deterrents and repellents, iii) metabolic state of the insect, including degree of deprivation of specific dietary factors and iv) learning resulting from previous feeding experience (Fig. 2) (Dethier 1982).

The first step of the acceptance process is exploration of plant after landing. Among piercing-sucking insects, auchenorrhynchans exhibit a unique behavior, namely dabbing (the insect repeatedly touches the plant with the tip of the labium, which bears chemo- and mechanoreceptors). Simultaneously, chemicals in the plant's waxy cuticle solubilize in the insect's saliva, producing a chemical mixture that is sensed by labial sensilla (Backus 1985). Basiconic sensilla with supposed olfactory function have recently been described on the labium of *P. spumarius* (Ranieri et al. 2020).

Besides chemical cues, the tissue topography of the plant is another essential cue in the process of recognizing a suitable host. While sharpshooters and aphids insert their stylets through flat surfaces, spittlebugs prefer a rounded support to grab with the forelegs, pulling it toward their labium while pushing the stylets in (Cornara et al. 2019). Besides providing stimuli for host recognition and acceptance, the plant anatomy could represent a first line of defense against spittlebug settling. For example, glandular trichomes function as feeding barriers in the case of *P. spumarius* nymphs (Hoffman and McEvoy 1985; Smith and Chuang 2014). On the other hand, once the insect has access to the plant, probing occurs, in that the stylets are inserted into the plant tissues searching for gustatory cues driving ingestion and

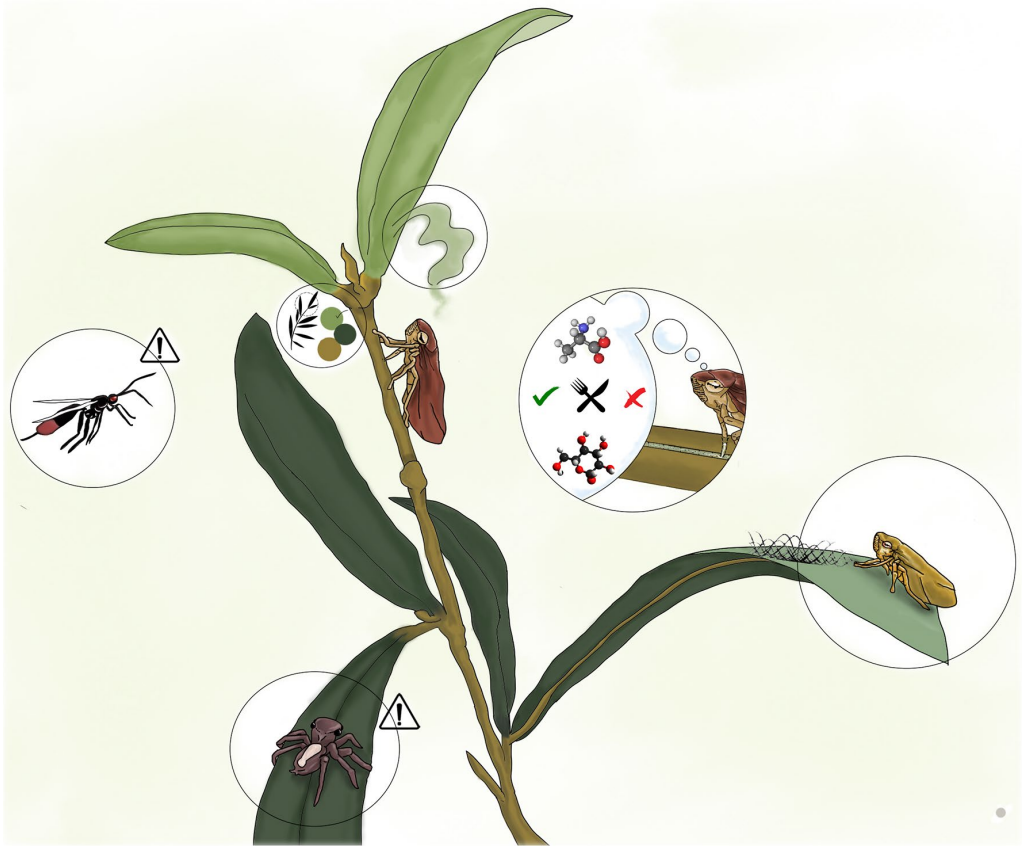


Fig. 2 The image depicts some of the cues that may influence the settling of the spittlebug *Philaenus spumarius* on a plant. Semiochemicals include plant volatiles **A** and composition of the xylem sap **B**, which provide information regrading the suitability of the plant as food source. Semiophysicals comprise vibrational signals emitted by **C** conspecifics and **D** predators, as well as acoustic signals produced by approaching parasitoids **E** and **F** visual cues such as color and plant structure and shape

final acceptance. It is important to point out that probing and feeding should be considered separate behaviors since each is triggered by a primary set of specific gustatory stimuli (Sogawa 1976). Among *X. fastidiosa* vectors, trial ingestion and sensing/tasting of xylem sap through the sensilla lining the precibarium are pivotal steps underlying host plant acceptance (Backus and McLean 1985; Cornara et al. 2018b; Ranieri et al. 2020). If the plant is not considered an "acceptable host" after probing, the stylets are withdrawn and feeding does not occur.

Precibarial sensilla are crucial for host acceptance as they detect extremely diluted metabolites, such as amino acids, organic acids and sugars, present in the xylem sap (Prokopy and Owens 1983; Miller and Strickler 1984; Visser 1986). The latter is an incredibly challenging food source, given that it is dilute, nutritionally unbalanced and under negative tension. Unique features permit xylem feeders to live on this nutritionally poor diet, including i) capacity of ingesting voluminous throughput; ii) ability of efficiently extracting organic nutrients; iii) assets of symbionts that reprocess the amino acid repertoire; and iv) mechanisms for disposal of excess nitrogen. As an example, large sucking pump (cibarial) muscles allow xylem feeders to process hundreds of body weight equivalents of xylem fluid daily. This high throughput is then secreted from the anus and is used by spittlebugs nymphs to produce the spittle mass, while adults (and nymphs of other species) excrete the liquid as a succession of droplets. The flow is so rapid and evident that sharpshooters owe their name to this behavior. Besides managing the voluminous input of sap, efficient extraction of nutrients is crucial and is accomplished by means of a bypass. The filter chamber, in which the anterior and posterior extremities of the midgut are closely apposed, shuts most xylem sap into the lower reaches of the digestive track. This permits the intestines to extract remaining organic compounds more efficiently, resulting in assimilation of more than 99% of the nutrients (Andersen et al. 1989). In addition, to cope with the fact that xylem-sap organics are dominated by a handful of nonessential amino acids, typically glutamine and asparagine, xylem feeders have evolved close symbiotic relationships with bacteria that convert these compounds into essential amino acids (Ankrah et al. 2020a). The carbon skeletons of these organic nitrogen compounds are source of energy and building blocks for carbohydrates and fats. Overall, these factors enable xylem feeders, despite the low absolute levels of nitrogen in xylem sap, to have a surfeit of nitrogen which they dispose in form of ammonia, a toxic compound. However, diluted ammonia is excreted within the copious fluid output (the process is known as ammonotelism and is common among insects' aquatic forms) (Ankrah et al. 2020b).

Even if xylem sap is a challenging feeding source, polyphagy and frequent host switching enable xylem feeders to take advantage of the temporal and spatial dynamic of rapidly changing xylem stream nutrients (Mizell III et al. 2008). Once a xylem vessel is reached and sap flows through the food canal and the precibarium, chemical components of the xylem sap should trigger the ingestion, acting as phagostimulants. Several studies on both sharpshooters and spittlebugs point toward amino acids, particularly the amides glutamine and asparagine, as the main drivers of host plant acceptance, shaping xylem-feeders' abundance and feeding patterns (Brodbeck et al. 1990; Andersen et al. 1992; Thompson 1994, 2004). Availability and ratios of amides are potentially the most relevant indicators of a host's nutritional value in xylem feeders and other species as well (Andersen et al. 1992). Glutamine, for instance, is an essential component of insect artificial diets, while asparagine deficiencies likely represent a host resistance mechanism against brown planthoppers (Auclair and Cartier 1963; Mittler 1972). Although not essential, proline can influence feeding preferences as well, because it is presumably indispensable for flying (Weeda et al. 1979).

Xylem feeders have to overcome another obstacle: xylem sap is energetically costly to extract, due to negative pressure within xylem vessels (Raven 1983). Studies on the relationship between xylem-sap tension and feeding in *P. spumarius* suggest that negative tension may not be a limiting factor, as far as the ratio between the volumetric energy density of sap ingested and the energy spent to withdraw and ingest it remains positive. Moreover, cibarial dilator muscles are able to overcome tensions up to -15 bar, well above the normal range of tensions spittlebugs are expected to encounter under field conditions (Bergman et al. 2021). In addition, Beckett et al. (2019) reported that the metabolic rate of actively feeding *P. spumarius* is just 20% greater than during resting, indicating a relatively low cost of xylem-sap extraction. Similarly, xylem tension does not seem to affect the feeding rates of *H. vitripennis*, provided that the nutritional value of the host exceeds the costs of feeding under xylem negative pressures (Brodbeck et al. 1990; Andersen et al. 1992). The net result is that xylem feeders profitably extract the exceptionally large quantities of xylem sap required for adequate nutrition, as attested by their evolutionary success and status as abundant and consequential pests and vectors.

Overall, more precise knowledge of the sensory perception and specific stimuli that mediate feeding may aid plant geneticists in designing cultivars resistant to spittlebugs and sharpshooters. Moreover, the role played by xylem-sap metabolites in shaping host plant acceptance opens the possibility for managing populations of xylem feeders by manipulating exogenous factors (such as water, fertilizers, etc.) that might influence xylem-sap chemistry, potentially reducing the host suitability for the insects.

Substrate-borne vibrational signals as semiophysicals

From a vibrational point of view, plants are noisy environments, where both biotic and abiotic factors provide specific constraints that shape insect communication systems and host preferences (Šturm et al. 2022). Many arthropods use substrate-borne vibrations in combination with other modalities to gather information from the environment, and it is estimated that at least 150,000 insect species rely exclusively on vibrational communication (Virant-Doberlet and Cokl 2004; Cocroft and Rodríguez 2005). As noted above, this is the case in Auchenorrhyncha, whose mechanoreceptors are tuned to perceive species-specific signals emitted by co- and heterospecifics (Virant-Doberlet and Cokl 2004; Hill and Wessel 2016). Consequently, substrate-borne vibrations are semiophysicals that travel through plants and convey essential information regarding the environment (e.g., for determining whether the plant is a favorable host for living, feeding, mating and ovipositing), while reducing the risks of being predated (Cocroft and Rodríguez 2005; Virant-Doberlet et al. 2019; Nieri et al. 2021).

Given that communication can take place only when signal prevails over noise (Wiley 1983), the signal transmission properties of plants can ultimately influence how insects select and use their hosts (Joyce et al. 2014; Mortimer 2017). In fact, even if wind and rain are ubiquitous sources of abiotic noise, the vibrations that they induce in the plant structure can differ depending on the plant species or even between parts of the same plant (Barth et al. 1988; McVean and Field 1996). A plant influences insect communication not only by conveying noise but also by acting as a signal filter, framing which signal features are transmitted and how (Michelsen et al. 1982; Magal et al. 2000). In some cases, closely related insect species or subspecies have evolved distinct signals as a result of adaptation to host plants with different signal transmission properties (McNett and Cocroft 2008). Indeed, such coevolution between insects and plants mechanical features usually occurs in insect specialist species, whose life cycle occurs on the same host (Čokl et al. 2005).

Given the wide and varied nature of their hosts, polyphagous species such as *P. spumarius* are likely not adapted to particular plant structures. While other biotic factors are important in shaping the relationships between the spittlebug and plants, the presence of signaling co- and heterospecifics could be used by *P. spumarius* to evaluate the suitability of plants as hosts, not only as a food source, but also as mating and oviposition sites. In this species, mating success accordingly depends upon the establishment of a vibrational duet between a male and a female, in that female vibrational signals trigger pair formation and guide the male toward the partner (Avosani et al. 2020). Even so, females call only when sexually mature, thus after the breakage of the ovarian parapause, when eggs start to develop in their ovaries. Depending on the geographical range, the sexually maturity occurs from late summer or autumn until oviposition and death of the female (Avosani et al. 2021b; Cornara et al. 2018a). Given that female signals may influence the persistence of males on plant substrates, especially at the end of summer, they could be used in the framework of BM approaches. Moreover, female could use signals emitted by other females on the same plant to evaluate suitable oviposition sites given that aggregation could be common even among *P. spumarius* adults, and not only among nymphs aggregating and sharing their spittle to enhance their probability of survival (Mangan and Wutz 1983; Wise et al. 2006; Bodino et al. 2021a, b). Even if a recent study indicates a prevailing role of intraspecific volatiles in aggregating ovipositing females (Sevarika et al. 2022), we cannot exclude that vibrational signals could concur to it. Likewise, if vibrational signals could attract or hold spittlebugs on plants, it might be possible to keep infective spittlebugs inside "trap" crops, reducing their movement toward olive trees, especially when applied to plants that are preferred by *P. spumarius*. In this regard, it has been shown that *Lavandula angustifolia* and, in particular, *Taraxacum officinale* are more suitable hosts for spittlebugs nymphs and adults compared to other species common in olive groves (Morente et al. 2022). The authors suggested avoiding the two plant species as ground cover plants in crops vulnerable to *X. fastidiosa*, but there could be room for evaluating them as trap crop, potentially integrating other (e.g., vibrational) stimuli.

Another possible approach is to interfere with the mating communication in order to reduce male–female encounters. Attempt of vibrational mating disruption already proved to be effective in several leafhoppers and planthoppers in laboratory conditions but also at the field level, as in the case of the leafhoppers *S. titanus* and *H. vitripennis* (Krugner and Gordon 2018; Mazzoni et al. 2019, Nieri and Mazzoni 2019, Feng et al. 2022). Similarly, trials conducted under laboratory conditions demonstrated a significant reduction of *P. spumarius* mating when virgin pairs were exposed to synthetic vibrational signals that masked the frequency pattern of the natural mating signals (Avosani et al. 2022). Given the promising results, research should unveil whether the synthetic vibrational signal affects multiple behaviors simultaneously, as in the case of *S. titanus*, in which the disturbance noise used for mating disruption significantly decreased the time spent by males and females on vibrated plants and reduced oviposition (Zaffaroni Caorsi et al. 2022).

These approaches based on vibrations are promising because they manipulate highly conserved communication modalities that mediate behaviors crucial for the species survival, such as reproduction. However, a deep knowledge of

insect phenology in association with the vibrational behavior of the species are required. As mentioned before, in *P. spumarius* vibrational signals have different roles in the course of the summer because females start to be active and sensitive to male signals only in correspondence of the ovariole development (Akassou et al. 2021; Avosani et al. 2021a). For this reason, methods of interference and/or capture are more likely to succeed only if applied in the proper time windows, similarly to semiochemicals in field applications (Suckling 2000a, 2000b).

As mentioned above, the plant is an intricate network of signaling and eavesdropping arthropods, thus an insect has to overcome the noise coming from conspecifics and heterospecifics, while avoiding being detected by unwanted receivers such as predators and parasitoids (Virant-Doberlet et al. 2011, 2019). In some species, vibrations are used to cooperate, while in others to compete, or to mediate spacing (Greenfield 1994, 2014). Though parasitoids and predators can exploit vibrations emitted by preys to locate them, the opposite is also true (Virant-Doberlet et al. 2019): prey can discriminate between approaching predators and non-predators and respond accordingly. Depending on the species, escaping a predator could mean jumping or dropping off the plant, startling the predator, freezing or engaging in warning displays (Tsubaki et al. 2014; Kang et al. 2017; Takanashi et al. 2019). *Philaenus spumarius* is equipped with a large and complex array of abdominal mechanoreceptors that may serve to perceive environmental vibrations (Ehlers et al. 2022). If *P. spumarius* detects predators through vibrations and avoids them by leaving the host plant, similar vibrational signals could be used as repellent stimuli, potentially reducing the suitability of hosts. For instance, nonspecific vibrations (white noise) induce aphids to leave host plants consistently reducing the population density (Parent et al. 2022). A specific signal, such as a predator or an intraspecific distress signal, could be used to develop an effective behavioral manipulation strategy that can overcome the risk of habituation (Foster and Harris 1997), although even predator signals, not coupled with actual threat, could be soon become ineffective, as suggested by Aflitto and Thaler (2020). Overall, the combination of attractive and repellent substrate-borne signals could lead to the development of push-and-pull vibrational approaches to reduce the settling of adult spittlebugs into olive trees.

Interestingly, in addition to deterring permanent settlement on the plant, substrate-borne vibrations could affect spittlebugs probing and feeding behaviors, as recently demonstrated by Avosani et al. (2021b). In their pioneering study, vibrational stimuli employed against *P. spumarius* reduced its feeding activity on an herbaceous plant, although the same stimuli had no effect when applied to olive plantlets, probably because the signal was filtered differently by the olives structure. Nonetheless, this proof-of-concept demonstrated that the spittlebug host acceptance is susceptible to vibrational interference, and paves the way for further research (Avosani et al. 2021b). Considering that *P. spumarius* is a native and abundant species in all the Palearctic region, a feeding deterrent strategy is promising in light of the fight against *X. fastidiosa*, as it could reduce the transmission of the bacterium by lowering the number of vectors feeding on the plant and the contact between insect stylets and plant xylem. The signal used by Avosani and colleagues (2021a) consisted in a modified female distress signal, but it would be interesting to assess whether a fright stimulus, such as vibrations coming from a predator, could be even more effective in deterring feeding or in repelling *P. spumarius* from hosts.

Conclusions

The aim of this review was to outline the primary interactions between host plants and pests that govern attraction toward the host, acceptance and settling, and to suggest approaches to disrupt these interactions in the frame of an integrated pest management strategy. We were particularly interested in profiling environmentally and economically sustainable strategies that could prevent further spread of the bacterium *X. fastidiosa* in Europe based on the interference with the processes governing host finding and settling in its vector, *P. spumarius*.

As mentioned before, the epidemiology of the *X. fastidiosa* depends on the completion of steps (host plant detection, acceptance and feeding) by its insect vectors in order to spread to healthy plants and, consequently, to survive and evolve (Chatterjee et al. 2008; Retchless et al 2014; Sicard et al 2018). Transmission of the bacterium is mainly associated with two factors, namely vector transmission efficiency and vector activity, the latter being the combination of the abundance of insect vectors on the host plant and their time of permanence. Considering the transmission of the strain ST53 to olive by the meadow spittlebug, a relatively low efficiency is compensated by high population densities residing on olive canopies for months (Cornara et al. 2017; Bodino et al. 2019, 2021a). Another aspect to consider is that bacterial cells inoculation seems to occur a few minutes after stylets insertion into the host plant and contact with a xylem vessel (Cornara et al. 2020). Therefore, a successful and sustainable strategy aimed at disrupting *X. fastidiosa* transmission and spread should aim at (i) avoiding vector-host plant contact, and (ii) reducing host plant suitability.

Compelling evidence suggests that odors might indeed play a role in host localization by adult spittlebugs, even though developing attractive semiochemicals and trapping devices seems still challenging, considering that more information are needed regarding the candidate compounds'

active space and concentration needed to successfully guide *P. spumarius* toward the odor source (Ranieri et al. 2016; Anastasaki et al. 2021; Cascone et al. 2022; Sevarika et al. 2022). On the other hand, if repellent or deterrent odors are identified (i.e., from emissions of plants unsuitable for *P. spumarius*), there is room for designing push-and-pull techniques, even if practical aspects such as application and persistence of the chemical compounds on the olive canopy and/or coexisting alternative hosts must be ascertained.

Visual stimuli might be essential within host finding and have been greatly neglected in the case of *X. fastidiosa* vectors. For example, yellow sticky traps are inadequate for monitoring *P. spumarius* in olive orchards, especially if insect densities are low (Morente et al. 2018). Instead of focusing on colors as perceived by humans, sticky traps could be improved by changing the shape, the use of contrasting colors, and spectral reflectance relevant for the insects (Farnier et al. 2014). Furthermore, the landscape composition of olive orchards could contribute to orientating *P. spumarius* toward the host plants. In our opinion, it would be crucial to assess whether the contrast between the bare soil and the plant canopy could encourage adult spittlebugs to move toward olive trees and other woody plants when the ground cover is removed. In such scenario, the practice of removing the herbaceous cover to reduce nymphal populations may be a further trigger for spittlebug dispersal toward olive canopies. If this is the case, diverse types of mulches could be tested in olive groves, in order to evaluate if any of them could lower the number of adult spittlebugs landing on olive trees. Mulches within the olive orchards could be for example coupled with colored sticky traps and other attractants designed to draw *P. spumarius* out of the orchards.

Another interesting approach to test is represented by intercropping, since it may potentially reduce the colonization of plants susceptible to *X. fastidiosa* by adult spittlebugs, as the latter could find shelter and feeding sources alternative to olive trees. At any rate, woody hosts that tolerate summer drought, and are not hosts of the bacterium would be the most suitable ones. An additional benefit of intercropping relies on the potential increase in predators and parasitoids within the orchard (Ju et al. 2019), which could enhance biocontrol and/or reduce settling of spittlebugs. Even so, intercropping may influence pest management in truly diverse ways, based on local conditions and specific interactions within the agroecosystem. Tests are therefore needed to assess whether this would be a feasible approach for the European outbreaks, and whether it could be used to reduce the transmission of *X. fastidiosa*.

Besides interfering with host plant location and landing, effective BM strategies should include measures aimed at reducing the chances for a spittlebug to settle on the target plant. If chemical compounds are not used by *P. spumarius* as olfactory cues in host localization, they likely convey pivotal information on host suitability during probing. There may be deterrent compounds involved in host resistance that reduce suitability of certain plant species or cultivars, thus decreasing vector feeding activities and bacterium transmission. By elucidating the compounds and the mechanisms involved in resistance to both *X. fastidiosa* and the vector, it may be possible to breed resistant or tolerant olive varieties or to stimulate similar responses in susceptible cultivars. Screening for resistant genotypes is of crucial importance, as olive cultivars expressing repellent and deterrent traits are urgently needed to allow infected areas to be replanted and us to witness the blossoming of new olive trees resistant to the vector, the pathogen, or both. Several decades of effort to breed pasture grass cultivars resistant to Neotropical spittlebug pests offers a note of caution. Although there was some success in breeding and screening for nymph resistant strains, this resistance did not carry over to adults (Aguirre et al. 2013). In practice, succession of new tolerant and resistant plantings in pastures did not result in control of spittlebugs, partly because tolerance engendered high pest populations. The case of *P. spumarius* is not directly analogous, since this spittlebug function as a vector and neither it harms directly by feeding nor olives are nymphal hosts. Nonetheless, the large literature on Neotropical spittlebug pests of sugarcane and cultivated pasture grasses probably harbors useful lessons for *P. spumarius* control in Europe.

Finally, vibrations, as well as chemical compounds, play a key role in the short-range communication and host acceptance of insects (Strauß et al. 2021). As suggested by several authors, hi-jacking insect vibrational communication makes it possible to manipulate behaviors, including mating, permanence on hosts, oviposition and feeding (Eriksson et al. 2012; Takanashi et al. 2019; Avosani et al. 2021a, b; Nieri et al. 2021; Zaffaroni-Caorsi et al. 2022). *Philaenus spumarius* relies on substrate-borne vibrations to communicate with conspecifics, to perceive the environment and, potentially, to detect natural enemies. By achieving deeper knowledge regarding the role of vibrations in determining host suitability, it may be possible to identify semiophysicals that can be used in concomitance to other cues to attract spittlebugs toward trap crops, repel them from olive trees and/or deter feeding, overall reducing interactions with the plant and constraining the spread of *X. fastidiosa.*

To conclude, there are diverse BM strategies targeting insect–plant interactions that can be evaluated in the future and that could, if effective, be combined with other integrated pest management tools and approaches, such as biocontrol, biopesticides and cultural practices, to protect olive groves from inoculation with the fastidious bacterium.

Author contribution

SA and DC wrote the first draft. SA prepared the figures. All the authors edited and reviewed the manuscript. DC coordinated the review.

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Declarations

Conflict of interest The authors declare no competing interests.

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CHAPTER II

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

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

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ORIGINAL PAPER

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 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

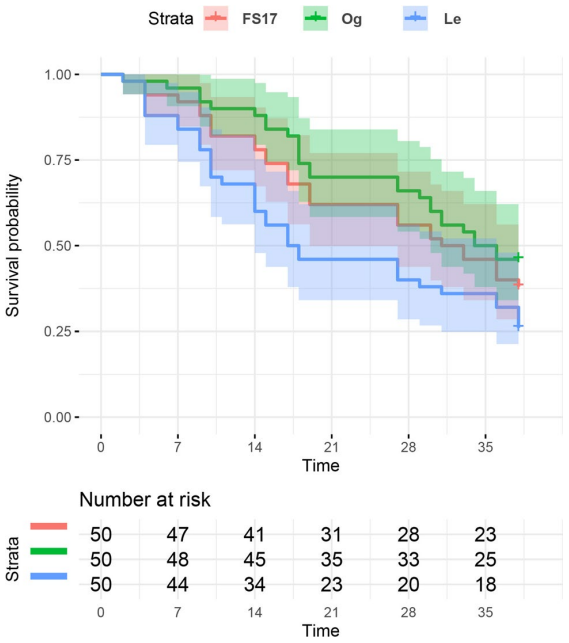


Fig. 1 Kaplan–Meier plot describing the survival curves for *Philaenus spumarius* at three olive varieties (FS17, Og, Le). The curves represent the estimated survival probability at each time point. *Y*-axis shows the probability of being alive at each time point, while *X*-axis shows the time-points. 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)

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 $[M\text{-}H]^-$ 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		

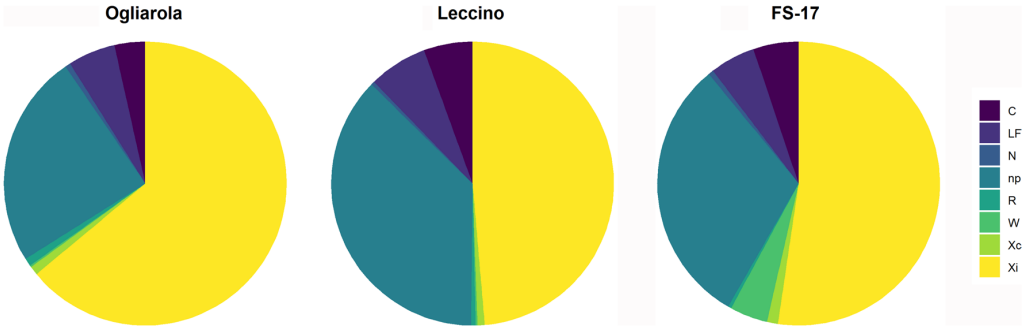


Fig. 2 Duration of each probing behavioral pattern/waveform (WDI) during the 4 h access to the three olive varieties. EPG WAVEFORMS = np: non-probing; C: pathway (stylets outside xylem); Xc: xylem contact (tasting of the xylem sap, and testing the mechanical strength of the sealing); Xi (xylem ingestion); 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

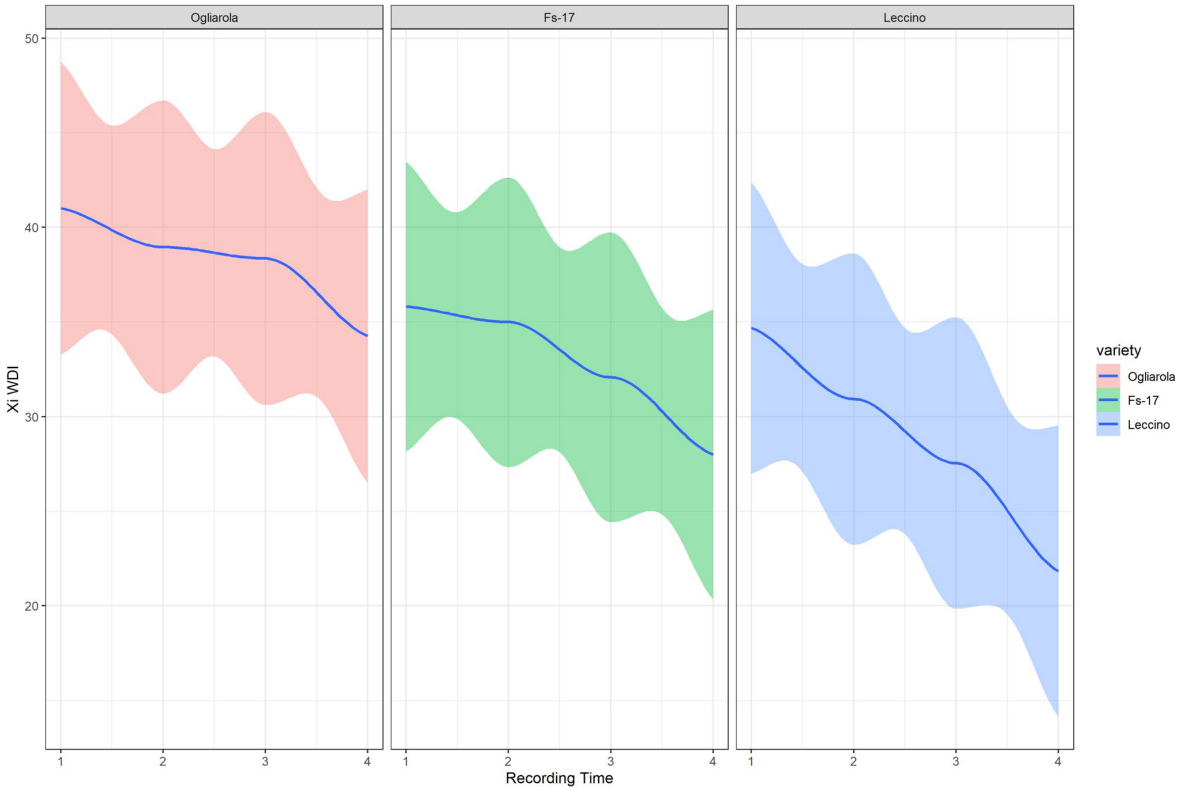


Fig. 3 Xylem ingestion trends in the three olive varieties during the 4 h EPG. *x* axis: duration of the xylem ingestion expressed in minutes. *y* axis: hour of the recording

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.

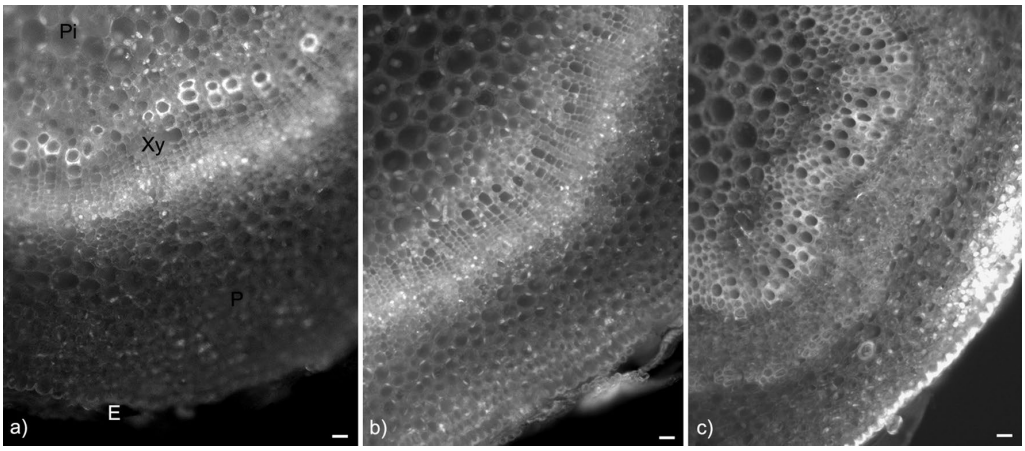


Fig.4 Micrographs of the stem cross sections of FS17 **a**, Leccino **b** and Ogliarola **c** cultivars. Pi: pith; Xy: xylem vessels; P: parenchima; E: epicuticle. Scale bars: 50 mm

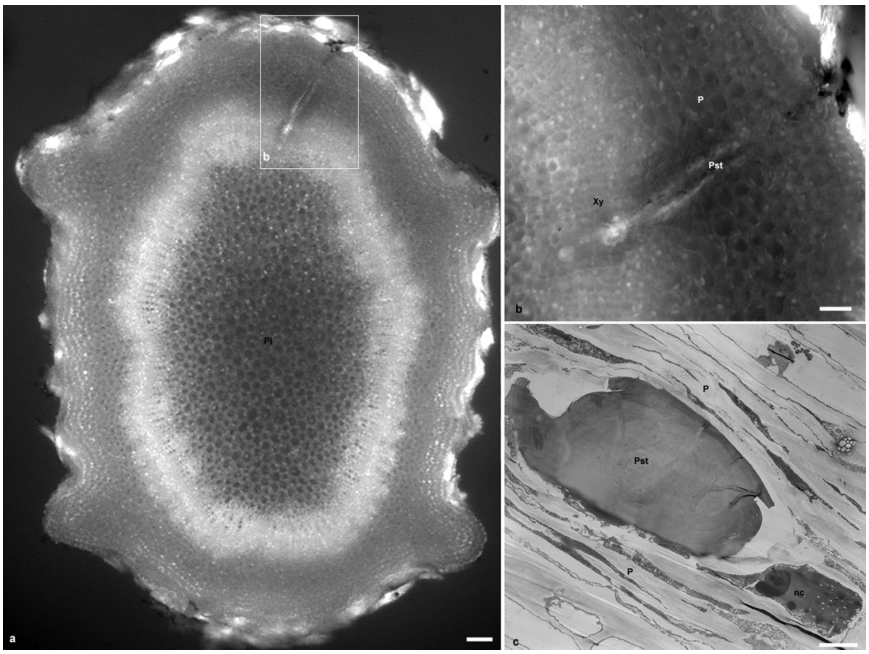


Fig. 5 Micrographs of the stylets trace (Pst) of *Philaenus spumarius* in the stem of olive plants var. Leccino observed under optical (**a**) and electron (**c**) microscopy. White boxed area in **a** is enlarged in (**b**). **b** Close-up of distal part of the salivary sheath as it passes the parenchymal 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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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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Supplementary Information

Online resource 1

Table 1. Summary of *Philaenus spumarius* distribution encounters among the three olive varieties per strata and tissue (no-choice experiment).

Total number *Philaenus spumarius*																
	Apical portion					*Middle portion*					*Basal portion*					
Variety	stem	petiole	leaf up	leaf down	Total	stem	petiole	leaf up	leaf down	Total	stem	petiole	leaf up	leaf down	Total	Cage
FS17	567	88	245	63	963	62	6	19	28	115	10	1	13	32	56	77
Ogliarola	601	100	302	95	1098	66	7	19	29	121	10	2	10	30	52	86
Leccino	443	97	139	115	794	50	5	19	25	99	13	2	5	32	52	70

Table 2. Mixed model for zero-inflated data of *Philaenus spumarius* cage/plant choice for each different variety (no-choice experiment). Reference level: apical.

Model	**Coefficients**	**Estimate**	**Std. Erros**	**z-value**	**Pr(>|z|)**
FS17	(Intercept)	0.9983	0.0422	23.7	<2e-16 ***
	middle	-2.2151	0.1137	-19.5	<2e-16 ***
	basal	-2.8535	0.1485	-19.2	<2e-16 ***
	cage	-2.7393	0.1463	-18.7	<2e-16 ***
Ogliarola	(Intercept)	1.1220	0.0325	34.5	<2e-16 ***
	middle	-2.2504	0.1054	-21.4	<2e-16 ***
	basal	-3.0802	0.1530	-20.1	<2e-16 ***
	cage	-2.6877	0.1316	-20.4	<2e-16 ***
Leccino	(Intercept)	0.7729	0.0962	8.04	9.2e-16 ***
	middle	-2.1644	0.1193	-18.14	< 2e-16 ***
	basal	-2.7520	0.1529	-18.00	< 2e-16 ***
	cage	-2.5568	0.1432	-17.85	< 2e-16 ***

Table 3. GLMM of *Philaenus spumarius* tissue choice for each different variety (no-choice experiment). Reference level: stem/female.

Model	Coefficients	Estimate	Std. Erros	z-value	Pr(>|z|)
FS17	(Intercept)	0.653770	0.085260	7668	1.75e-14 ***
	abaxial	-0.711737	0.167020	-4261	2.03e-05 ***
	adaxial	-0.024812	0.128950	-192	0.8474
	petiole	-1.240681	0.209894	-5911	3.40e-09 ***
	day	-0.003136	0.003548	-884	0.3767
	sexMale	-0.098201	0.085577	-1148	0.2512
	abaxial:day	-0.024690	0.011002	-2244	0.0248 *
	adaxial:day	-0.034473	0.006973	-4944	7.67e-07 ***
	petiole:day	0.004316	0.010632	406	0.6848
Ogliarola	(Intercept)	0.680727	0.078106	8715	< 2e-16 ***
	abaxial	-0.497597	0.152259	-3268	0.00108 **
	adaxial	-0.319304	0.127376	-2507	0.01218 *
	petiole	-0.826116	0.189183	-4367	1.26e-05 ***
	day	-0.002171	0.003454	-628	0.52970
	sexMale	-0.057040	0.062194	-917	0.35908
	abaxial:day	-0.027748	0.010030	-2767	0.00566 **
	adaxial:day	-0.007243	0.006126	-1182	0.23706
	petiole:day	-0.016685	0.010488	-1591	0.11163
Leccino	(Intercept)	0.587440	0.135518	4335	1.46e-05 ***
	abaxial	-0.282825	0.149724	-1889	0.058895
	adaxial	-0.767978	0.162909	-4714	2.43e-06 ***
	petiole	-0.588813	0.191868	-3069	0.002149 **
	day	-0.013985	0.004015	-3483	0.000496 ***
	sexMale	-0.084210	0.204508	-412	0.680508
	abaxial:day	-0.020543	0.009949	-2065	0.038947 *
	adaxial:day	-0.007594	0.008452	-898	0.368943
	petiole:day	-0.020470	0.011518	-1777	0.075523

Table 4. Survival table of *Philaenus spumarius* among the three olive varieties.

Variety	Number at risk (Number of events)	Median OS† (95% CI)
FS17	50 (31)	32 (19 - NA)
Ogliarola	50 (27)	35 (30 - NA)
Leccino	50 (37)	17.5 (14 - 36)

†: Days. NA: Not Available.

Figures

Figure 1. Experimental design of no-choice (A) and choice (host preference) (B) trials. Plant hypothetically into MS (wooden basal part of approx 30 cm) and GSS (green shot above the wooden basal part, approx 30 cm), and subdivided in three portions 10 cm each (apical, median, basal) with the corresponding plant tissue for each (petiole, stem, leaf abaxial or adaxial).

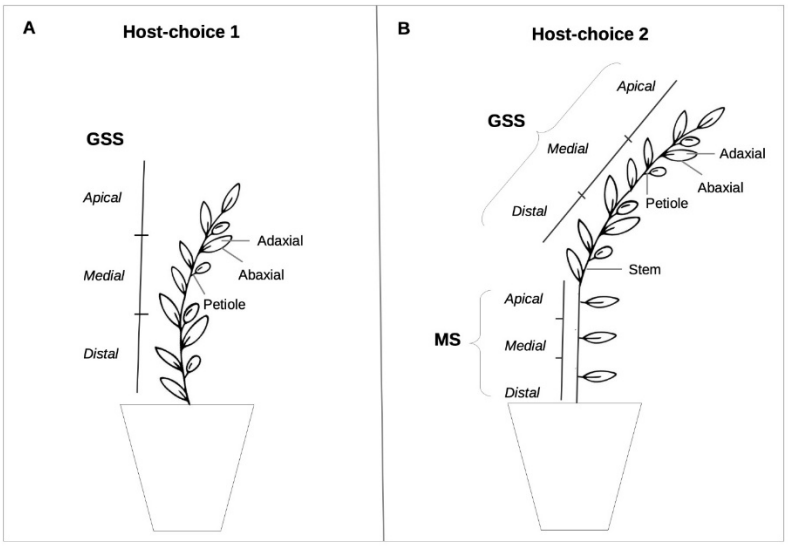


Figure 2. Total encounter of Philaenus spumarius individuals along the 38 days of experiment per variety and strata (FS17-top; Og-middle; Le-bottom plot)

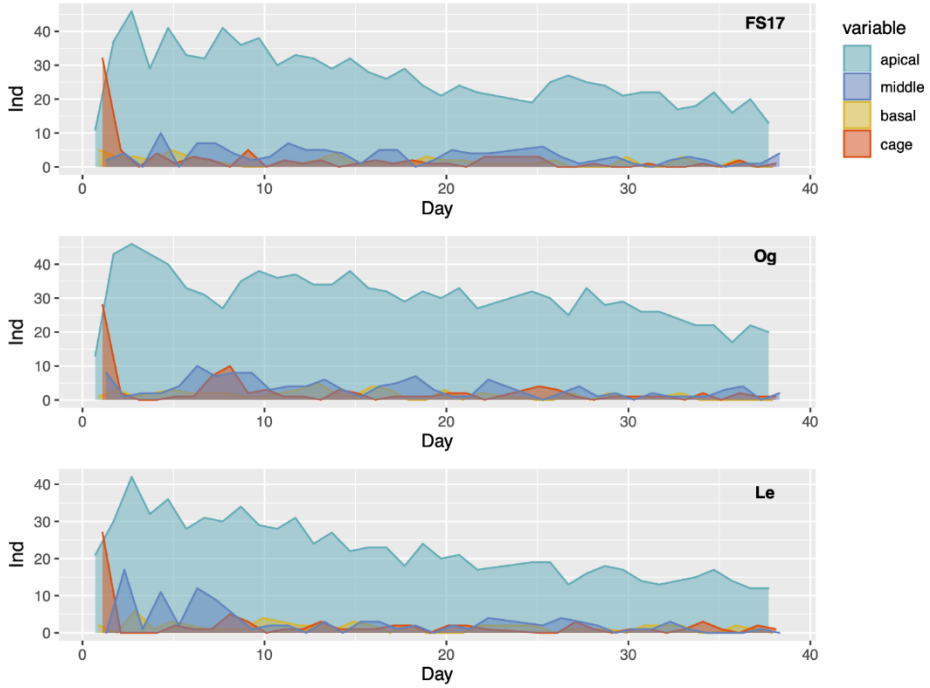


Figure 3. Total encounter of *Philaenus spumarius* individuals along the 38 days of experiment distributed in the apical part of the plant divided by tissue (leaf abaxial; leaf adaxial; petiole; stem) on the three olive varieties (FS17-top; Ogliarola-middle; Leccino-bottom plot).

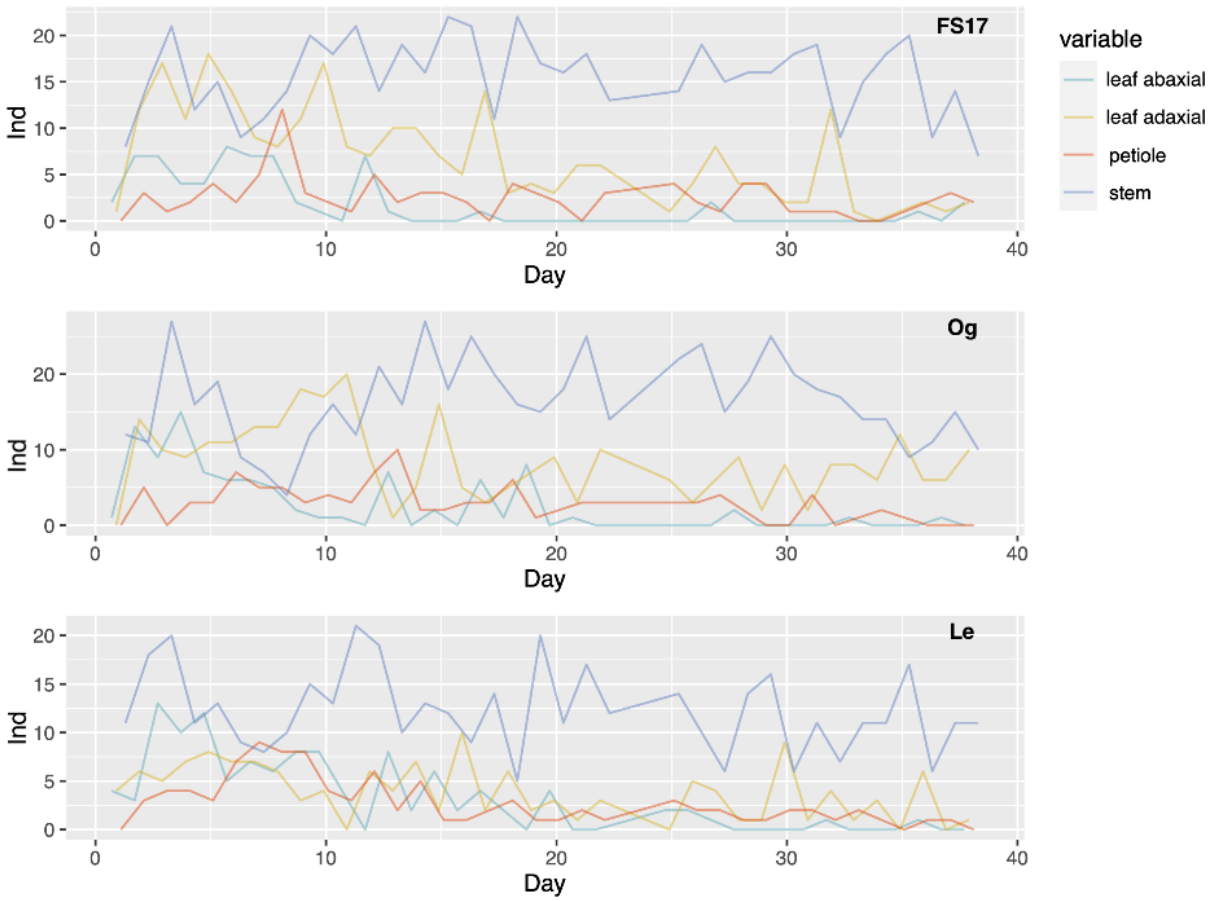


Figure 4. Total encounter of *Philaenus spumarius* individuals along the 72 hours of experiment distributed along the three olive varieties (FS-17; Leccino, Ogliarola).

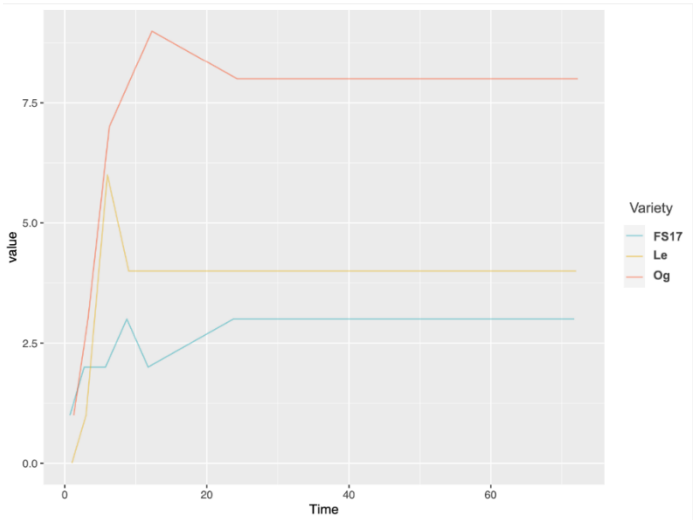
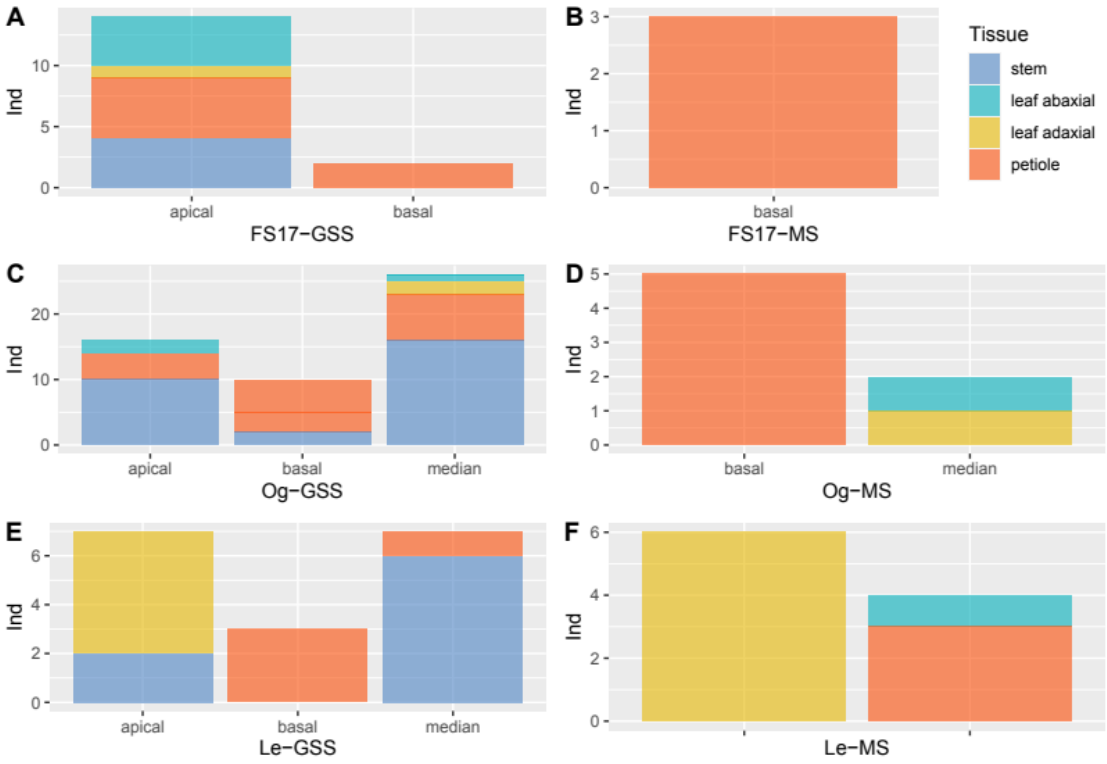


Figure 5. Total encounter of *Philaenus spumarius* individuals per variety, portion and plant tissue (choice trial)


A
Ind
10
5
0
apical
basal
FS17−GSS
B
Ind
3
2
1
0
basal
FS17−MS
Tissue
stem
leaf abaxial
leaf adaxial
petiole
C
Ind
20
10
0
apical
basal
median
Og−GSS
D
Ind
5
4
3
2
1
0
basal
median
Og−MS
E
Ind
6
4
2
0
apical
basal
median
Le−GSS
F
Ind
6
4
2
0
Le−MS

Online resource 2

Table 1. Summary statistics of non-sequential variables of olive varieties

	Varieties	N	Min	Max	Mean	Std. error	Stand. dev	Median
Tot_pr	Ogliarola	32	1	9	3.69	0.40	2.24	3.5
	Leccino	36	1	14	4.64	0.53	3.16	4
	Fs-17	31	1	12	4.55	0.47	2.59	4
Unsucc_pr	Ogliarola	32	0	7	2.00	0.38	2.16	1
	Leccino	36	0	13	3.06	0.53	3.15	2
	Fs-17	31	0	7	2.48	0.40	2.25	2
Initial_pr	Ogliarola	32	0	2	0.13	0.07	0.42	0
	Leccino	36	0	1	0.03	0.03	0.17	0
	Fs-17	31	0	2	0.19	0.09	0.48	0
Succ_pr	Ogliarola	32	1	4	1.56	0.14	0.80	1
	Leccino	36	1	4	1.56	0.13	0.77	1
	Fs-17	31	1	5	1.87	0.17	0.96	2
Sustained_pr	Ogliarola	32	1	3	1.50	0.12	0.67	1
	Leccino	36	1	3	1.50	0.12	0.70	1
	Fs-17	31	1	3	1.74	0.13	0.73	2
np_NWEI	Ogliarola	32	1	9	3.72	0.39	2.20	3.5
	Leccino	36	1	14	4.69	0.53	3.17	4
	Fs-17	31	1	12	4.55	0.47	2.59	4
C_NWEI	Ogliarola	32	2	13	5.81	0.58	3.30	5.5
	Leccino	36	1	18	6.03	0.61	3.65	6
	Fs-17	31	1	18	8.00	0.80	4.43	7
Xc_NWEI	Ogliarola	32	1	12	4.44	0.54	3.06	3
	Leccino	36	1	10	3.19	0.35	2.11	3
	Fs-17	31	1	14	5.06	0.60	3.35	4
Xi_NWEI	Ogliarola	32	2	31	10.56	1.12	6.35	9
	Leccino	36	1	22	9.44	0.87	5.23	8
	Fs-17	31	2	33	12.06	1.23	6.86	11
LF_NWEI	Ogliarola	32	0	12	2.34	0.53	2.98	1
	Leccino	36	0	18	2.72	0.64	3.84	2
	Fs-17	31	0	12	2.94	0.60	3.37	2
N_NWEI	Ogliarola	32	1	23	7.75	0.97	5.51	7
	Leccino	36	0	21	5.97	0.85	5.10	5
	Fs-17	31	0	27	8.00	1.00	5.58	8
R_NWEI	Ogliarola	32	0	4	0.38	0.15	0.87	0
	Leccino	36	0	2	0.17	0.08	0.51	0
	Fs-17	31	0	3	0.13	0.10	0.56	0
Unk_NWEI	Ogliarola	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	0	0.00	0.00	0.00	0
	Fs-17	31	0	0	0.00	0.00	0.00	0
Xe_NWEI	Ogliarola	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	2	0.08	0.06	0.37	0

	Fs-17	31	0	0	0.00	0.00	0.00	0
W_NWEI	**Ogliarola**	32	0	3	0.94	0.15	0.84	1
	Leccino	36	0	3	1.11	0.15	0.89	1
	Fs-17	31	0	5	1.39	0.18	0.99	1
Xi_LF_NWEI	**Ogliarola**	32	2	31	12.91	1.27	7.17	11
	Leccino	36	1	40	12.17	1.24	7.44	11
	Fs-17	31	2	34	15.00	1.45	8.09	13
np_WDI	**Ogliarola**	32	0.8	186.7	58.06	10.87	61.50	38.7
	Leccino	36	1.2	232.1	87.79	12.92	77.53	48.85
	Fs-17	31	0.4	200.2	76.80	10.80	60.11	59.8
C_WDI	**Ogliarola**	32	2.1	20.2	8.32	0.88	4.98	8.55
	Leccino	36	1.3	68.1	13.25	2.47	14.85	8.6
	Fs-17	31	0.6	50	12.98	1.72	9.58	11.3
Xc_WDI	**Ogliarola**	32	0.7	7.1	2.31	0.33	1.84	1.6
	Leccino	36	0.5	6.7	2.01	0.22	1.30	1.6
	Fs-17	31	0.7	10.7	3.28	0.45	2.51	2.7
Xi_WDI	**Ogliarola**	32	33.1	232.5	152.58	11.34	64.16	164.9
	Leccino	36	5.3	232.3	114.99	12.84	77.02	114.1
	Fs-17	31	15.5	226.6	130.88	10.67	59.38	134.8
LF_WDI	**Ogliarola**	32	0	176	12.89	5.65	31.98	2.6
	Leccino	36	0	121.9	15.91	5.15	30.91	4.4
	Fs-17	31	0	69.1	13.07	3.62	20.17	2.7
N_WDI	**Ogliarola**	32	0.1	3.8	1.50	0.17	0.97	1.35
	Leccino	36	0	3.9	1.14	0.16	0.96	0.9
	Fs-17	31	0	4.1	1.51	0.16	0.89	1.7
R_WDI	**Ogliarola**	32	0	20.6	2.20	1.00	5.64	0
	Leccino	36	0	27.8	1.32	0.87	5.24	0
	Fs-17	31	0	34.7	1.13	1.12	6.23	0
Unk_WDI	**Ogliarola**	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	0	0.00	0.00	0.00	0
	Fs-17	31	0	0	0.00	0.00	0.00	0
Xe_WDI	**Ogliarola**	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	2.4	0.07	0.07	0.40	0
	Fs-17	31	0	0	0.00	0.00	0.00	0
W_WDI	**Ogliarola**	32	0	6.4	0.50	0.21	1.18	0.2
	Leccino	36	0	5.6	0.56	0.20	1.20	0.1
	Fs-17	31	0	2.3	0.35	0.09	0.51	0.2
Xi_LF_WDI	**Ogliarola**	32	43.7	234.7	165.47	11.26	63.71	175.65
	Leccino	36	5.3	234	130.90	12.69	76.13	138.5
	Fs-17	31	24.2	227.2	143.95	10.46	58.25	152.7
max_Xi	**Ogliarola**	32	43.7	234.7	165.47	11.26	63.71	175.65
	Leccino	36	5.3	234	130.90	12.69	76.13	138.5
	Fs-17	31	24.2	227.2	143.95	10.46	58.25	152.7
max_LF	**Ogliarola**	32	0	46.4	5.47	1.60	9.04	2.1
	Leccino	36	0	48.6	6.98	1.92	11.52	2.9

	Fs-17	31	0	34.1	6.06	1.66	9.23	1.3
np_WDIp	**Ogliarola**	32	0.3	77.8	24.34	4.52	25.56	16.15
	Leccino	36	0.5	96.7	37.01	5.39	32.33	20.6
	Fs-17	31	0.2	83.4	32.00	4.50	25.05	24.9
C_WDIp	**Ogliarola**	32	0.9	8.4	3.51	0.36	2.06	3.55
	Leccino	36	0.6	28.4	5.67	1.06	6.38	3.6
	Fs-17	31	0.2	20.8	5.40	0.72	3.99	4.7
Xc_WDIp	**Ogliarola**	32	0.3	3	0.98	0.13	0.76	0.7
	Leccino	36	0.2	2.8	0.84	0.09	0.56	0.7
	Fs-17	31	0.3	4.5	1.37	0.19	1.04	1.1
Xi_WDIp	**Ogliarola**	32	13.8	96.9	64.42	4.80	27.16	70.4
	Leccino	36	2.2	96.8	48.49	5.32	31.92	48.85
	Fs-17	31	6.5	94.4	54.54	4.44	24.74	56.2
LF_WDIp	**Ogliarola**	32	0	73.3	5.38	2.35	13.32	1.1
	Leccino	36	0	50.8	6.71	2.15	12.88	1.85
	Fs-17	31	0	28.8	5.45	1.51	8.41	1.1
N_WDIp	**Ogliarola**	32	0	1.6	0.63	0.07	0.41	0.55
	Leccino	36	0	1.6	0.48	0.07	0.40	0.4
	Fs-17	31	0	1.7	0.62	0.07	0.36	0.7
R_WDIp	**Ogliarola**	32	0	1.6	0.63	0.07	0.41	0.55
	Leccino	36	0	1.6	0.48	0.07	0.40	0.4
	Fs-17	31	0	1.7	0.62	0.07	0.36	0.7
Unk_WDIp	**Ogliarola**	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	0	0.00	0.00	0.00	0
	Fs-17	31	0	0	0.00	0.00	0.00	0
Xe_WDIp	**Ogliarola**	32	0	0	0.00	0.00	0.00	0
	Leccino	36	0	0	0.00	0.00	0.00	0
	Fs-17	31	0	0	0.00	0.00	0.00	0
W_WDIp	**Ogliarola**	32	0	2.7	0.21	0.09	0.50	0.1
	Leccino	36	0	2.3	0.23	0.08	0.51	0.05
	Fs-17	31	0	1	0.13	0.04	0.22	0.1
Xi_LF_WDIp	**Ogliarola**	32	18.2	97.8	69.79	4.74	26.80	76.15
	Leccino	36	2.2	97.5	55.20	5.25	31.51	58.5
	Fs-17	31	10.1	94.7	59.97	4.36	24.27	63.6
np_WDEImd	**Ogliarola**	32	0.1	93.3	8.63	3.09	17.50	2.25
	Leccino	36	0.2	88.3	9.49	2.72	16.34	4.4
	Fs-17	31	0.3	83.2	13.09	3.16	17.61	5.9
C_WDEImd	**Ogliarola**	32	0.3	3.4	1.17	0.14	0.80	0.95
	Leccino	36	0.3	3.9	1.24	0.15	0.92	1
	Fs-17	31	0.4	3	1.07	0.12	0.65	0.9
Xc_WDEImd	**Ogliarola**	32	0.3	3.4	1.17	0.14	0.80	0.95
	Leccino	36	0.3	3.9	1.24	0.15	0.92	1
	Fs-17	31	0.4	3	1.07	0.12	0.65	0.9
Xi_WDEImd	**Ogliarola**	32	3.2	86.7	16.54	3.42	19.35	9.45
	Leccino	36	1.5	54.3	10.68	1.69	10.16	7.6

	Fs-17	31	1.8	40.8	9.91	1.56	8.71	7.5
LF_ WDElmd	**Ogliarola**	22	0.9	15.6	3.43	0.76	3.55	2.45
	Leccino	23	0.6	13.1	4.10	0.75	3.61	2.8
	Fs-17	22	0.7	5.7	2.28	0.31	1.46	2.1
N_ WDElmd	**Ogliarola**	32	0.1	0.3	0.20	0.01	0.06	0.2
	Leccino	33	0.1	0.3	0.21	0.01	0.06	0.2
	Fs-17	30	0.1	0.3	0.20	0.01	0.05	0.2
R_ WDElmd	**Ogliarola**	7	1.1	13.1	5.76	1.89	4.99	3.6
	Leccino	4	0.5	13.9	6.45	2.89	5.77	5.7
	Fs-17	2	0.4	11.9	6.15	5.75	8.13	6.15
W_ WDElmd	**Ogliarola**	22	0	6.4	0.64	0.30	1.38	0.2
	Leccino	27	0	4	0.46	0.17	0.91	0.2
	Fs-17	28	0	1.1	0.24	0.05	0.27	0.1
np_WDEI mn	**Ogliarola**	32	0.5	93.3	15.58	3.21	18.16	10.2
	Leccino	36	0.6	88.3	22.79	3.72	22.29	16.75
	Fs-17	31	0.3	83.2	21.04	3.66	20.40	15.9
C_ WDElmn	**Ogliarola**	32	0.4	4.1	1.58	0.15	0.84	1.3
	Leccino	36	0.4	14.4	2.26	0.42	2.52	1.45
	Fs-17	31	0.4	8.3	1.71	0.24	1.36	1.4
Xc_ WDElmn	**Ogliarola**	32	0.2	2.4	0.61	0.07	0.41	0.5
	Leccino	36	0.2	3.4	0.78	0.11	0.63	0.6
	Fs-17	31	0.3	4.6	0.79	0.14	0.77	0.6
Xi_ WDElmn	**Ogliarola**	32	5.6	77.5	19.52	3.06	17.33	13.45
	Leccino	36	1.6	54.5	13.44	1.75	10.49	11.1
	Fs-17	31	3.1	55.5	13.52	1.83	10.17	12.3
LF_ WDElmn	**Ogliarola**	22	0.9	19.6	4.08	0.89	4.19	2.75
	Leccino	23	0.6	16.4	5.04	0.89	4.24	3.6
	Fs-17	22	0.8	13.8	3.55	0.68	3.17	2.85
N_ WDElmn	**Ogliarola**	32	0.1	0.3	0.21	0.01	0.05	0.2
	Leccino	33	0.1	0.3	0.21	0.01	0.05	0.2
	Fs-17	30	0.1	0.3	0.21	0.01	0.04	0.2
R_ WDElmn	**Ogliarola**	7	1.1	13.1	5.74	1.89	5.00	3.5
	Leccino	4	0.5	13.9	6.45	2.89	5.77	5.7
	Fs-17	2	0.4	11.6	6.00	5.60	7.92	6
W_WDElm n	**Ogliarola**	22	0	6.4	0.64	0.29	1.38	0.2
	Leccino	27	0	4	0.52	0.18	0.95	0.2
	Fs-17	28	0	1.1	0.25	0.05	0.27	0.15

Table 2. Summary statistics of sequential variables of olive varieties

	Varieties	N	Min	Max	Mean	Std. error	Stand. dev	Median
np to first Xc	Ogliarola	32	1.6	63	9.28	2.01	11.37	6.85
	Leccino	36	2.5	43.4	11.02	1.32	7.94	9.05
	Fs-17	31	1.2	68	12.16	2.95	16.42	7.5
np to first Xi	Ogliarola	32	3.2	63.5	11.20	1.97	11.14	8.35
	Leccino	36	3.4	43.9	12.49	1.34	8.05	10.8
	Fs-17	31	2.3	69.4	14.61	2.90	16.14	10.7
np to first LF	Ogliarola	22	3.8	108.9	33.94	6.18	28.97	22.9
	Leccino	23	7.2	219.1	63.50	12.53	60.07	47.5
	Fs-17	22	3	108.8	39.78	7.30	34.25	26.15
first C to first Xc	Ogliarola	32	0.6	22	4.83	0.82	4.62	3.35
	Leccino	36	0.6	18.9	5.79	0.87	5.19	3.15
	Fs-17	31	0.4	29.5	5.83	1.35	7.53	2.5
first C to first Xi	Ogliarola	32	1.5	23.5	6.77	0.85	4.82	5.8
	Leccino	36	1.5	20.1	7.26	0.90	5.41	4.9
	Fs-17	31	1.8	30.4	8.30	1.35	7.50	6.7
first C to first LF	Ogliarola	22	3.3	108	28.88	5.88	27.60	17.45
	Leccino	23	6.3	216	59.62	12.24	58.70	45.7
	Fs-17	22	2.4	91.3	34.38	6.54	30.65	24.35
np to first sustained Xi	Ogliarola	32	3.7	82.6	16.24	2.76	15.64	11.75
	Leccino	35	3.4	52.9	15.07	1.60	9.48	13.4
	Fs-17	31	2.3	71.4	19.34	3.30	18.36	11.5
first C to first sustained Xi	Ogliarola	32	2.5	43.2	11.79	1.79	10.15	7.8
	Leccino	35	1.5	20.1	9.87	1.02	6.03	11
	Fs-17	31	1.9	63.6	13.02	2.08	11.57	9.4
time to first probe	Ogliarola	32	0.4	53.5	4.44	1.66	9.39	1.55
	Leccino	36	0.5	41.9	5.23	1.30	7.80	2.15
	Fs-17	31	0.2	49.7	6.31	1.96	10.89	1.9
time to first probe with Xi	Ogliarola	32	0.4	59	7.31	1.89	10.69	5
	Leccino	36	0.5	41.9	9.11	1.41	8.47	7
	Fs-17	31	0.2	65.6	11.09	2.89	16.09	6.2
time to first probe with LF	Ogliarola	22	0.5	95.1	16.45	5.11	23.99	6.45
	Leccino	23	0.6	156.3	29.97	9.18	44.03	14
	Fs-17	22	0.2	69.1	16.85	4.59	21.53	7.5
time to first probe with sustained Xi	Ogliarola	32	0.4	59	7.31	1.89	10.69	5
	Leccino	35	0.5	41.9	9.00	1.45	8.56	6.6
	Fs-17	31	0.2	65.6	11.09	2.89	16.09	6.2

Online resource 3

Table 1. Number of xylem vessel counted and analyzed portions per leaf and variety.

Countings							
	Portion	Ogliarola		FS17		Leccino	
		Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Number of xylem vessels analyzed	Stem	653	371	303	338	420	320
	Petiole	312	194	160	240	199	168
	Midrib	340	251	245	432	188	284
Number of xylem vessels analyzed	Stem	105	108	115	113	217	77
	Petiole	64	85	57	60	105	60
	Midrib	62	75	66	61	90	60
Number of areas (mm2) analyzed	Stem	7	12	10	20	6	14
	Petiole	10	8	10	6	10	9
	Midrib	8	10	10	9	8	10

Table 2. Summary statistics of xylem vessels diameter across varieties and plant portions.

Xylem vessels diameter													
		n	mean	sd	median	trimmed	mad	min	max	range	skew	kurtosis	se
Midrib	Ogliarola	591	23.27	5.09	23	23.32	5.93	10	36	26	-0.07	-0.61	0.21
	FS17	676	30.44	5.98	30	30.26	5.93	15	52	37	0.3	0.02	0.23
	Leccino	472	23.37	4.28	23	23.19	4.45	13	39	26	0.4	0.24	0.2
Petiole	Ogliarola	506	23.43	3.54	23	23.35	2.97	13	37	24	0.23	0.11	0.16
	FS17	400	30.31	4.74	30.5	30.28	5.19	17	46	29	0.08	0.02	0.24
	Leccino	367	21.59	3.58	21	21.44	2.97	11	34	23	0.43	0.68	0.19
Stem	Ogliarola	1024	25.27	4.04	25	25.21	4.45	13	43	30	0.21	0.36	0.13
	FS17	641	37.05	5.93	37	37.08	5.93	19	58	39	-0.08	-0.09	0.23
	Leccino	740	25.7	4.85	25	25.61	4.45	14	39	25	0.18	-0.37	0.18

Table 3. Estimated marginal means of xylem vessels diameter across varieties and plant portions.

Species	Portion	emmean	SE	df	lower.CL	upper.CL
Ogliarola	Midrib	4.79	0.019	5407	4.76	4.83
FS17	Midrib	5.49	0.0177	5407	5.46	5.53
Leccino	Midrib	4.81	0.0212	5407	4.77	4.86
Ogliarola	Petiole	4.83	0.0205	5407	4.79	4.87
FS17	Petiole	5.49	0.0231	5407	5.44	5.53
Leccino	Petiole	4.63	0.0241	5407	4.58	4.68
Ogliarola	Stem	5.01	0.0144	5407	4.98	5.04
FS17	Stem	6.07	0.0182	5407	6.03	6.1
Leccino	Stem	5.05	0.017	5407	5.01	5.08

Table 4. Pairwise comparisons of xylem vessels diameter between varieties within each plant portion.

contrast	estimate	SE	df	t.ratio	p.value
Ogliarola Midrib - FS17 Midrib	-0.69649	0.026	5407	-26.804	<.0001
Ogliarola Midrib - Leccino Midrib	-0.02	0.0285	5407	-0.702	0.9988
FS17 Midrib - Leccino Midrib	0.67649	0.0277	5407	24.442	<.0001
Ogliarola Petiole - FS17 Petiole	-0.66109	0.0309	5407	-21.414	<.0001
Ogliarola Petiole - Leccino Petiole	0.19627	0.0316	5407	6.204	<.0001
FS17 Petiole - Leccino Petiole	0.85735	0.0334	5407	25.706	<.0001
Ogliarola Stem - FS17 Stem	-1.05575	0.0232	5407	-45.43	<.0001
Ogliarola Stem - Leccino Stem	-0.03541	0.0223	5407	-1.59	0.8104
FS17 Stem - Leccino Stem	1.02034	0.0249	5407	40.983	<.0001

Table 5. Summary statistics of xylem vessels depth across varieties and plant portions.

Xylem vessels depth													
		n	mean	sd	median	trimmed	mad	min	max	range	skew	kurtosis	se
Midrib	Ogliarola	137	114.34	20.49	112	112.11	20.76	82	186	104	1.1	1.41	1.75
	FS17	127	124.12	19.13	123	123.31	19.27	84	180	96	0.46	-0.15	1.7
	Leccino	150	106.63	18.89	107	106.75	19.27	58	151	93	-0.11	-0.39	1.54
Petiole	Ogliarola	149	153.95	22.11	149	153.03	23.72	112	205	93	0.37	-0.91	1.81
	FS17	117	165.16	22.05	163	164.96	23.72	119	217	98	0.1	-0.63	2.04
	Leccino	165	110.88	31.36	101	108.73	34.1	60	192	132	0.53	-0.8	2.44
Stem	Ogliarola	213	91.99	10.79	93	92.89	10.38	57	115	58	-0.75	0.47	0.74
	FS17	228	180.59	75.97	202.36	177.32	122.11	81	332.54	251.54	0.18	-1.61	5.03
	Leccino	294	98.68	16.58	96	97.12	14.83	64	162	98	1	1.34	0.97

Table 6. Estimated marginal means of xylem vessels depth across varieties and plant portions.

Species	Portion	emmean	SE	df	lower.CL	upper.CL
Ogliarola	Midrib	4.73	0.0198	1570	4.69	4.76
FS17	Midrib	4.81	0.0206	1570	4.77	4.85
Leccino	Midrib	4.65	0.0189	1570	4.62	4.69
Ogliarola	Petiole	5.03	0.019	1570	4.99	5.06
FS17	Petiole	5.1	0.0214	1570	5.06	5.14
Leccino	Petiole	4.67	0.018	1570	4.64	4.71
Ogliarola	Stem	4.52	0.0159	1570	4.48	4.55
FS17	Stem	5.1	0.0153	1570	5.07	5.13
Leccino	Stem	4.58	0.0135	1570	4.55	4.61

Table 7. Pairwise comparisons of xylem vessels depth between varieties within each plant portion.

contrast	estimate	SE	df	t.ratio	p.value
Ogliarola Midrib - FS17 Midrib	-0.08506	0.0285	1570	-2.98	0.0718
Ogliarola Midrib - Leccino Midrib	0.07157	0.0274	1570	2.614	0.1815
FS17 Midrib - Leccino Midrib	0.15663	0.0279	1570	5.606	<.0001
Ogliarola Petiole - FS17 Petiole	-0.07143	0.0286	1570	-2.496	0.2347
Ogliarola Petiole - Leccino Petiole	0.35648	0.0262	1570	13.613	<.0001
FS17 Petiole - Leccino Petiole	0.42791	0.028	1570	15.279	<.0001
Ogliarola Stem - FS17 Stem	-0.58716	0.0221	1570	-26.591	<.0001
Ogliarola Stem - Leccino Stem	-0.06443	0.0208	1570	-3.09	0.0524
FS17 Stem - Leccino Stem	0.52273	0.0204	1570	25.564	<.0001

Table 8. Summary statistics of xylem vessels aggregation across varieties and plant portions.

Xylem vessels aggregation													
		n	mean	sd	median	trimmed	mad	min	max	range	skew	kurtosis	se
Midrib	Ogliarola	17	4710.88	2253.14	4915	4630	2432.95	1350	9285	7935	0.43	-0.84	546.47
	FS17	19	2955.63	1074.3	3079	2950.12	1146.05	1219	4786	3567	-0.3	-1.23	246.46
	Leccino	16	3291.81	1894.25	3027	3047.79	898.46	1138	8862	7724	1.45	2.1	473.56
Petiole	Ogliarola	18	3241.89	1700.3	3260.5	3219.06	2120.86	768	6081	5313	0	-1.54	400.76
	FS17	16	710.81	342.73	650.5	691.93	230.54	169	1517	1348	0.6	-0.15	85.68
	Leccino	17	2043.59	1139.44	1616	1912.73	830.26	920	5130	4210	1.19	0.62	276.35
Stem	Ogliarola	19	4336.47	1490.83	4235	4311.12	2109.74	2425	6679	4254	0.04	-1.64	342.02
	FS17	30	1916.5	1143.7	1889	1823.12	1339.53	315	4536	4221	0.48	-0.64	208.81
	Leccino	20	2909.2	1396.84	2775	2707.75	1090.45	1341	7533	6192	1.71	3.34	312.34

Table 9. Estimated marginal means of xylem vessels aggregation across varieties and plant portions.

Species	Portion	emmean	SE	df	lower.CL	upper.CL
Ogliarola	Midrib	66.7	3.06	162	60.7	72.8
FS17	Midrib	53.4	2.9	162	47.7	59.1
Leccino	Midrib	55.5	3.16	162	49.2	61.7
Ogliarola	Petiole	54.8	2.98	162	48.9	60.6
FS17	Petiole	25.9	3.16	162	19.7	32.1
Leccino	Petiole	43.8	3.06	162	37.7	49.8
Ogliarola	Stem	64.9	2.9	162	59.2	70.6
FS17	Stem	41.7	2.3	162	37.2	46.3
Leccino	Stem	52.7	2.82	162	47.2	58.3

Table 10. Pairwise comparisons of xylem vessels aggregation between varieties within each plant portion.

contrast	estimate	SE	df	t.ratio	p.value
Ogliarola Midrib - FS17 Midrib	13.329	4.21	162	3.163	0.0475
Ogliarola Midrib - Leccino Midrib	11.256	4.4	162	2.56	0.2122
FS17 Midrib - Leccino Midrib	-2.073	4.28	162	-0.484	0.9999
Ogliarola Petiole - FS17 Petiole	28.857	4.34	162	6.653	<.0001
Ogliarola Petiole - Leccino Petiole	10.967	4.27	162	2.569	0.2083
FS17 Petiole - Leccino Petiole	-17.89	4.4	162	-4.068	0.0023
Ogliarola Stem - FS17 Stem	23.181	3.7	162	6.262	<.0001
Ogliarola Stem - Leccino Stem	12.159	4.04	162	3.006	0.073
FS17 Stem - Leccino Stem	-11.021	3.64	162	-3.024	0.0697

Figure 1. Distribution of xylem vessel diameter in stem, midrib, and petiole of three olive varieties.

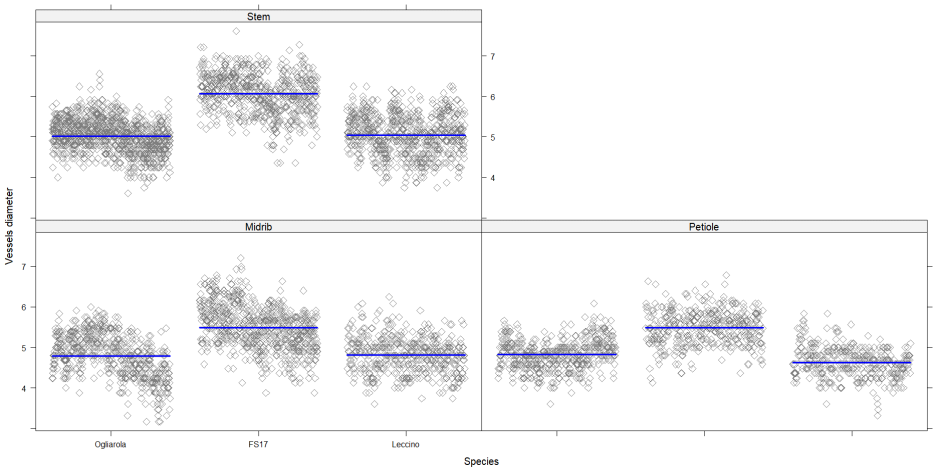


Figure 2. Distribution of xylem vessel depth in stem, midrib, and petiole of three olive varieties.

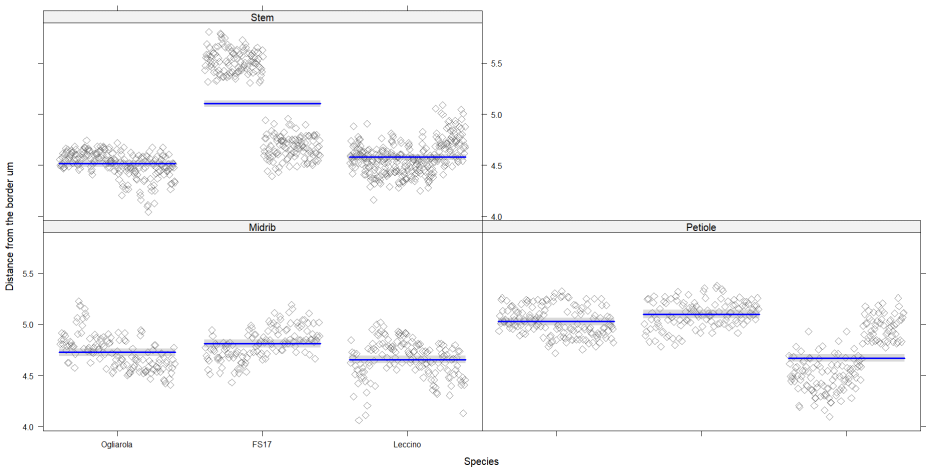
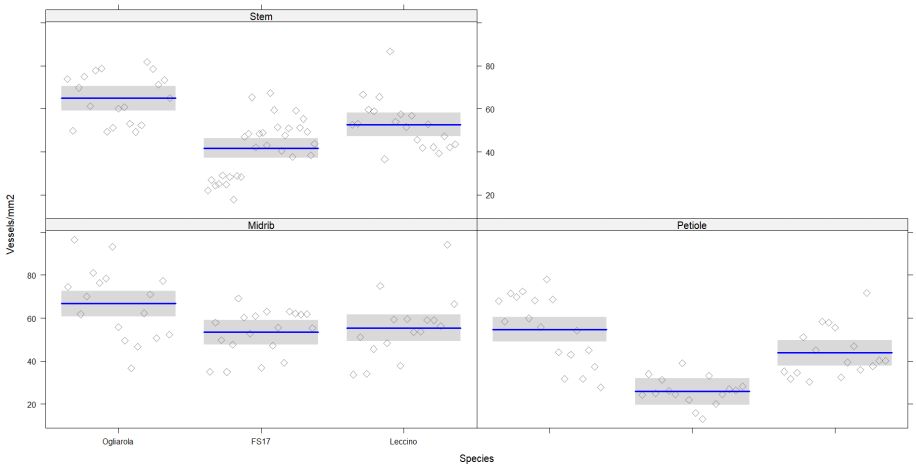


Figure 3. Distribution of xylem vessel density in stem, midrib, and petiole of three olive varieties.



CHAPTER III

Unravelling xylem sap chemical traits mediating spittlebug-olive interaction

4. Unravelling xylem sap chemical traits mediating spittlebug-olive interaction through a metabolomics approach

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Under review

Abstract

- Xylem sap chemistry is crucial for host plant acceptance and settling by xylem feeders as *Philaenus spumarius*, the main European vector of *Xylella fastidiosa (Xf)* to olive. Knowledge on specific metabolites underlying host suitability for vectors is limited.
- We combined spittlebug probing behavior characterization using Electropenetrography and UHPLC-Q-Exactive-MS/MS analysis of xylem sap of four olive varieties differing in their susceptibility to *Xf* (Coratina, FS-17, Leccino and Ogliarola) to identify compounds putatively affecting host plant acceptance. Thereafter, we extended probing behavior analysis to 14 olive varieties (including the previously studied), calculated the xylem ingestion index, quantified the compounds selected in the first experiment (biomarkers), and inferred their role in shaping plant suitability.
- Partial least squares regression analysis revealed the compounds with the greatest influence on plant suitability for the spittlebug, namely glutamine, malic acid, and daucic acid acting as potential phagostimulants, and gluconic acid, salidroside, oleuropein diglucoside, and D-manno-2-heptulose discouraging feeding. Additionally, independently of the effect of single organic acids on plant suitability for spittlebugs, least suitable olive varieties were characterized by higher concentrations of all the selected biomarkers belonging to this chemical class.
- Our findings represent a crucial step for *Xf* management spread through either the selection of germplasm resistant to the vector, or agronomic practices reducing plant suitability and vector activity by manipulating xylem chemistry.

Key words: Antixenosis, Host acceptance, Meadow spittlebug, Phagostimulant, Phagodeterrent, *Xylella fastidiosa*.

Introduction

Insects rely on a variety of external sensory inputs, such as visual, olfactory and gustatory cues, to locate food sources and assess their suitability. Notably, taste plays a crucial role in the ultimate decision to accept or reject a plant the insect landed on (Jaenike, 1990; Chapman, 2003). Piercing-sucking insects possess specialized gustatory receptors enabling the recognition of chemicals that might promote (phagostimulants) or discourage (phagodeterrents) feeding (Andersen *et al.,* 1989, 1992; Brodbeck *et al.,* 1990; Tjallingii 1995; Pompon *et al.,* 2011; Kordan *et al.,* 2012). Sensilla perceiving these stimuli have been found on labial and precibarial territories in Hemipterans (Wensler & Filshie, 1969; Backus & McLean, 1985; Backus, 1988).

Phagodeterrent metabolites are part of a wide array of strategies adopted by plants to minimize the damage caused by herbivores. Anatomical and/or chemical plant traits interfering with the dynamics of insect-plant interaction, by hampering landing and/or host recognition upon alighting, and/or reducing the plant's palatability, are defined antixenotic traits (Kogan & Ortman, 1978; Kordan *et al.,* 2019). Chemical traits play a key role in mediating antixenosis by altering the plant's suitability and nutritional value, thereby affecting herbivore feeding behavior and survival (Mitchell *et al.,* 2016). For examples, alkaloids (*e.g.,* lupanine and quinine), flavonoids (*e.g.,* luteolin and genistein) and terpenoids (*e.g.,* β-damascone and gossypol) were reported to interfere with aphid nervous system and affect taste perception, rendering the plants unpalatable (Simmonds, 2001; Goławska & Łukasik, 2012; Kordan *et al.,* 2012; Gabryś *et al.,* 2015). Additionally, organic acids (*e.g.,* oxalic acid and trans-aconitic acid) and flavonoids (*e.g.,* apigenin-C-glycosides) were observed to discourage feeding by planthoppers as *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) (Yoshihara, 1980; Grayer *et al.,* 1993; Kang *et al.,* 2023).

In the case of insect vectors of plant pathogens, antixenotic traits play a pivotal role in shaping the spread of the pathogen, given they affect a key epidemiological parameter as vector activity. According to Purcell (1981) definition, vector activity is the number of insect vectors on a host plant per time they spend on that host. For some vector-borne plant pathogens, like the xylem-limited bacterium *Xylella fastidiosa* Well*,* the greater the number of insects on a plant, the longer the time they spend on it, and the higher the likelihood of pathogen transmission to occur (Daugherty & Almeida, 2009). A plant resistant or less susceptible to an insect vector, *i.e.,* displaying anatomical and chemical traits reducing palatability for the insect, could theoretically be a poor source/recipient for a pathogen as *X. fastidiosa*, given the expected low

vector activity. Specifically, by deterring or delaying probing and feeding activities fundamental for pathogen transmission and shortening the insect-plant interaction, antixenotic defenses can limit the ability of the vector to acquire pathogens from infected plants (source) and subsequently inoculate them to recipient ones (Le Roux *et al.,* 2010; Kordan *et al.*, 2012, 2019). On the other hand, a plant providing all the necessary cues for location and acceptance and recognized by the insect vector as a suitable host, could play an essential role in pathogen spread given the expected higher vector activity. In this context, understanding the role of plant traits in host location and acceptance by an insect vector is crucial to characterize the epidemiology of a vector-borne plant pathogen.

The meadow spittlebug *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) is the preeminent European vector of *X. fastidiosa* subsp. *pauca* (ST53), the causal agent of the Olive Quick Decline Syndrome, a disease resulting in a massive socio-economic impact on the Southern Italian olive heritage (Schneider *et al.,* 2020). This xylem sap feeding insect spreads the bacterium, making it the primary target of current control strategies, which mainly rely on ground cover removal and pesticides' applications. Such control strategy yielded quite inconsistent results in terms of limiting the bacterium spread besides posing serious threat to the agroecosystems and human health (Lago *et al.,* 2022; Labadessa *et al.*, 2024). Given the dynamics of *X. fastidiosa* transmission by spittlebugs, with a relatively low transmission efficiency compensated by high vector activity (Cornara *et al.,* 2020), better results in limiting pathogen spread could be achieved by reducing vector-plant interaction and interfering with host plant location and acceptance, thus curbing vector activity. However, vector and transmission control strategies aimed at interfering with host location and acceptance require an in-depth knowledge of visual, olfactory and gustatory cues underlying these processes (Avosani *et al.,* 2024). Host plant location in spittlebugs has been recently proven to be possibly mainly mediated by vision (Lazar *et al.,* 2025). Upon landing (*i.e.*, host location), tasting of xylem sap and recognition of a favorable chemical profile underlie host acceptance. As recently reported by Cornara *et al.,* (2024), a more favorable xylem sap chemical profile independently of xylem anatomy might explain the higher preference for some olive varieties over others. The aforementioned study focused solely on primary metabolites (*i.e.,* amino acids, organic acids and sugars) overlooking the potential role of secondary metabolites in influencing spittlebugs' host acceptance. Secondary metabolites, such as phenols and terpenes, play a significant role in reducing susceptibility and/or enhancing tolerance to a range of biotic and abiotic stresses in olive trees (Novelli *et al.,* 2019; Vergine *et al.,* 2020; Boutaj *et al.,* 2021;

Anguita-Maeso *et al.,* 2023). Overall, knowledge of xylem sap metabolites' role in plant acceptance or rejection by spittlebugs is crucial for informing breeding programs and sustainable bacterium management strategies based on the adoption of germplasm less susceptible to the spittlebugs, thus theoretically less prone to serve as source/recipient of the bacterium given the reduced vector activity.

Here, coupling spittlebug's probing and feeding behavior analysis through Electropenetrography (EPG) with xylem sap metabolome characterization using UHPLC-(HR)-MS and UHPLC-(HR)-MS/MS, we aimed at identifying the xylem sap chemical components underlying olive plants suitability for *P. spumarius*, thus eventually influencing also the spread of *X. fastidiosa* within agroecosystems.

Materials and Methods

1) Insects and plants rearing

Philaenus spumarius 3rd/4th instar nymphs were collected in an *X. fastidiosa*-free area in Apulia region (Southern Italy) and reared on ten days old vetch (*Vicia sativa* L.) and fava bean (*Vicia faba* L.) plants until adulthood with host plants replaced on a weekly basis. Newly molted adults were collected and caged on a mix of potted grapevine (*Vitis vinifera* L.), common amaranth (*Amaranthus retroflexus* L.), fava bean and vetch with a sex ratio 1:1. Both nymphs and adults were reared in Bugdorms inside an insect-proof shaded screenhouse located in the premises of the Department of Soil, Plant and Food Sciences (DiSSPA), University of Bari Aldo Moro, at temperature 22 ± 2°C and relative humidity 60 ± 5% (no photoperiod control) (Bari, South-Eastern Italy). Herbaceous plants were replaced weekly; grapevines were pruned monthly. Plants were watered every other day.

Olive plants used for EPG-assisted observations were one-year old seedlings grown in Typical Brill substrate (Brill® Substrate 70 L, Italia) and perlite (PERALIT25—Perlite Italiana srl, MI, Italia) (2:1) inside 1 L pots. Plants were maintained inside an insect proof greenhouse, at temperature 22 ± 2°C and relative humidity 60 ± 5% (no photoperiod control), watered every other day and fertilized once a week with Plantafol 20:20:20 (NPK, Valagro) 1 g L^{-1}. To avoid interference with spittlebug probing behavior, no pesticides were applied.

2) Characterization of xylem sap metabolites mediating olive plants suitability

The goal was to characterize the olive xylem sap metabolome, and to select the compounds most likely to play a role in plant suitability for the spittlebug (biomarkers) and to be quantified and correlated with feeding behavior in the second trial (see details below). The initial qualitative screening was conducted using three olive varieties, namely Ogliarola Salentina ("hereafter Ogliarola"), Leccino and FS-17 with a different degree of suitability for the spittlebug (Cornara *et al.,* 2024) and for *X. fastidiosa* (Saponari *et al.,* 2017), in addition to a fourth variety, *i.e.,* Coratina, whose acceptability for the spittlebug is currently unknown.

2.1) EPG-assisted analysis of *Philaenus spumarius* probing behavior

The probing behavior of both females and males *P. spumarius* on the four olive varieties (namely, Coratina, FS-17, Leccino, and Ogliarola), was assessed by means of EPG (Electrical Penetration Graph) technique, following the protocol described by Markheiser *et al.* (2023). Recordings were carried out during two temporal replicates, namely May-June 2024 (spring) and September-October 2024 (fall), corresponding to the two spittlebug population peaks observed on olive plants under field conditions in the Apulia Region (Cornara *et al.,* 2017).

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 (EPG Systems, Wageningen, Netherlands (epgsystems.eu)). For the connection to the EPG, one to two (spring recordings) and four to five (fall recordings) months old spittlebug adults were anesthetized by storing them at -20°C for 2 minutes, then immobilized with a vacuum device and tethered to 18 µm gold wire (EPG-Systems, Wageningen, Netherlands) with a double layer of silver conductive paint (Pelco®, no. 16062, Ted Pella, USA). Wired spittlebugs were, then, starved inside aerated Petri-dishes (where they were able to move and get accustomed to the wire) for about one hour. At the end of the starvation period, the wire-electrode connection on the insect was strengthened with a drop of conductive silver paint, before plugging the electrode with the insect into the EPG probe. The probing behavior of each spittlebug was recorded for 6 h. The recordings were performed inside a Faraday cage, under controlled conditions (23 ± 1°C, 60 ± 5% HR, supplemented with artificial lights 1,000 ± 200 lx). The insects were offered the apical 5 cm portion of olive plants, with access to stem and at least one petiole/leaf. A minimum of ten replicates/recordings per sex per variety and per season (spring and fall) were carried out, with single combinations of insect/olive plant (one plant per spittlebug). The position of each variety was switched at each replicate to avoid any positional bias.

EPG waveforms were manually marked using 'Stylet+a' software (EPG Systems, Wageningen, Netherlands) and then analyzed by "XylFeed" MS Excel macro (Markheiser *et al.,* 2023). Following the protocol described by Spadavecchia-Hamouche *et al.* (2024), EPG waveforms considered were 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) N, non-pathway interruption of xylem activity; vii) N2, second type of non-pathway xylem interruption; viii) R, resting; ix) Xe, behavior associated with *X. fastidiosa* inoculation; x) W,

stylets withdrawal; xi) Esc, escaping of the individual from the host plant. EPG variables considered were: i) Total probing time - TPT (time spent by the spittlebugs with the stylets inserted in plant tissues over the 6 h); ii) Total residence time - TRT (time spent by the spittlebugs on the plant without escaping over the 6 h); iii) Probes: number of probes without (unsuccessful) and with xylem ingestion (successful); number of probes with only xylem vessel contact (initial); number of probes with a xylem ingestion event longer than 5 min (sustained); iv) NWEI: number of waveform events per insect; v) WDEI: median duration of the single waveform event per insect; vi) WDI/TPT: total duration of the waveform per insect over the total probing time; vii) WDI/TRT: total duration of the waveform per insect over the total residence time; viii) Sequential: time required to perform a certain waveform calculated either from the beginning of the EPG recording or from the first probe. To account for "escape" events, the ratios WDI/TPT and WDI/TRT were analyzed instead of considering WDI alone (*i.e.*, total time spent by spittlebugs engaging in a specific waveform).

A detailed explanation of the EPG variables analyzed is provided in Table 1.

Statistical analysis of the EPG data was carried out using R software (Version 4. 3. 1). Variety-associated probing behavioral differences (*i.e.,* total probing time, total residence time, probes, non-sequential and sequential variables) were investigated with Generalised Linear Mixed Models using Template Model Builder (GLMM.TMB). GLMMs were chosen for their flexibility in handling non-normal response distributions while incorporating both fixed and random effects, allowing for the modeling of complex relationships and providing a robust framework for comparing treatments while accounting for potential sources of variation. Olive variety and sex were used as covariates, with the day of the recording as random factor. EPG datasets generated during spring and fall were analyzed separately. The data obtained from the EPG were transformed with ln (x+1) or $\sqrt{}$ (x) to reduce heteroscedasticity and improve normal distribution. Models were run using the "glmmTMB" package (Venables & Ripley, 2002), while residual distribution was checked using the "DHARMa" package (Fox & Weisberg, 2019). An analysis of variance (ANOVA) was performed using the "car" package to assess the overall significance of fixed effects in the model. For statistically significant effects (p<0.05), pairwise comparisons were conducted by Tukey's Honest Significant Differences (HSD) test, using the "emmeans" package (Lenth, 2021). A summary of the raw datasets was prepared by "psych" package (Revelle, 2021). Plots were generated using visreg package (Breheny & Burchett, 2017).

Table 1. Definition of EPG variables analyzed in the study.

	Variable abbreviation	Variable definition
Probes	**Unsucc_pr**	Unsuccessful probe (probe where the insect does not reach the xylem)
	Initial_pr	Initial probe (probe where the insect reaches the xylem but does not ingest); this excludes Xi and LF
	Succ_pr	Successful probe (probe where the insect reaches xylem ingestion); this includes Xi and LF
	Sustained_pr	Probes with sustained ingestion: Xi or LF longer than 5min; part of Succ_probe
	Tot_pr	Total number of probes per experiment (unsuccessful + initial + successful)
	TPT	Total probing time (time spent by the spittlebugs with the stylets inserted in plant tissues over the 6h)
	TRT	Total residence time (time spent by the spittlebugs on the plant without escaping over the 6h)
Non-sequential variables	**np_NWEI**	non-probing - number of events per individual
	C_NWEI	pathway - number of events per individual
	Xc_NWEI	xylem contact - number of events per individual
	Xi_NWEI	xylem ingestion - number of events per individual
	LF_NWEI	low frequency xylem ingestion - number of events per individual
	N_NWEI	xylem non-pathway interruption - number of events per individual
	R_NWEI	resting - number of events per individual
	Unk_NWEI	unknown - number of events per individual
	Esc_NWEI	escapes from the EPG - number of events per individual
	Xe_NWEI	xylem egestion - number of events per individual
	W_NWEI	stylets withdrawal - number of events per individual
	np_WDEImd	non-probing – duration per event per individual (median)
	C_WDEImd	pathway – duration per event per individual (median)
	Xc_WDEImd	xylem contact – duration per event per individual (median)
	Xi_WDEImd	xylem ingestion – duration per event per individual (median)
	LF_WDEImd	low frequency xylem ingestion – duration per event per individual (median)
	N_WDEImd	non-pathway interruption – duration per event per individual (median)
	R_WDEImd	resting – duration per event per individual (median)
	Unk_WDEImd	unknown waves – duration per event per individual (median)
	Esc_WDEImd	escapes from the EPG – duration per event per individual (median)
	Xe_WDEImd	xylem egestion – duration per event per individual (median)
	W_WDEImd	stylets withdrawal – duration per event per individual (median)
	np_WDI/TRT	Percentage of non-probing duration per total residence time per individual

	C_WDI/TPT	Percentage of pathway duration per total probing time per individual
	C_WDI/TRT	Percentage of pathway duration per total residence time per individual
	Xc_WDI/TPT	Percentage of xylem contact duration per total probing time per individual
	Xc_WDI/TRT	Percentage of xylem contact duration per total residence time per individual
	Xi_WDI/TPT	Percentage of xylem ingestion duration per total probing time per individual
	Xi_WDI/TRT	Percentage of xylem ingestion duration per total residence time per individual
	LF_WDI/TPT	Percentage of low-frequency xylem ingestion duration per total probing time per individual
	LF_WDI/TRT	Percentage of low-frequency xylem ingestion duration per total residence time per individual
	Xi_LF_WDI/TPT	Percentage of total xylem ingestion duration (Xi+LF) per total probing time per individual
	Xi_LF_WDI/TRT	Percentage of total xylem ingestion duration (Xi+LF) per total residence time per individual
	R_WDI/TPT	Percentage of resting duration per total probing time per individual
	R_WDI/TRT	Percentage of resting duration per total residence time per individual
Sequential variables	**np to 1st Xc**	time from the beginning of the recording to the first xylem contact
	np to 1st Xi	time from the beginning of the recording to the first xylem ingestion
	np to 1st LF	time from the beginning of the recording to the first low frequency xylem ingestion
	1st C to 1st Xc	time from the first probe to the first xylem contact
	1st C to 1st Xi	time from the beginning of the first probe to the first xylem ingestion
	1st C to 1st LF	time from the beginning of the first probe of the recording to the first low frequency xylem ingestion
	np to 1st sustained Xi	time from the beginning of the recording to the start of the first sustained Xi
	1st C to 1st sustained Xi	time from the first absolute probe to the start of the first sustained Xi
	Time to the 1st probe	time from the beginning of the recording to the first probe
	Time to the 1st probe with Xi	time from the beginning of the recording to the first probe with xylem ingestion
	Time to the 1st probe with sustained Xi	time from the beginning of the recording to the first probe with sustained Xi

2.2) Identification of metabolites from different xylem sap samples by UHPLC-Q-Exactive-MS/MS analysis

At the end of the 6 h EPG recordings, xylem sap was extracted from half of the plants tested [10 randomly selected samples per variety per season (5 per sex)], following the protocols described in Anguita-Maeso *et al.* (2021) and Cornara *et al.* (2024) using a Scholander pressure chamber (PMS Instruments). Briefly, at the end of the EPG assay, plants spittlebugs fed on were excised at the base; two basal cm of the main stem was debarked and disinfected with sterile paper moistened in absolute ethanol. Thereafter, the cutting was inserted upside down into the disinfected chamber. Ethanol was left to evaporate before sap extraction. The applied pressure was gradually increased up to a maximum of 25 bars, to avoid external contamination from cell rupture. Xylem sap was collected in 5 mL sterile Eppendorf tubes containing 100 µL of absolute ethanol placed on ice. An average of 2-3 mL of xylem sap was collected from each tested plant. Samples were stored at -80°C until the chemical analysis.

Olive xylem sap samples were first dried under nitrogen and later dissolved in sterile water containing 0.1% formic acid at 1 mg mL^{-1} concentration, then centrifuged at 13,200 rpm for 2 min. The supernatants were transferred to LC-MS microvials for an untargeted analysis to identify and characterize the main metabolites by means of a UHPLC-Q-Exactive (Thermo Fisher Scientific, Bremen/Germany). The mobile phases used for chromatographic separation were water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), with the following increasing linear gradient (v/v) at a flow rate of 0.200 mL min^{-1} of solvent B: 0-20 min, from 2 to 65%; 20-25 min, from 65 to 98%; 25-26 min, from 98 to 2% and another 4 min to 2%. Analyses were performed in negative ion mode. A volume of 10 µL of each sample was injected into the instrument. A Kinetex EVO 5.0 µm (150 mm × 2.1 mm) chromatography column (Phenomenex, Aschaffenburg, Germany) was used. The ESI source parameters were as follows: ion source temperature 300 °C; sheath and auxiliary gas flow (N2), 50 and 10; sweep gas 0. The full range *m/z* adapted for MS spectrum acquisition was 66.7-1000. For the fragmentation study, a data-dependent scan was set up. In developing a data-dependent scan for MS/MS experiments, five precursor ions corresponding to the most intense peaks in the LC-MS analysis were fragmented at a collision energy of 30%. The metabolic profiles obtained from the LC-ESI-Qexactive-MS/MS analysis of olive xylem samples were elaborated using Xcalibur 2.2 software, which provided the accurate mass for each feature (ppm≤5). From the study of HR mass value and MS/MS fragmentation patterns of the molecular ions, a putative identification was made by comparing the data with those available both in literature and

specific databases such as Knapsack (http://www.knapsackfamily.com), MassBank (https://massbank.eu/MassBank/) and FoodB (https://foodb.ca/). Furthermore, the identification of metabolites from sap samples was also performed using Compound Discoverer 3.3SP3 software.

The UHPLC-Q-Exactive chromatograms were subjected to chemometric analyses such as Principal Component Analysis (PCA) (data not shown) and Partial Least Square Discriminant Analysis (PLS-DA) to classify the samples and evaluate the different expressions of metabolites in the xylem sap sample classes, specifically between the four olive varieties (Coratina, FS-17, Leccino, and Ogliarola) studied. This analysis allows, firstly, the assessment of metabolic differences within a large dataset and, secondly, the identification of "chemical markers" for each sample class, *i.e.,* metabolites that are more highly expressed in one sample class than in the others analyzed. Each chromatogram was processed using MZmine (http://mzmine.sourceforge.net/). This software allowed retention time compensation and noise removal from LC-MS profiles by setting the noise level to 5.0×10^6, so that data points below this value were not included in the multivariate analysis. Only the *m/z* of metabolites identified by metabolite profiling were included in the analyses using a pseudo-targeted approach (Crescenzi *et al.,* 2025). The data set was exported and processed by SIMCAP+ software 12.0 (Umetrix AB, Umea Sweden) for performing PCA (preliminary analysis, data not shown), grouping samples based on similarity in metabolite composition. Before submitting to multivariate data analysis, the data were scaled to Unit Variance. PLS-DA was subsequently performed for the classification of samples to identify the different metabolites characterizing the different groups of samples. In this analysis, to use the regression algorithm necessary to classify the information on the different types of samples, numerical values were assigned to the classes of samples (Leccino, -2; Coratina, -1; FS-17, 1; Ogliarola, 2). In addition, the model was validated by cross-validation techniques and permutation test according to standardized practicals to obtain a statistically robust method.

A data matrix was thus obtained for the spring samples, with rows representing the individual samples analyzed (40 samples) and columns representing the integrated and normalized peak areas of the identified metabolites (75 variables), using a pseudo-targeted approach. The first component explained 63% of the variance, and the third explained 5% of the variance, with a Q2 of 81% for the PLS-DA model characteristic of the data collected for xylem sap samples.

Raw data from xylem sap samples collected in fall generated a data matrix with 80 samples and 64 variables. The PLS-DA model representative of the data obtained for the fall xylem sap samples showed that the first component explained 41% of the variance and the fourth explained 2%, with a Q2 of 63%.

3) Identification of phagostimulant and phagodeterrent olive xylem sap metabolites

The goal was to identify the role played by each of the metabolites identified in the first trial as relevant for determining olive plants' suitability for the meadow spittlebug, *i.e.,* the biomarkers. We thus extended the characterization of *P. spumarius* probing and feeding behavior to 14 tested olive varieties commonly cultivated or newly introduced to Apulia region, South of Italy, (Arbequina, Bella di Spagna, Cima di Melfi, Coratina, Frantoio, FS-17, Lecciana, Leccino, Leccio del Corno, Nocellara Messinese, Nociara, Ogliarola Salentina "hereafter Ogliarola", Pendolino and Picholine), in order to increase the variability of the xylem sap metabolome. We used the EPG to calculate the xylem ingestion index (overall time spent by the insect ingesting xylem sap (Xi+LF) over the total probing time) for each of the 14 olive varieties. We quantified for each variety the biomarkers identified in the first trial and evaluated the influence (negative or positive) of each metabolite on the xylem index, thus on olive plant suitability for the spittlebug.

3.1) EPG-assisted analysis of *Philaenus spumarius* probing and feeding behavior

The probing and feeding behavior of adult *P. spumarius* (both males and females) on 14 olive varieties was assessed using the EPG. We used EPG data to calculate the xylem ingestion index, *i.e.,* overall duration of xylem ingestion with respect to the total probing time (Xi+LF WDI/TPT). We carried out 20 recordings per variety, sex ratio 1:1, with single combination inset-plant (one plant per insect), during spring (May-June, as in the first trial). The EPG recording and the subsequent extraction of the xylem sap were conducted following the protocol described above. Xylem sap was extracted from ten randomly selected plants per variety, five per sex.

3.2) Quantitative chemical analysis of the xylem sap metabolites

The standards used for the optimization of the methodology that are commercially available at the purity required to enable accurate quantitative analysis are gluconic acid, malic acid, cis aconitic acid, fumaric acid, citric acid, sucrose, mannitol, glutamine, were purchased from LGC Standards (Teddington, UK); salidroside and elenolic acid glucoside were purchased from Sigma-Aldrich (Milan, Italy).

Instrumental parameters for the mass spectrometer were optimized via direct infusion of standardized metabolite solutions (1 μg mL^{-1} in 0.1% formic acid). Employing an AB Sciex 6500 QTRAP system (Foster City, CA, USA) equipped with an electrospray ionization (ESI) source, analyses were conducted in negative ion mode. A syringe pump delivered the standard at a constant flow rate of 20 μL min^{-1}. Mass spectrometric detection was performed using full-scan and product ion scan modes, with fragmentation achieved through collision-induced dissociation (CID).

Before LC-MS analysis, the xylem sap samples were subjected to a drying process under a nitrogen stream. The resulting residues were reconstituted in an aqueous solution of 0.1% formic acid at a concentration of 1 mg mL^{-1}. Following centrifugation at 13,200 rpm for 2 min, the clarified supernatants were carefully aliquoted into LC-MS vials for subsequent targeted metabolomic analysis to quantify primary and secondary metabolites (glutamine, sucrose, gluconic acid, fumaric acid, cis aconitic acid, citric acid, 2-furoic acid, salidroside, elenolic acid glucoside, ribonic acid, oleuropein diglucoside, mannitol, 2-*O*-methylxylose, malic acid, D-manno-2-heptulose, and daucic acid). Metabolite separation was achieved using a Shimadzu Nexera UHPLC system equipped with a Gemini-NX C18 column (150 × 2.0 mm, 5.0 μm; Phenomenex, Germany). The mobile phase consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The following gradient was applied at a constant flow rate of 0.250 mL min^{-1}. The percentage of mobile phase B was held at 1% for 0.1 minutes, then linearly increased to 6% by 10.0 minutes, and further raised to 25% by 15.0 minutes. The gradient continued to 40% B at 18.0 minutes, followed by a rapid re-equilibration to the initial conditions of 1% B at 20.0 min, where it was held to stabilize the column for the subsequent injection. The injection volume was 5 μL, and the column temperature was maintained at 40 °C. Eluted compounds were detected using a Sciex 6500 QTRAP mass spectrometer operating in negative electrospray ionization (ESI) mode. The instrument was configured for Multiple Reaction Monitoring (MRM) to ensure high sensitivity and specificity

for quantitative analysis. The ion source parameters were optimized as follows: ion spray voltage, -4500 V; temperature, 350 °C; curtain gas, 35 psi; ion source gas 1 and 2, 25 psi each; collision gas, medium. For each target metabolite, optimized MRM transitions were defined with specific parameters for declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). A dwell time of 20 ms per transition ensured sufficient data points across chromatographic peaks, with a total cycle time of 0.3 seconds. Data were acquired using Analyst software version 1.6.2 (AB Sciex, USA). Multiquant software 3.0.3 was used for processing (ABSciex, Foster City, CA, USA).

The ultra-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-QTRAP-MS/MS) method was rigorously validated in compliance with the International Council for Harmonisation (ICH) Q2(R1) guideline on validation of analytical procedures, as adopted by the European Medicines Agency (EMA) (ICH, 2022). The validation study assessed key parameters, including specificity, linearity, accuracy, precision, and robustness. The experimental approach for the validation protocol was adapted from the methodology previously established by Crescenzi *et al.* (2023).

The quantitative results from the targeted analysis of xylem metabolites were imported into the MetaboAnalyst 5.0 web-based platform (https://www.metaboanalyst.ca/) for multivariate statistical analysis. The data matrix, consisting of metabolite concentrations (rows) across the different olive varieties (columns), was log-transformed (log10) and mean-centered to improve normality and reduce heteroscedasticity. The quantitative results from the targeted analysis of xylem metabolites were subjected to a comprehensive statistical analysis. A one-way analysis of variance (ANOVA) was performed for each metabolite to test the null hypothesis that its mean concentration was equal across all olive varieties.

3.3) Effect of xylem metabolites on olive plants suitability

We investigated the relationship between the xylem ingestion index and xylem sap metabolites, through Partial Least Squares Regression (PLSR) using the pls package. The xylem index was used as response variable (Y), with metabolite concentrations were used as predictors (X). Data were mean-centered and scaled prior to modeling. Model complexity was assessed by cross-validation using the validationplot() function, evaluating the root mean squared error of prediction (RMSEP) across components. The model with two components was retained, as it provided a substantial reduction in RMSEP compared with one component, while additional components yielded only marginal improvements. Variable Importance in Projection (VIP)

scores were calculated using a function adapted for pls::plsr models, and metabolites with VIP > 1 were considered influential for explaining xylem ingestion time. Regression coefficients were extracted to determine the direction (positive or negative) of metabolite effects. All analyses were carried out in R software (Version 4. 5. 1) using the packages pls (Mevik & Wehrens, 2007), plsVarSel (Mehmood *et al.,* 2012), and ggplot2 (Wickham, 2016).

Results

1) Characterization of xylem sap metabolites mediating olive plants suitability

1.1) EPG-assisted analysis of *Philaenus spumarius* probing behavior

EPG experiments aimed to characterize seasonal and variety-dependent differences in the probing behavior of males and females *P. spumarius* on four olive varieties, namely Coratina, FS-17, Leccino and Ogliarola, served to qualitatively characterize the metabolites (biomarkers) most likely to play a role in olive plants suitability for the spittlebug. To streamline the results of EPG, we present only the significant differences in spittlebugs probing behavior, reporting the mean raw values. Anova results and pairwise comparisons of the statistically significant EPG variables are reported in Table 2.

Spring EPG recordings

The number of pathway events (C NWEI) was significantly higher on FS-17 (9.86±1.08) compared to Leccino (6.5±0.78). The number of xylem ingestion (Xi NWEI) and non-pathway (N NWEI) events was significantly higher on Ogliarola (21.68±2.83; 16.05±2.35, respectively) compared to Leccino (15.04±2.11; 9.88±1.9, respectively).

The total duration of xylem ingestion, relative to either the total probing or residence times, was significantly shorter on Leccino (Xi WDI/TPT: 53.44±5.75; Xi WDI/TRT: 45.25±5.34) compared to Coratina (Xi WDI/TPT: 69.21±4.62; Xi WDI/TRT: 59.33±4.69), FS-17 (Xi WDI/TPT: 71.17±4.47; Xi WDI/TRT: 62.77±4.49), and Ogliarola (Xi WDI/TPT: 76.22±4.39; Xi WDI/TRT: 68±4.5). Similarly, the overall duration of the total xylem ingestion (Xi+LF) relative to both the total probing time and the total residence time was significantly shorter on Leccino (Xi+LF WDI/TPT: 68.53±5.21; Xi+LF WDI/TRT: 59.71±5.57) compared to Coratina (Xi+LF WDI/TPT: 82.49±3.16; Xi+LF WDI/TRT: 71.72±4.26), FS-17 (Xi+LF WDI/TPT: 81.79±3.43; Xi+LF WDI/TRT: 72.69±4.15), and Ogliarola (Xi+LF WDI/TPT: 85.67±1.91; Xi+LF WDI/TRT: 76.87±3.03) (Fig. **1**).

The total duration of non-pathway event with respect to the total residence time (N WDI/TRT) was significantly longer on Ogliarola (0.93±0.11) compared to Leccino (0.53±0.08).

The time required for the stylets to reach xylem vessels and for the onset of xylem sap ingestion was significantly longer on FS-17 (np to 1st Xc: 17.31±6.85 min; 1st C to 1st Xc: 12.61±4.4 min; np to 1st Xi: 18.2±6.81 min; 1st C to 1st Xi: 13.48±4.38 min) compared to Leccino (np to

1st Xc: 5.32±0.75 min; 1st C to 1st Xc: 3.31±0.63 min; np to 1st Xi: 5.8±0.75 min; 1st C to 1st Xi: 3.8±0.65 min).

Independently of varieties, female spittlebugs exhibited significantly fewer non-probing events (np NWEI) (3.43±0.33) compared to males (4.73±0.51). Males spent a longer time engaging with pathway activities relative to the total probing time (C WDI/TPT) (8.69±1.71) compared to females (6.49±1.63). The median duration of single LF event (LF WDEImd) was significantly longer in females (10.58±1.21 min) compared to males (5.94±0.69 min).

The waveform Xe (pattern leading to *X. fastidiosa* inoculation) was observed only in two EPG recordings, one on Coratina and one on Leccino (both performed by female spittlebugs).

Fall EPG recordings

In contrast with the spring observations, no variety-associated differences in the behavior among the four varieties were observed for fall recordings. The only significant differences were sex-associated (EPG results regarding sex-associated differences are reported in Supporting Information Table **S1**).

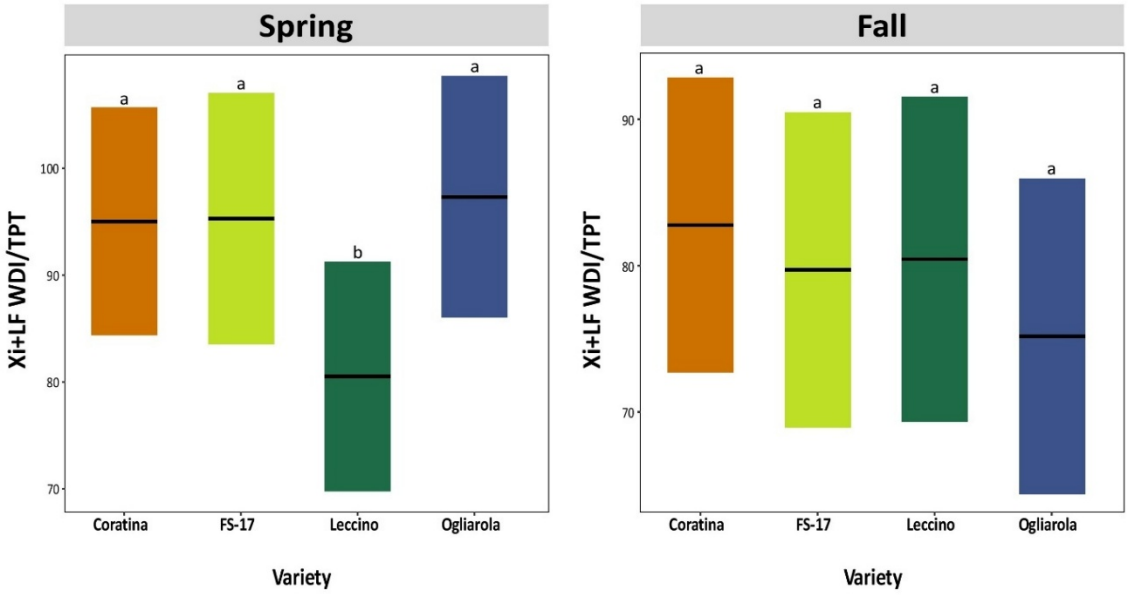


Figure 1. Xylem ingestion index: Overall duration of xylem ingestion (Xi+LF) over the total probing time recorded on the four olive varieties tested in spring and fall. Varieties indicated with different letters are statistically different between each other ($p < 0.01$).

Table 2. Anova results and pairwise comparisons of the statistically significant EPG variables in the olive varieties: Ogliraola, FS-17, Leccino and Coratina during Spring (*p*-values: * < 0.05; ** < 0.01; *** < 0.001).

EPG Variables	Anova					Tukey Test				
		Chisq	Df	Pr(>Chisq)		contrast	estimate	SE	t ratio	*p* value
C NWEI	Variety	8.948	3	0.02999	*	FS17 - Leccino	0.185	0.068	2.734	0.038
	Sex	0.051	1	0.82103						
Xi NWEI	Variety	9.834	3	0.02003	*	Leccino - Ogliarola	-0.42	0.155	-2.7	0.041
	Sex	1.652	1	0.19866						
N NWEI	Variety	8.847	3	0.03139	*	Leccino - Ogliarola	-0.29	0.102	-2.89	0.025
	Sex	3.231	1	0.07225	.					
Xi WDI/TPT	Variety	21.419	3	8.62E-05	***	Coratina - Leccino	0.109	0.033	3.341	0.0046
	Sex	0.513	1	0.4738		FS17 - Leccino	0.130	0.034	3.825	0.0008
						Leccino - Ogliarola	-0.142	0.035	-4.112	0.0002
Xi WDI/TRT	Variety	25.136	3	1.45E-05	***	Coratina - Leccino	0.085	0.025	3.386	0.004
	Sex	1.441	1	0.23		FS17 - Leccino	0.109	0.026	4.132	0.0002
						Leccino - Ogliarola	-0.120	0.027	-4.458	<.0001
Xi+LF WDI/TPT	Variety	21.177	3	9.67E-05	***	Coratina - Leccino	0.055	0.015	3.665	0.0014
	Sex	1.094	1	0.2957		FS17 - Leccino	0.056	0.016	3.471	0.0029
						Leccino - Ogliarola	-0.065	0.016	-3.97	0.0004
Xi+LF WDI/TRT	Variety	18.091	3	0.000421	***	Coratina - Leccino	0.065	0.021	3.112	0.0101
	Sex	1.810	1	0.178573		FS17 - Leccino	0.073	0.022	3.312	0.0051
						Leccino - Ogliarola	-0.086	0.023	-3.809	0.0008
N WDI/TRT	Variety	10.426	3	0.01527	*	Leccino - Ogliarola	-0.33	0.105	-3.18	0.011
	Sex	0.425	1	0.51443						
np to 1st Xc	Variety	7.846	3	0.0493	*	FS17 - Leccino	0.615	0.224	2.742	0.037
	Sex	0.819	1	0.3656						
np to 1st Xi	Variety	7.861	3	0.04898	*	FS17 - Leccino	0.352	0.127	2.78	0.033
	Sex	0.691	1	0.40568						
1st C to 1st Xc	Variety	10.885	3	0.01236	*	FS17 - Leccino	0.747	0.228	3.282	0.008
	Sex	1.838	1	0.17522						
1st C to 1st Xi	Variety	10.667	3	0.01367	*	FS17 - Leccino	0.745	0.228	3.264	0.009
	Sex	1.243	1	0.265						
np NWEI	Variety	1.761	3	0.62341		Female - Male	-0.27	0.135	-2	0.0487
	Sex	4.001	1	0.04548	*					
C WDI/TPT	Variety	1.458	3	0.69196		Female - Male	-0.41	0.202	-2.02	0.047
	Sex	4.079	1	0.04341	*					
LF WDEImd	Variety	0.079	3	0.994276		Female - Male	0.463	0.163	2.832	0.006
	Sex	8.021	1	0.004624	**					

1.2) Identification of metabolites from xylem sap samples by UHPLC-Q-Exactive-MS/MS analysis

Metabolite profiling through high-resolution mass spectrometry experiments enabled the identification of 85 metabolites (91 including the isomers) in the xylem of olive plants (Supporting Information, Table **S2**), primarily belonging to the classes of organic acids, amino acids, sugars, terpenes, and phenols. The identified primary and specialized metabolites were present in the xylem samples of all the analyzed olive varieties. Most organic acids were detected early in the chromatographic run, with peaks of highest intensity for malic acid (**14**), citric acid (**16a**, **16b**), and daucic acid (**19**) (*m/z* 133.01295, 191.01876, 203.01917). A few amino acids (L-aspartic acid, asparagine, glutamine) and sugars (2-*O*-methylxylose, gluconic acid, sucrose, D-manno-2-heptulose, D-mannose) were also identified in the initial part of the chromatogram. Mannitol (**5**) showed the most intense peak in all samples. Xylem samples were rich in phenolic compounds, notably hydroxytyrosol 4-*O*-glucoside (compound **32**, *m/z* 315.10849), the most representative phenolic compound identified. Other phenolics, such as esculin (**42**) and esculetin (**47**), were identified with fragments at *m/z* 177.0 and 101.0, respectively, confirming previous reports (Tóth *et al.,* 2015). Secoiridoids were abundant in xylem sap, such as oleoside and elenolic acid glucoside (compounds **33** and **35**), along with other secoiridoids like hydroxyoleuropein (**42**), oleuropein diglucoside (**48**), oleuropein (**58**), and ligustroside (**61**).

Chemical profiling of olive xylem sap

After the identification of the primary and specialized metabolites in the olive xylem sap samples, a multivariate statistical analysis was conducted to evaluate differences in the metabolic profiles of xylem sap from the four olive varieties studied, collected in both spring and fall time.

Spring samples: the PLS-DA performed on the xylem sap samples collected in spring revealed distinct metabolic expression patterns among the four olive varieties analyzed. In the score scatter plot (Fig. **2a**), the closer the observations (the individual samples) appear to each other, the more similar they are according to the model. Indeed, samples from the different varieties occupy well-defined and separate regions within the plot, except for Ogliarola and Coratina xylem samples, which are positioned closer together, suggesting a quite similar metabolic composition. All samples of Leccino variety, which was the least preferred by the spittlebug (see spring EPG results), form a distinct cluster in the plot, indicating a characteristic metabolic

expression. The loading scatter plot (Fig. **2b**) identifies the chemical markers for each variety, as indicated in Table 3.

The xylem sap of FS-17 was considerably rich in phenols, such as esculetin, esculin and hydroxytyrosol, as well as terpenes such as aucubin, ligustroside and lamiidoside. FS-17 variety was also enriched by specific metabolic markers, including the amino acid asparagine and the organic acids L-threonic acid, quinic acid and sinapinic acid.

Leccino exhibited a metabolomic profile dominated by organic acids particularly 2-oxobutyrix acid, 2-furoic acid, fumaric acid, malic acid, ribonic acid, cis-aconitic acid, citric acid and daucic acid. Leccino xylem sap was also characterized by elevated levels of sugars (D-mannose, mannitol, gluconic acid, D-manno-2-heptulose and sucrose), phenolic compounds (*i.e.,* salidroside and vanillin) and terpenes (*e.g.,* elenolic acid glucoside and oleuropein diglucoside).

Ogliarola and Coratina displayed highly similar metabolic profiles, both characterized by high expression of the two amino acids glutamine and oxoproline along with several phenols (*e.g.,* glucovanillin and koaburaside) and terpenes (*e.g.,* lamiol and barlerin).

Fall samples: xylem sap extracted from the four olive varieties after the fall EPG recordings exhibited a distinct metabolic expression in the PLS-DA. The four analyzed varieties formed separate clusters in the score scatter plot (Fig. **2c**).

The xylem sap samples of Coratina variety were the richest in gein (phenylpropanoid), as well as adinoside A, ligustroside, oleuropein, and 10-hydroxyoleuropein (terpenes).

Lignan olivil 4''-*O*-glucoside, esculin, esculetin (phenols), and eriodictyol-7-*O*-glucoside, as well as oleoside and loganic acid (terpenes) were the most highly expressed metabolites in FS-17.

The xylem sap of Leccino was rich in hydroxytyrosol, hydroxytyrosol 4-*O*-glucoside, and glucovanillin (phenols), as well as in aucubin and oleuropein diglucoside (terpenes).

Organic acids such as fumaric acid, ribonic acid, azelaic acid, and quinic acid, along with phenols like vanillin and terpenes such as lamiol and barlerin were the chemical markers for Ogliarola variety collected during fall (Fig. **2d**).

The sugars mannitol, sucrose, 2-*O*-methylxylose, gluconic acid, and D-manno-2-heptulose, the phenols verbascoside and decaffeoylverbascoside, the amino acids oxoproline, asparagine, L-

aspartic acid, and glutamine, the organic acids 2-furoic acid, malic acid, citric acid, daucic acid, L-threonic acid, cis-aconitic acid, and sinapinic acid, the lignan 8-acetoxypinoresinol 4-glucoside, and the terpene elenolic acid glucoside were similarly expressed in the fall xylem sap across the four olive varieties screened.

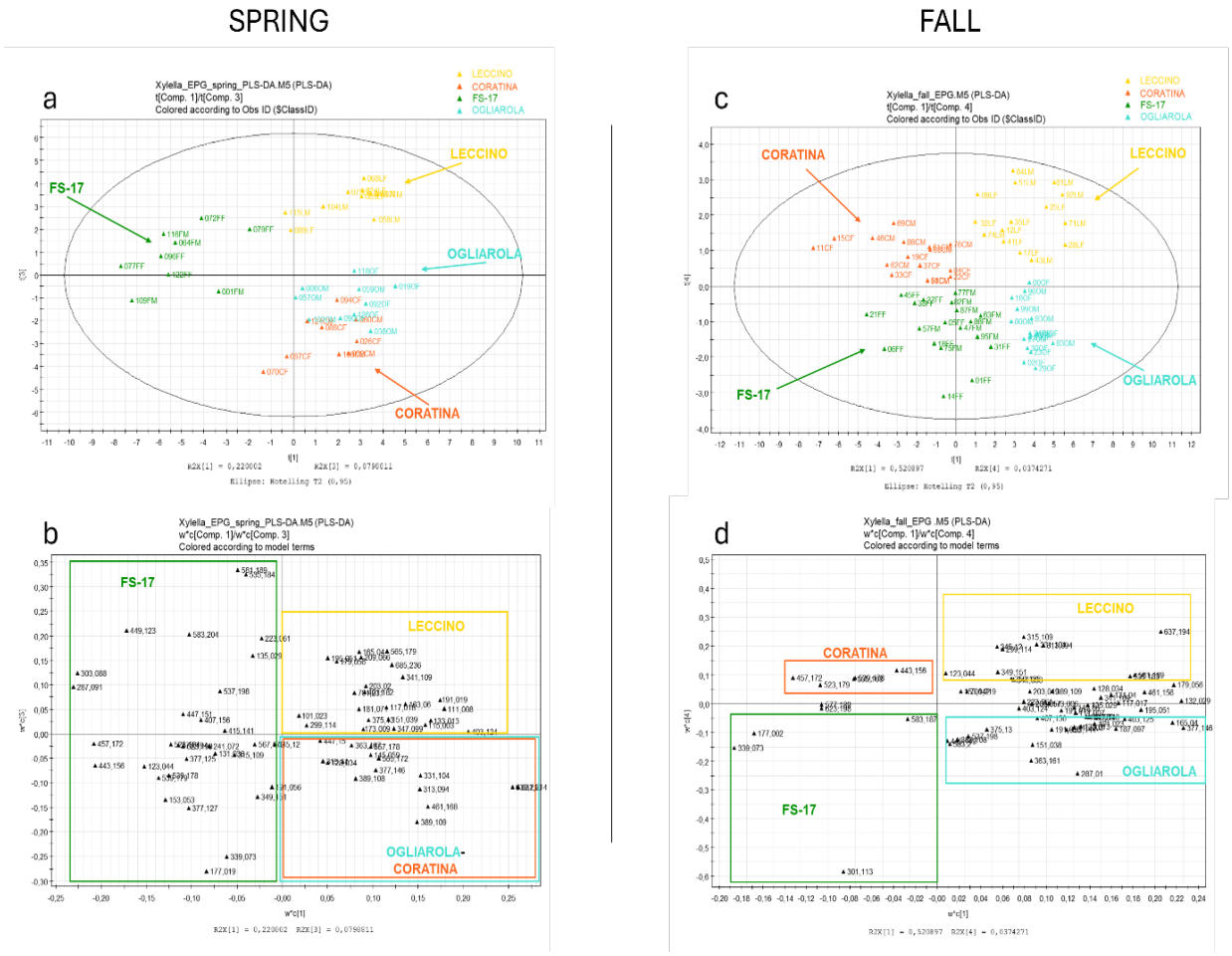


Figure 2. Partial Least Squares-Discriminant Analysis (PLS-DA) of spring and fall xylem sap samples from the four varieties with a pseudo-targeted approach; **a** and **c** score scatter plots and **b** and **d** loading scatter plots.

Table 3. Chemical markers detected by PLS-DA in the xylem of the four olive varieties collected during the spring and fall periods. X: not present; nd: not discriminant. *Mass identification was performed on accurate mass measurement and fragmentation spectra; RMS errors (ppm) were reported in Table **S2**.

[M-H]$^-$ (*m/z*)	Molecular Formula	Identification*	Class Compounds	Spring	Fall
128.034	$C_5H_7O_3N$	oxoproline	Amino acids	Ogliarola/Coratina	nd
131.036	$C_4H_8N_2O_3$	asparagine	Amino acids	FS-17	nd
132.029	$C_4H_8NO_4$	L-aspartic acid	Amino acids	X	nd
145.059	$C_5H_{10}N_2O_3$	glutamine	Amino acids	Ogliarola/Coratina	nd
123.044	$C_7H_9O_2$	3-hydroxybenzyl alcohol	Benzenoids	FS-17	Leccino
577.194	$C_{28}H_{34}O_{13}$	8-acetoxypinoresinol 4-glucoside	Lignans	FS-17	nd
583.203	$C_{27}H_{36}O_{14}$	(-)-olivil 4″-*O*-glucoside	Lignans	FS-17	FS-17
111.008	$C_5H_5O_3$	2-furoic acid	Organic acids	Leccino	nd
115.003	$C_4H_4O_4$	fumaric acid	Organic acids	Leccino	Ogliarola
133.013	$C_4H_6O_5$	malic acid	Organic acids	Leccino	nd
135.029	$C_4H_9O_5$	L-threonic acid	Organic acids	FS-17	nd
165.040	$C_4H_{10}O_6$	ribonic acid	Organic acids	Leccino	Ogliarola
173.009	$C_6H_7O_6$	cis-aconitic acid	Organic acids	Leccino	nd
187.097	$C_9H_{16}O_4$	azelaic acid	Organic acids	X	Ogliarola
191.019	$C_6H_8O_7$	citric acid	Organic acids	Leccino	nd
191.056	$C_7H_{12}O_6$	quinic acid	Organic acids	FS-17	Ogliarola
203.020	$C_7H_8O_7$	daucic acid	Organic acids	Leccino	nd
223.061	$C_{11}H_{13}O_5$	sinapinic acid	Organic acids	FS-17	nd
301.113	$C_{14}H_{22}O_7$	butyl 3-(2-butoxy-2-oxoethyl)-3-hydroxy-4-oxooxetane-2-carboxylic acid	Organic acids	X	FS-17
457.172	$C_{21}H_{30}O_{11}$	gein	Phenilpropanoids	FS-17	Coratina
151.039	$C_8H_8O_3$	vanillin	Phenols	Leccino	Ogliarola
153.053	$C_8H_{10}O_3$	hydroxytyrosol	Phenols	FS-17	Leccino
177.019	$C_9H_6O_4$	esculetin	Phenols	FS-17	FS-17
287.091	$C_{15}H_{12}O_6$	2-hydroxyflavanone naringenin	Phenols	FS-17	Ogliarola
299.114	$C_{14}H_{20}O_7$	salidroside	Phenols	Leccino	Leccino
303.088	$C_{15}H_{12}O_7$	nigrescin	Phenols	FS-17	X
313.094	$C_{14}H_{18}O_8$	glucovanillin	Phenols	Ogliarola/Coratina	Leccino
315.110	$C_{14}H_{20}O_8$	hydroxytyrosol 4-*O*-glucoside	Phenols	FS-17	Leccino
331.104	$C_{14}H_{20}O_9$	koaburaside	Phenols	Ogliarola/Coratina	Leccino
339.073	$C_{15}H_{16}O_9$	esculin	Phenols	FS-17	FS-17
415.141	$C_{22}H_{25}O_8$	acetoxypinoresinol	Phenols	FS-17	X
449.123	$C_{21}H_{22}O_{11}$	eriodictyol-7-*O*-glucoside	Phenols	FS-17	FS-17
461.168	$C_{20}H_{31}O_{12}$	decaffeoylverbascoside	Phenols	Ogliarola/Coratina	nd
535.184	$C_{26}H_{32}O_{12}$	8-hydroxypinoresinol 4′-*O*-*β*-D-glucopyranoside	Phenols	FS-17	Leccino
581.188	$C_{27}H_{34}O_{14}$	naringin dihydrochalcone	Phenols	FS-17	Leccino

623.199	$C_{29}H_{36}O_{15}$	verbascoside	Phenols	FS-17	nd
637.214	$C_{30}H_{38}O_{15}$	4'-hydroxy-5,7,2'-trimethoxyflavanone 4'-rhamnosyl- (1->6) -glucoside	Phenols	Ogliarola/Coratina	Leccino
639.181	$C_{29}H_{36}O_{16}$	β-hydroxyacteoside	Phenols	Ogliarola/Coratina	X
163.060	$C_6H_{12}O_5$	2-*O*-methylxylose	Sugars	Leccino	nd
179.056	$C_6H_{12}O_6$	D-mannose	Sugars	Leccino	Leccino
181.071	$C_6H_{14}O_6$	mannitol	Sugars	Leccino	nd
195.051	$C_6H_{12}O_7$	gluconic acid	Sugars	Leccino	nd
209.066	$C_6H_{11}O_8$	D-manno-2-heptulose	Sugars	Leccino	nd
341.109	$C_{12}H_{22}O_{11}$	sucrose	Sugars	Leccino	nd
347.099	$C_{14}H_{21}O_{10}$	1,2,3,4-tetra-*O*-acetyl-*β*-D-glucopyranose	Sugars	Leccino	Leccino
363.167	$C_{16}H_{28}O_9$	α-L-galactopyranose, 6-deoxy-3-*O*-(2,6-dideoxy-4-*O*-methyl-α-D-arabino-hexopyranosyl)-2-*O*-methyl-, 4-acetate (9CI, ACI)	Sugars	Ogliarola/Coratina	Ogliarola
401.182	$C_{19}H_{30}O_9$	benzyl *β*-primeveroside	Sugars	Leccino	X
407.156	$C_{17}H_{28}O_{11}$	dimethyl-4-(α-D-mannopyranosyloxy)-5-methoxy-1,2-cyclohexanedicarboxylate	Sugars	FS-17	Ogliarola
241.072	$C_{11}H_{15}O_6$	elenolic acid	Terpenes	FS-17	X
345.120	$C_{15}H_{22}O_9$	aucubin	Terpenes	FS-17	Leccino
349.151	$C_{15}H_{26}O_9$	eucommioside	Terpenes	FS-17	Leccino
359.135	$C_{16}H_{25}O_9$	plantarenaloside	Terpenes	X	Leccino
375.130	$C_{16}H_{24}O_{10}$	loganic acid	Terpenes	Leccino	FS-17
377.125	$C_{19}H_{22}O_8$	oleuropein-aglycone	Terpenes	FS-17	X
377.146	$C_{16}H_{26}O_{10}$	lamiol	Terpenes	Ogliarola/Coratina	Ogliarola
389.109	$C_{16}H_{22}O_{11}$	oleoside	Terpenes	Ogliarola/Coratina	FS-17
403.125	$C_{17}H_{24}O_{11}$	elenolic acid glucoside	Terpenes	Leccino	ND
435.144	$C_{18}H_{28}O_{12}$	alpigenoside	Terpenes	X	Ogliarola
443.157	$C_{20}H_{28}O_{11}$	adinoside A	Terpenes	FS-17	Coratina
447.150	$C_{19}H_{28}O_{12}$	barlerin	Terpenes	Ogliarola/Coratina	Ogliarola
523.184	$C_{25}H_{32}O_{12}$	ligustroside	Terpenes	FS-17	Coratina
537.198	$C_{26}H_{36}O_{12}$	diosbulbinoside F	Terpenes	FS-17	FS-17
539.178	$C_{25}H_{32}O_{13}$	oleuropein	Terpenes	FS-17	Coratina
555.172	$C_{25}H_{32}O_{14}$	10-hydroxyoleuropein	Terpenes	Ogliarola/Coratina	Coratina
557.178	$C_{26}H_{38}O_{13}$	octenoyloxy secologanoside	Terpenes	Ogliarola/Coratina	X
565.179	$C_{23}H_{34}O_{16}$	glucopyranosyl methyloleoside	Terpenes	Leccino	X
567.173	$C_{26}H_{32}O_{14}$	lamiidoside	Terpenes	FS-17	X
685.236	$C_{31}H_{42}O_{17}$	nuzhenide	Terpenes	Leccino	X
701.231	$C_{31}H_{42}O_{18}$	oleuropein diglucoside	Terpenes	Leccino	Leccino

Biomarkers: Overall, comparing spring and fall EPG and metabolomic data, we isolated the differentially expressed metabolites (biomarkers) possibly underlying plant suitability for the spittlebug. The biomarkers, quantified in the 14 varieties xylem sap in the second trial, are reported in Table 4.

Table 4. Chemical markers identified in olive xylem sap with potential phagodeterrent or phagostimulant effect; nd: not discriminant. Expression in spring = olive variety with the highest expression of the compound in the xylem sap among the four varieties tested during spring; Expression in fall = olive variety with the highest expression of the compound in the xylem sap among the four varieties tested during fall.

Metabolites	Class	Expression in Spring	Expression in Fall
2-furoic acid	Organic acids	Leccino	nd
fumaric acid	Organic acids	Leccino	Ogliarola
malic acid	Organic acids	Leccino	nd
ribonic acid	Organic acids	Leccino	Ogliarola
cis-aconitic acid	Organic acids	Leccino	nd
citric acid	Organic acids	Leccino	nd
daucic acid	Organic acids	Leccino	nd
glutamine	Amino acids	Ogliarola	nd
salidroside	Phenols	Leccino	Leccino
oleuropein diglucoside	Terpenes	Leccino	Leccino
elenolic acid glucoside	Terpenes	Leccino	nd
Mannitol	Sugars	Leccino	nd
2-*O*-methylxylose	Sugars	Leccino	nd
gluconic acid	Sugars	Leccino	nd
D-manno-2-heptulose	Sugars	Leccino	nd
sucrose	Sugars	Leccino	nd

2) Identification of phagostimulant and phagodeterrent olive xylem sap metabolites

2.1) EPG-assisted calculation of xylem index in 14 olive varieties

The lowest xylem ingestion index, *i.e.,* the overall duration of the total xylem ingestion (Xi + LF) relative to the total probing time (Xi + LF WDI/TPT), among the olive varieties tested was observed in Pendolino, Leccino and Picholine. Specifically, the index in Leccino (69.14 ± 5.08) was significantly lower than in Bella di Spagna (84.28 ± 4.28, t=2.46, p=0.014), Coratina

(82.65 ± 3.04, t=3.42, p<0.001), Frantoio (81.65 ± 3.56, t=2.99, p=0.003), FS-17 (79.65 ± 3.91, t=2.62, p=0.009), Leccio del Corno (79.04 ± 4.4, t=-2.15, p=0.032), Nociara (85.38 ± 4.35, t=-2.97, p=0.003), and Ogliarola (83.28 ± 2.74, t=-3.29, p=0.001). Similarly, spittlebugs offered Pendolino plants displayed significantly shorter ingestion (67.15 ± 5.56) compared to Coratina (t=2.75, p=0.006), Frantoio (t=3.12, p=0.002), FS-17 (t=2.20, p=0.029), Leccio del Corno (t=2.22, p=0.027), Nociara (t=3.15, p=0.002) and Ogliarola (t=2.80, p=0.005). Finally, xylem index in Picholine (70.74 ± 5.31) was significantly lower than in Bella di Spagna (t=2.04, p=0.041), Coratina (t=2.33, p=0.02), Frantoio (t=2.70, p=0.007), Nociara (t=2.58, p=0.01) and Ogliarola (t=2.40, p=0.016).

2.2) Xylem sap metabolites effect on olive plants suitability

Through targeted mass spectrometry experiments, 16 metabolites, namely, glutamine, sucrose, gluconic acid, fumaric acid, cis aconitic acid, citric acid, 2-furoic acid, salidroside, elenolic acid glucoside, ribonic acid, oleuropein diglucoside, mannitol, 2-*O*-methylxylose, malic acid, D-manno-2-heptulose, and daucic acid, were quantified in xylem samples from 14 different olive varieties that had been in contact with *P. spumarius*. The aim was to evaluate the relationship between the xylem ingestion index expressing host plant suitability, and the biomarkers concentration in the xylem sap. Experiments were conducted in MRM mode by analyzing 6 replicates per variety.

The UPLC-ESI-QTRAP-MS/MS method was validated in accordance with the European Medicines Agency (EMA) guidelines. Calibration curves were constructed by plotting the peak area of each analyte against the corresponding concentration of the external standard. All standards were analyzed in duplicate, and excellent linearity was observed across the validated concentration range, with correlation coefficients (R^2) ranging from 0.997 to 0.999. The sensitivity of the method was assessed through the determination of the limit of detection (LOD) and limit of quantitation (LOQ), defined as signal-to-noise ratios of 3:1 and 10:1, respectively. The LOD values ranged from 0.04 to 0.20 µg L^{-1}, while LOQ values ranged from 0.04 to 0.10 µg L^{-1}, confirming the high sensitivity of the assay. Precision was evaluated through repeatability (intra-day) and intermediate precision (inter-day) experiments. Two replicates of each sample were analyzed within a single day and again over three consecutive days. Precision was expressed as relative standard deviation (RSD, %). Recovery studies were conducted to evaluate extraction efficiency at three concentration levels (low, medium, and high), each analyzed in duplicate. The method demonstrated satisfactory accuracy and

precision, with recovery rates ranging from 2% to 4% and RSD values within acceptable limits. The results of the quantitative analysis (mean ± standard deviation) of the quantified metabolites of xylem olive samples are reported in Supporting Information Table **S3**.

To visualize the overall metabolic differences and similarities between the samples, the concentration data was mean-centered and unit-variance scaled (auto-scaled) to ensure all metabolites contributed equally to the analysis. A heatmap (Fig. 3) was subsequently generated using Euclidean distance and the Ward clustering algorithm as a similarity measure. This unsupervised approach allowed for the intuitive assessment of inherent patterns, grouping both metabolites and samples based on their abundance profiles, thereby facilitating the identification of distinct metabolic signatures among the olive varieties exposed to spittlebugs.

Briefly, clustering analysis revealed two major metabolite-based groups among the 14 olive varieties (Fig. **3**). The first group, including Cima di Melfi, Leccino, Frantoio, Nociara, Pendolino, and Picholine, was characterized by generally higher metabolite abundance, though with distinct metabolic specializations. For example, Leccino accumulated high levels of elenolic acid glucoside, salidroside, mannitol, and ribonic acid but was poor in glutamine. Cima di Melfi stood out for exceptionally high cis-aconitic and daucic acids, while Pendolino was enriched in fumaric acid, oleuropein diglucoside, ribonic acid, and 2-O-methylxylose. In contrast, Frantoio and Nociara were marked by a lack of salidroside. The second group, composed of Ogliarola, Coratina, FS-17, Arbequina, Nocellara Messinese, Lecciana, Bella di Spagna, and Leccio del Corno, displayed an overall lower metabolite abundance. Within this cluster, Ogliarola and Coratina showed the lowest biomarkers content overall, though they were relatively rich in glutamine (Ogliarola) and sugars (Leccino). Arbequina and Nocellara Messinese shared higher levels of glutamine and gluconic acid, while Lecciana diverged by exhibiting strong organic acid enrichment (cis-aconitic and 2-furoic acids), coupled with low levels of phenolics such as elenolic acid glucoside and salidroside.

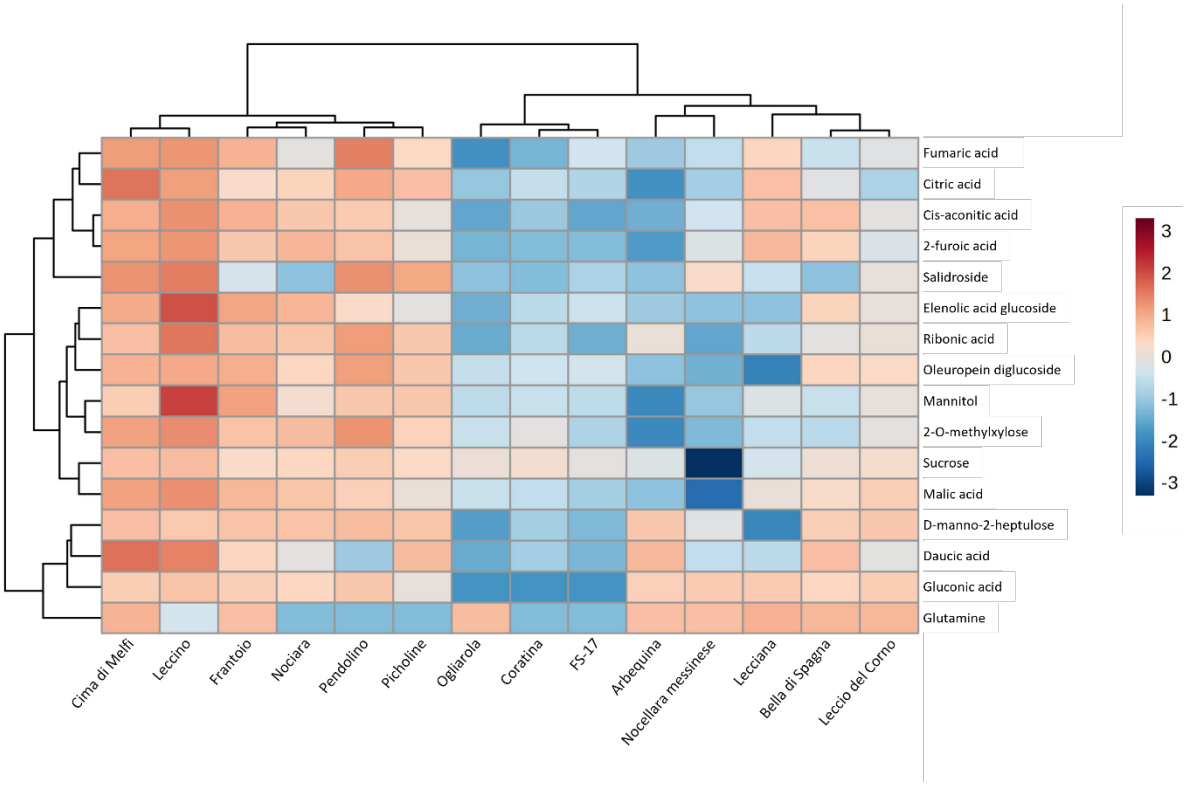


Figure 3. Heatmap of xylem sap metabolite profiles across olive varieties. The heatmap displays the relative abundance of identified metabolites across the tested olive varieties, with rows corresponding to individual metabolites and columns to varieties.

Partial least squares regression (PLSR) was performed to investigate the relationship between xylem sap metabolite composition and the xylem index, the latter used to express olive plants suitability for the spittlebug. The first two components of the PLS models explained 63.71% of the variance in metabolite profiles (R^2X) and 22.1% of the variance in xylem ingestion (R^2Y), respectively, indicating that metabolite composition accounted for a measurable but partial fraction of the observed behavioral variation. The regression coefficients showed that glutamine, malic acid and daucic acid were the top positive contributors, highlighting their role as phagostimulants. In contrast, salidroside, gluconic acid, D-manno-2-heptulose and oleuropein diglucoside exhibited strongly negative coefficients, consistent with a potential phagodeterrent effect. Variable Importance in Projection (VIP) analysis confirmed that all metabolites contributed to the model (all VIP > 1), but highlighted malic acid, daucic acid, sucrose, 2-O-methylxylose, cis aconitic acid, mannitol, citric acid and elenolic acid glucoside as the most influential predictors (VIP > 3). The remaining metabolites exhibited moderate influence, with VIP scores ranging between two and three (Fig. **4**).

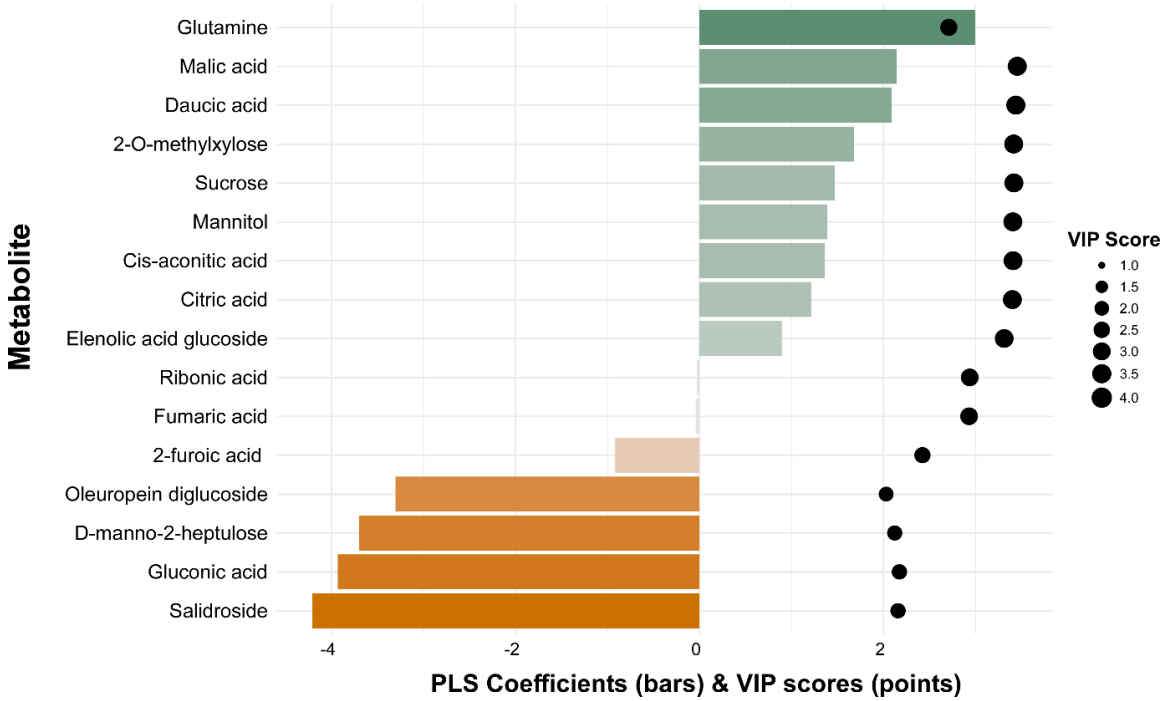


Figure 4. Metabolite contributions to PLS Model (Coefficients and VIP scores). The regression coefficients showed that glutamine, malic acid and daucic acid were the top positive contributors, suggesting that higher levels of these metabolites promoted feeding activity. Salidroside, gluconic acid, D-manno-2-heptulose and oleuropein diglucoside exhibited strong negative coefficients, consistent with a potential deterrent effect. Variable Importance in Projection (VIP) analysis confirmed that all metabolites contributed to the model (all VIP > 1).

Discussion

In the present study, we identified the chemical components of olive xylem sap promoting or discouraging host plant acceptance and settling by the meadow spittlebug *P. spumarius*. First, we characterized the xylem sap metabolome and identified the metabolites (biomarkers) most likely to influence insect's probing and feeding behaviors underlying host plant acceptance using four olive varieties with a different degree of suitability for the meadow spittlebug (Cornara *et al.,* 2024). These biomarkers were then quantified in a second trial where the observation of *P. spumarius* probing and feeding behavior was extended to 14 olive varieties, including the four previously tested, to increase the xylem chemical profile variability. We then assessed the contribution of each biomarker in determining plant suitability, expressed as xylem ingestion index.

Considering spittlebug's behavior during spring (May-June), consistently with previous findings (Cornara *et al.,* 2024), the variety-associated differences in probing and feeding regarded only the activities related to xylem, and mainly the duration of xylem sap ingestion. Leccino was the least preferred variety, as evidenced by its low xylem ingestion index (*i.e.,* the overall duration of xylem ingestion with respect to the total probing time) compared to the three other varieties. Given that xylem ingestion is a key indicator of host suitability for xylem-feeding insects (Markheiser *et al.,* 2020), these findings suggest that the xylem sap chemical profile of Leccino plants was the least favorable for the spittlebug. On the other hand, Ogliarola, *i.e.,* the variety with the longest xylem sap ingestion, had the preferred xylem sap composition among the varieties tested. The difference in the xylem sap ingestion duration was associated with a reduction in the number of xylem sap ingestion bouts, with fewer ingestion events in Leccino compared to Ogliarola. However, the duration of single ingestion events remained similar in all the tested varieties, indicating that although certain compounds in xylem sap might limit spittlebugs feeding, their deterrent effect may not be strong enough to trigger an immediate cessation of the sap uptake. Conversely, probing and feeding by spittlebugs was similar among the four olive varieties during fall recordings. Nonetheless, sex-dependent differences were observed, with females exhibiting longer total duration of xylem ingestion and longer single ingestion bouts compared to males. This increased feeding activity in females might reflect their higher physiological demands during this period, potentially associated to their increased need for energy for overwintering, mating, or egg production and maturation (Brodbeck *et al.,* 1993, 2011; Akassou *et al.,* 2021).

Once the stylets penetrate a xylem vessel, xylem feeders screen xylem sap chemical profile through precibarial chemosensilla, which permit the insects to assess the suitability of the host plant (Backus & McLean, 1985; Backus, 1988). According to Chapman (2003), chemical cues are not "immediately" processed, but rather the insect needs time after probing to evaluate whether the food source could provide the necessary nutritional requirement or whether the search for a new host is needed. If the nutritional requirements of the insect are not met, and the insect perceives chemical traits indicating plant unsuitability, it would abandon that plant searching for another suitable host. The reduction in host acceptance rate would in turn reduce vector activity, thereby lowering the likelihood of pathogen transmission (Purcell, 1981; Daugherty & Almeida, 2009). This aligns with the antixenosis resistance concept, where plant traits alter insects' behavior resulting in the failure of the plant to serve as a host. Plants antixenotic traits affecting Hemipterans probing and feeding behavior have been widely reported in literature. For instance, shorter duration of phloem feeding has been observed in the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) on grapevines cultivars resistant to *Flavescence dorée* (Ripamonti *et al.,* 2022). Additionally, Kleina *et al.* (2020) reported that the probing and feeding activities of the sharpshooter *Bucephalogonia xanthophis* Berg (Hemiptera: Cicadellidae) differed significantly between insects a plum genotype resistant to Plum Leaf Scald (PLS) disease (*i.e.,* SC13) and a PLS-susceptible one (*i.e.,* Laetitia) possibly due to antixenotic resistance of the SC13 genotype occurring at the level of xylem chemistry or physiology (Kleina *et al.,* 2020).

The metabolomic profile of Leccino samples collected in spring, when compared to the other tested varieties, showed high levels of organic acids, 2-furoic acid, fumaric acid, malic acid, ribonic acid, cis-aconitic acid, citric acid and daucic acid, of the phenol salidroside, and of the terpenes oleuropein diglucoside and elenolic acid glucoside. Additionally, Leccino sap during spring exhibited also higher levels of sugars (*e.g.,* sucrose and mannitol) compared to the other varieties.

The composition of xylem metabolome changes significantly through the seasons, influenced by various environmental factors and physiological processes. Changes in temperature, water availability and soil nutrient content affect the uptake and transport of metabolites in the xylem, leading to variations in the concentration of organic acids, amino acids, sugars, and secondary metabolites (Tromp, 1979; Ferguson *et al.,* 1983; Andersen *et al.,* 1989; Brodbeck *et al.,* 1990; Redak *et al.,* 2004). In consistence with the literature, seasonal fluctuations in the xylem

metabolome between spring and fall were reported in our study even though carried out on one-year old plants cultivated under controlled environmental conditions. Interestingly, most of the chemical markers of Leccino in spring, particularly the identified organic acids, sugars and the terpene elenolic acid glucoside, did not show specificity toward any variety during fall, as they were equally expressed in all the varieties tested. Furthermore, the four amino acids identified, namely oxoproline, asparagine, L-aspartic acid and glutamine, were also equally present in all varieties.

Therefore, we assumed these differentially expressed metabolites (biomarkers) to be the main determinants of olive plants suitability for spittlebugs. The biomarkers were thus quantified in the xylem sap of 14 olive varieties and correlated with host plant suitability for *P. spumarius* expressed as xylem ingestion index (or xylem index) retrieved from EPG data. The quantified metabolites were: the amino acid glutamine; the organic acids malic acid, daucic acid, fumaric acid, ribonic acid, 2-furoic acid, citric acid and cis-aconitic acid; the sugars sucrose, gluconic acid, mannitol, 2-O-methylxylose and D-manno-2-heptulose; the phenolic compound salidroside; the iridoid terpenes oleuropein glucoside and elenolic acid glucoside. Partial least squares regression analysis revealed that glutamine, malic acid, and daucic acid are the most relevant feeding promoters (phagostimulants), *i.e.,* their concentration was positively correlated with insect's feeding, whereas gluconic acid, salidroside, oleuropein diglucoside, and D-manno-2-heptulose discouraged feeding (phagodeterrents), *i.e.,* their concentration was negatively correlated with insect's feeding. Nonetheless, Variable Importance in Projection (VIP) analysis revealed that almost all the selected biomarkers had an influence on the xylem index, suggesting that host acceptance results from a complex interaction of nutritional and deterrent cues, where sugars, amino acids, organic acids and secondary metabolites each contribute to shaping ingestion dynamics.

Regarding organic acids, several studies highlighted their role as potential phagodeterrents in plant-insect interactions (Kim *et al.,* 1976; Yoshihara, 1980; Lateef, 1985; Kang *et al.,* 2023; Cornara *et al.,* 2024). However, our data indicate a positive correlation between the concentration of malic acid, daucic acid, and cis-aconitic acid and xylem ingestion, suggesting a potential phagostimulant effect of these compounds. However, it is worth noting that the least suitable varieties, Leccino and Pendolino, were overall characterized by high levels of all the quantified biomarkers belonging to this chemical class. These contradictory findings suggest that organic acids may act in a synergistic manner: while individual acids can stimulate

ingestion, their combined abundance might substantially lower the pH of xylem sap making it less palatable for the insects (Vega *et al.,* 1995; Bernays *et al.,* 1998; Hu *et al.,* 1999). Bernays *et al.* (1998) observed that low pH levels associated with the presence of plant acids inhibit the gustatory response of sugar-sensitive cells and trigger deterrent taste cells in the caterpillar *Manducta sexta* (Lepidoptera: Sphingidae). Additionally, the feeding propensity and duration of the apple maggot fly *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) were significantly reduced at pH levels below 4.0 (Hu *et al.,* 1999). Similarly, the corn planthopper *Peregrinus maidis* Ashmead (Hemiptera: Delphacidae) showed reduced feeding on diseased corn plants, where the phloem pH was lower (6.9) compared to that of healthy plants (7.6), suggesting that even moderate acidification could deter feeding (Vega *et al.,* 1995). These findings imply that the acidity of plant tissues could play a potential role in altering insects' feeding and consequently host acceptance (Chapman 2003). Further dedicated studies should investigate the correlation between pH and plant acceptance in spittlebugs.

Conversely, our results for glutamine intersect with the vast literature identifying this amino acid as a key phagostimulant for xylem feeders (Horsfield, 1977; Brodbeck *et al.,* 1990, 2004; Andersen *et al.,* 2005). In our analysis, glutamine emerged as the strongest positive contributor to xylem feeding, reinforcing its role as essential metabolite promoting host plant acceptance.

The phenolic compound salidroside was identified as the most consistent negative contributor to xylem ingestion in our models. Notably, the quantitative analysis of salidroside across the 14 olive varieties revealed that Leccino and Pendolino, the least suitable varieties for spittlebugs, contained the highest concentrations of salidroside. The phenol is known to possess potent α-glucosidase inhibitory activity and to cause structural damage to insect midgut tissues (Shao *et al.,* 2018; Yu *et al.,* 2021). Since α-glucosidase is a key enzyme required for sugar digestion in insects (Dillon & Kordy, 1997), the inhibition of this enzyme could interfere with the utilization of the high sugar levels identified in both varieties, Leccino and Pendolino, thereby reducing feeding efficiency. Such effects align with the concept of phagodeterrence: a phagodeterrent chemical might indeed either directly stimulate deterrent receptors or disrupt the neural pathways responsible for detecting phagostimulants such as sugars (Chapman, 2003; Koul, 2008).

Similarly to salidroside, the sugar gluconic acid and the iridoid oleuropein diglucoside were found to negatively influence the xylem ingestion index, thus the plant suitability for spittlebugs. To our knowledge, gluconic acid has not previously been reported as

phagodeterrent for insects, although a recent study demonstrated that it could impair larval growth of *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) (Bing *et al.,* 2024). Regarding oleuropein diglucoside, iridoids are well known for their defensive role in plant-insect interactions, often acting as potent feeding deterrents. Their bitter taste and potential toxicity could significantly reduce food intake and impede sustained feeding (Konno *et al.,* 1999; Biere & Marak, 2004; Eyles *et al.,* 2007).

Overall, this study provides the first insight into the xylem sap metabolites underlying host plant acceptance by *P. spumarius*, *i.e.,* a pivotal step for the transmission and spread of *X. fastidiosa*. Our findings may serve as a foundation for selecting germplasm potentially resistant to spittlebugs, thereby reducing the likelihood of these plants acting as important sources or recipients of *X. fastidiosa* and limiting pathogen spread. Additionally, our results pave the way for the development of strategies of interference with pathogen transmission by altering plant chemical profiles and reducing host plant suitability for insect vectors, thus vector activity, through agronomic approaches. Nevertheless, further studies extending this approach to additional olive varieties and experimentally manipulating plant chemical profiles will be necessary to validate and generalize the relationships identified in this work.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authors contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by ZH, MAC, GS, GC, and DC. EPG analysis was carried out by ZH and GS. Chemical analysis was performed by MAC and PM. Formal analysis was conducted by ZH, MAC, AT and DC. Methodology development involved ZH, AT, EdL and DC. The first draft of the manuscript was written by ZH, MAC and DC, and all authors commented on previous versions of the manuscript. Project administration and funding acquisition were managed by VV, EdL and DC. Supervision was provided by DC. All authors read and approved the final manuscript.

Data availability

Raw datasets and supplemental info are available from the corresponding author upon request.

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Supplementary Material

Table S1. Anova results, pairwise comparisons, and summary statistics of the statistically significant EPG variables in the olive varieties Ogliraola, FS-17, Leccino and Coratina during Fall (*p*-values: * < 0.05; ** < 0.01; *** < 0.001).

EPG Variables	Anova					Tukey test					Summary statistics							
		Chisq	Df	Pr(>Chisq)		contrast	estimate	SE	t ratio	*p* value	Sex	n	mean	sd	median	min	max	se
Xi WDI/TRT	Variety	1.1167	3	0.77305		Female - Male	0.237	0.103	2.306	0.024	Female	40	63.27	26.04	62.53	1.89	97.91	4.12
	Sex	5.3173	1	0.02111	*						Male	40	48.06	25.59	43.21	14.39	96.82	4.05
Xi WDEImd	Variety	0.4228	3	0.9355		Female - Male	0.214	0.0994	2.154	0.0345	Female	40	14.98	22.83	9.91	0.15	141.07	3.61
	Sex	4.6409	1	0.03122	*						Male	40	8.32	10.04	4.78	1.44	57.61	1.59
LF NWEI	Variety	4.5921	3	0.204224		Female - Male	-0.502	0.161	-3.119	0.0031	Female	22	4.32	3.24	4	1	12	0.69
	Sex	9.7273	1	0.001816	**						Male	33	7.33	4.18	8	1	16	0.73
LF WDI/TPT	Variety	1.6886	3	0.639465		Female - Male	-0.607	0.16	-3.802	0.0004	Female	22	13.32	19.44	7.04	0.33	79.83	4.15
	Sex	14.4541	1	0.000144	***						Male	33	30.26	20.73	24.64	0.68	74.57	3.61
LF WDI/TRT	Variety	1.8272	3	0.609		Female - Male	-1.14	0.259	-4.389	0.0001	Female	22	12.26	18.37	6.88	0.31	79.1	3.92
	Sex	19.2676	1	1.14E-05	***						Male	33	27.6	19.64	20.15	0.43	73.55	3.42
	Variety	1.2873	3	0.73214			-0.483	0.203	-2.385	0.0211	Female	22	10.83	29.52	3.72	1.12	142.38	6.29

LF WDElmd	Sex	5.6891	1	0.01707	*	Female - Male					Male	33	10.87	10.6	7.28	1.54	53.45	1.85
np to 1st LF	Variety	2.207	3	0.5306		Female - Male	0.509	0.229	2.22	0.0295	Female	40	224.61	139.44	241.65	10.2	360	22.05
	Sex	4.9298	1	0.0264	*						Male	40	142.49	123.55	102.75	15.6	360	19.53
first C to first LF	Variety	3.0725	3	0.38058		Female - Male	0.558	0.204	2.735	0.0078	Female	40	218.09	136.97	233.85	9	359.8	21.66
	Sex	7.4795	1	0.00624	**						Male	40	127.18	115.62	83.75	15.1	359	18.28

Table S2 Metabolites identified in olive xylem samples UHPLC-Q-Exactive/MS and UHPLC-Q-Exactive-MS/MS analysis.

N°	Rt (min)	[M-H]⁻ (*m/z*)	Molecular Formula	RMS error (ppm)	MS/MS (*m/z*)	Identification	Reference
1	1.01	132.02917	$C_4H_7NO_4$	-0.8	88.0/115.0/71.0	L-aspartic acid	CD
2	1.02	131.04482	$C_4H_7N_2O_3$	-2.3	114.0/88.0/70.0	asparagine	CD
3	1.02	163.06009	$C_6H_{11}O_5$	-0.1	85.0/59.0/101.0	2-*O*-methylxylose	CD
4	1.04	145.06068	$C_5H_9N_2O_3$	-0.1	127.0/109.0/102.0/84.0	glutamine	CD
5	1.04	181.07086	$C_6H_{13}O_6$	-0.9	101.0/89.0/59.0	mannitol	MB
6	1.04	195.05022	$C_6H_{11}O_7$	1.5	75.0/129.0/99.0	gluconic acid	CD
7	1.05	135.02887	$C_4H_8O_5$	-0.7	75.0/117.0/159.0	L-threonic acid	CD
8	1.12	165.03946	$C_5H_9O_6$	0.6	75.0/87.0/105.0/99.0/129.0/147.0	ribonic acid	(Zhu *et al.*, 2017)
9	1.12	191.05522	$C_7H_{11}O_6$	-0.1	85.0/127.0/111.0	quinic acid	(Difonzo *et al.*, 2022)
10	1.12	341.10873	$C_{12}H_{21}O_{11}$	1.9	59.0/89.0	sucrose	(Difonzo *et al.*, 2022)
11	1.13	209.06602	$C_6H_{10}O_8$	1.6	85.0/129.0/191.0	D-manno-2-heptulose	CD
12	1.16	179.05508	$C_6H_{11}O_6$	0.4	75.0/161.0	D-mannose	CD
13	1.26	115.00243	$C_4H_3O_4$	-1.3	71.0/87.0	fumaric acid	MB
14	1.26	133.01302	$C_4H_5O_5$	-2	115.0/71.0	malic acid	MB
15	1.47	111.00751	$C_5H_4O_3$	-1.4	85.0/71.0	2-furoic acid	CD
16a	1.47	191.01880	$C_6H_7O_7$	-0.3	111.0	citric acid isomer 1	(Difonzo *et al.*, 2022)
17	1.50	173.00861	$C_6H_6O_6$	0.7	111.0/85.0/129.0	cis-aconitic acid	CD
18	1.62	117.01805	$C_4H_5O_4$	-1.2	73.0/99.0	succinic acid	MB
16b	1.66	191.01880	$C_6H_7O_7$	-0.3	111.0	citric acid isomer 2	(Difonzo *et al.*, 2022)
19	1.66	203.01880	$C_7H_7O_7$	0.8	97.0/69.0/71.0/141.0	daucic acid	(Bagatini *et al.*, 2023)
20	1.73	128.03429	$C_5H_6O_3N$	0.6	82.0/84.0/65.7/106.4	oxoproline	CD
21	1.76	145.01329	$C_5H_6O_5$	-0.6	101.0/57.0	2-oxoglutaric acid	CD
22	1.81	101.02298	$C_4H_5O_3$	-3.4	57.0	2-oxobutyric acid	MB
23	2.08	331.10333	$C_{14}H_{19}O_9$	0.9	151.0	koaburaside	CD
24	3.04	347.09854	$C_{14}H_{20}O_{10}$	0.6	138.0/123.0	1,2,3,4-tetra-*O*-acetyl-*β*-D-glucopyranose	CD
25	3.47	227.05560	$C_{10}H_{11}O_6$	0.5	59.0/149.0	dimethyl succinylsuccinate	CD
26	4.39	217.07114	$C_9H_{13}O_6$	0.4	173.0/129.0/59.0	triacetin	CD
27	4.62	123.04390	$C_7H_8O_2$	-1.2	93.0/95.0/105.0	3-hydroxybenzyl alcohol	CD
28	4.65	375.12924	$C_{16}H_{23}O_{10}$	2.8	213.1/169.1/113.0	loganic acid	(Difonzo *et al.*, 2022)
29	4.75	245.06636	$C_{10}H_{13}O_7$	0.2	69.0/157.1/59.0	5-oxo-1,3,7-heptanetricarboxylic acid	CD
30	5.05	305.11430	$C_{15}H_{18}N_2O_5$	-0.1	176.1	carbobenzoxyglycyl-L-proline	CD
31	5.19	153.05464	$C_8H_9O_3$	0.1	123.0	hydroxytyrosol	(Tóth *et al.*, 2015)
32	5.19	315.10837	$C_{14}H_{19}O_8$	2.7	153.1	hydroxytyrosol 4-*O*-glucoside	(Difonzo *et al.*, 2022)
33	5.21	349.14987	$C_{15}H_{25}O_9$	1.6	59.0/169.1/89.0/101.0/187.1	eucommioside	(He *et al.*, 2018)

34	5.23	313.09296	$C_{14}H_{17}O_8$	2.1	151.0	glucovanillin	(Schwarz & Hofmann, 2009)
35 a	5.37	389.10873	$C_{16}H_{21}O_{11}$	2	59.0/121.1/165.1/183.1	oleoside isomer 1	(Difonzo *et al.,* 2022)
36	5.55	407.15573	$C_{17}H_{27}O_{11}$	1.6	71.0/151.0/101.0	dimethyl-4-(*α*-D-mannopyranosyloxy)-5-methoxy-1,2-cyclohexanedicarboxylate	CD
37	5.65	299.11365	$C_{14}H_{19}O_7$	3.6	59.0/89.0/101.02/119.04	salidroside	CD
38	5.71	137.02332	$C_7H_5O_3$	0.2	93.0/108.0/119.0	p-hydroxybenzoic acid	(Difonzo *et al.,* 2022)
39	5.76	345.11871	$C_{15}H_{21}O_9$	2.2	59.0/89.1	aucubin	CD
40	6.10	241.07150	$C_{11}H_{14}O_6$	2.0	95.1/139.0/127.0/101.0	elenolic acid	CD
41	6.29	359.13496	$C_{16}H_{24}O_9$	1.4	151.0	plantarenaloside	CD
42	6.38	339.07166	$C_{15}H_{15}O_9$	2.4	177.0	esculin	(Tóth *et al.,* 2015)
43	6.38	363.16600	$C_{16}H_{27}O_9$	2.9	169.1/59.0/101.0/115.0	*α*-L-galactopyranose, 6-deoxy-3-*O*-(2,6-dideoxy-4-*O*-methyl-*α*-D-arabino-hexopyranosyl)-2-*O*-methyl-, 4-acetate (9CI, ACI)	CD
44	6.44	151.03894	$C_8H_7O_3$	-0.2	123.0	vanillin	MB
45	6.68	565.17749	$C_{23}H_{33}O_{16}$	2	101.0/59.0/71.0/223.0/265.0/181.0/113.0	glucopyranosyl methyloleoside	(Liao *et al.,* 2018)
35 b	6.80	389.10910	$C_{16}H_{21}O_{11}$	2	59.0/121.1/165.1/183.1	oleoside isomer 2	(Difonzo *et al.,* 2022)
46	7.07	447.15060	$C_{19}H_{27}O_{12}$	0.7	269.1/161.0/401.1	barlerin	CD
47	7.18	177.01839	$C_9H_5O_4$	1.1	101.0/89.0/59.0	esculetin	(Tóth *et al.,* 2015)
48	7.31	223.06060	$C_{11}H_{12}O_5$	0.6	164.1/101.0/69.0/179.1	sinapinic acid	CD
49 a	7.32	403.12424	$C_{17}H_{23}O_{11}$	1.4	59.0/89.0/223.1	elenolic acid glucoside isomer 1	(Difonzo *et al.,* 2022)
50	7.56	377.14487	$C_{16}H_{25}O_{10}$	2.5	197.1/153.1	lamiol	(Alipieva *et al.,* 2007)
51 a	7.66	583.20251	$C_{27}H_{35}O_{14}$	0.4	179.1/195.1/375.1	(-)-olivil 4''-*O*-glucoside isomer 1	CD
52	7.76	537.19757	$C_{26}H_{35}O_{12}$	1.9	179.1/195.1/375.1	diosbulbinoside F	CD
53	7.86	215.09192	$C_{10}H_{15}O_5$	2.4	153.1/197.1/171.1	isoketocamphoric acid	CD
54	7.95	435.15009	$C_{18}H_{27}O_{12}$	0.9	101.0/157.0/89.0/119.0	alpigenoside	(Wang *et al.,* 2024)
49 b	8.00	403.12411	$C_{17}H_{23}O_{11}$	0.8	59.0/89.0/71.0	elenolic acid glucoside isomer 2	(Difonzo *et al.,* 2022)
55	8.22	461.16674	$C_{20}H_{30}O_{12}$	2.3	89.0	decaffeoylverbascoside	CD
56	8.35	639.19202	$C_{29}H_{35}O_{16}$	0.1	161.0/179.0/151.0/113.0	β-hydroxyacteoside	(Innocenti *et al.,* 2006)
57	8.37	401.18143	$C_{19}H_{29}O_9$	2	221.1/101.0/59.0/71.0	benzyl *β*-primeveroside	(Thakur *et al.,* 2024)

58	8.62	449.10791	$C_{21}H_{21}O_{11}$	1.4	287.1/259.1	eriodictyol-7-*O*-glucoside	(Sonmezdag *et al.*, 2018)
59 a	8.67	581.18750	$C_{27}H_{33}O_{14}$	2.9	89.0/59.0/295.0/355.1	naringin dihydrochalcone isomer 1	CD
60	8.87	555.17169	$C_{25}H_{31}O_{14}$	0.3	151.0	10-hydroxyoleuropein	(Tóth *et al.*, 2015)
61	9.45	609.14514	$C_{27}H_{29}O_{16}$	0.5	300.0	rutin	(Crescenzi *et al.*, 2021)
62	9.48	303.05121	$C_{15}H_{11}O_{7}$	3.3	125.0/285.0	nigrescin	CD
63	9.48	457.17126	$C_{21}H_{29}O_{11}$	2.8	149.1	gein	CD
64	9.49	443.15543	$C_{20}H_{27}O_{11}$	0.8	149.1	adinoside A	CD
65	9.59	535.18170	$C_{26}H_{31}O_{12}$	1.3	59.0/89.0/295.0/355.1	8-hydroxypinoresinol 4'-*O*-*β*-D-glucopyranoside	MB
59 b	9.59	581.18756	$C_{27}H_{33}O_{14}$	2.9	89.0/59.0/295.0/355.1	naringin dihydrochalcone isomer 2	CD
66	9.74	701.22992	$C_{31}H_{41}O_{18}$	0.8	315.1	oleuropein diglucoside	(Difonzo *et al.*, 2022)
67	10.03	415.13940	$C_{22}H_{24}O_{8}$	-1.2	151.0/280.0/295.1/375.1	acetoxypinoresinol	(Ali *et al.*, 2022)
68	10.09	187.09656	$C_{9}H_{15}O_{4}$	0.8	125.0	azelaic acid	CD
69	10.12	577.19220	$C_{28}H_{33}O_{13}$	1.1	151.0/415.1/295.1/325.1	8-acetoxypinoresinol 4-glucoside	CD
70	10.16	623.19824	$C_{29}H_{35}O_{15}$	1.4	415.1/151.0	verbascoside	(Tóth *et al.*, 2015)
71	10.31	567.17169	$C_{26}H_{31}O_{14}$	1.5	59.0/89.0/71.0/119.0/223.0/101.0/163.0/241.0	lamiidoside	CD
72	10.33	447.09329	$C_{21}H_{19}O_{11}$	0.9	285.0	luteolin 7-*O*-*β*-D-glucoside	(Difonzo *et al.*, 2022)
73	10.35	433.11157	$C_{21}H_{21}O_{10}$	1.1	113.0	naringenin hexoside	(Difonzo *et al.*, 2022)
74	10.61	287.05606	$C_{15}H_{11}O_{6}$	4.1	125.0	2-hydroxyflavanone naringenin	CD
75	10.63	539.17706	$C_{25}H_{31}O_{13}$	-0.4	275.1/149.0	oleuropein	(Difonzo *et al.*, 2022)
76	11.20	301.12906	$C_{14}H_{21}O_{7}$	3.5	185.1/71.0/115.0	butyl 3-(2-butoxy-2-oxoethyl)-3-hydroxy-4-oxooxetane-2-carboxylic acid	CD
51 b	11.26	583.20233	$C_{27}H_{35}O_{14}$	0.4	179.1/195.1/375.1	(-)-olivil 4''-*O*-glucoside isomer 2	CD
77	11.31	637.21338	$C_{30}H_{37}O_{15}$	1.9	203.1/429.2/59.1	4'-hydroxy-5,7,2'-trimethoxyflavanone 4'-rhamnosyl- (1->6) -glucoside	CD
78	11.53	523.18140	$C_{25}H_{31}O_{12}$	-0.4	291.0873/101.0231	ligustroside	(Tóth *et al.*, 2015)
79	11.71	395.20709	$C_{21}H_{31}O_{7}$	2	287.2/331.2	3-*O*-[[4-(3-methylbutoxy)phenyl]methyl]-1,2-*O*-(1-methylethylidene	CD

						)-α-D-glucofuranose	
80	11.84	685.23535	$C_{31}H_{41}O_{17}$	1	101.0/453.1/299.1	nuzhenide	(Difonzo *et al.,* 2022)
81a	11.96	377.12354	$C_{19}H_{21}O_8$	2.4	95.0/111.0/139.0/149.0/275.0	oleuropein-aglycone isomer 1	(Tóth *et al.,* 2015)
82	11.96	557.22363	$C_{26}H_{37}O_{13}$	-2.9	121.1/227.1/199.1/185.1	octenoyloxy secologanoside	(Araújo *et al.,* 2021)
81b	12.5	377.12411	$C_{19}H_{21}O_8$	2.4	95.0/111.0/139.0/149.0/275.0	oleuropein-aglycone isomer 2	(Tóth *et al.,* 2015)
83	13.15	327.21759	$C_{18}H_{31}O_5$	1.2	211.1/229.1/171.0	(10E,15Z)-9,12,13-trihydroxy-10,15-octadecadienoic acid	(Crescenzi *et al.,* 2021)
84	13.91	329.23310	$C_{18}H_{33}O_5$	1.3	211.1/229.1/171.0	(15Z)-9,12,13-trihydroxy-15-octadecenoic acid	(Crescenzi *et al.,* 2021)
85	16.02	293.17581	$C_{17}H_{25}O_4$	3.9	221.1/236.1	gingerol	(Crescenzi *et al.,* 2021)

Table S3 Targeted quantitative analysis of the quantified metabolites of xylem olive samples by UHPLC-ESI-QTrap-MS/MS analysis in MRM (Multiple Reaction Monitoring) mode (Concentration of metabolites in olive xylem (µg/mL), mean ± standard deviation (For metabolites that were not detected, a value of 0.000001 was assigned).

	Amino acid	Organic acids							Sugars					Terpenes		Phenol
Variety	Glutamine	Daucic acid	2-furoic acid	Cis aconitic acid	Citric acid	Fumaric acid	Malic acid	Ribonic acid	Gluconic acid	Sucrose	2-O-methylxylose	D-manno-2-heptulose	Mannitol	Oleuropein diglucoside	Elenolic acid glucoside	Salidroside
Arbequina	5.58±4.35	67.56±19.78	17.86±8.06	10.86±6.5	10.84±4.44	5.78±2.0	6.04±2.52	19.48±4.37	3.05±1.61	5.02±2.61	3.14±1.35	9.56±4.31	20.59±6.31	3.6±2.69	7.32±2.92	0.000001
Bella di Spagna	10.22±3.1	65.02±16.18	39.91±12.05	22.43±4.44	25.12±5.46	7.58±2.81	12.98±4.16	17.69±3.79	1.65±1.31	9.31±4.27	6.39±2.51	5.09±2.78	39.47±14.23	19.02±7.46	14.97±5.33	0.000001
Cima di Melfi	17.13±9.47	95.86±29.75	52.12±17.67	56.51±20.67	29.24±12.29	17.29±8.23	20.66±7.67	30.5±5.03	4.88±3.3	32.96±8.73	16.8±7.67	19.61±6.67	56.14±5.32	38.02±20.3	19.42±6.78	2.36±1.45
Coratina	0.000001	32.99±9.53	20.0±3.47	18.48±3.76	12.74±4.8	5.09±2.62	9.11±5.14	12.55±4.6	0.000001	10.71±4.62	8.8±3.58	1.07±0.79	38.43±10.01	7.45±3.62	8.52±2.46	0.52±0.33
FS-17	0.000001	28.03±8.75	19.87±3.32	16.82±4.53	9.75±1.49	7.52±0.87	6.58±1.25	7.24±2.21	0.000001	7.1±3.89	5.66±1.18	1.4±1.79	35.55±4.78	7.96±3.53	9.18±2.14	0.21±nan
Frantoio	4.54±3.59	61.29±30.59	42.76±12.26	28.65±9.15	30.76±20.78	14.16±4.1	17.93±7.38	31.53±7.56	4.66±3.73	16.73±9.79	13.89±8.05	15.09±6.9	72.62±17.66	35.92±9.79	20.14±6.53	1.48±1.34
Lecciana	27.82±24.27	40.74±21.64	44.74±7.25	36.67±15.52	27.92±13.88	11.95±6.4	11.98±6.37	12.7±3.98	5.84±3.03	4.23±3.17	6.68±2.36	0.000001	42.64±10.34	1.07±1.02	6.53±1.75	0.16±0.16
Leccino	2.4±2.02	89.24±32.25	52.89±7.55	42.66±7.91	31.96±6.79	16.43±3.12	22.31±3.21	55.09±15.14	11.29±3.91	33.72±7.47	18.72±7.82	6.83±1.17	108.48±14.82	37.82±7.51	31.88±10.49	8.2±2.28
Leccio del Corno	11.74±6.3	44.86±4.26	30.65±10.4	16.78±7.36	18.6±6.21	8.57±1.9	15.87±8.99	19.52±5.78	4.57±2.91	12.59±6.43	8.54±2.78	8.74±2.63	46.28±13.65	18.16±8.12	11.62±2.26	0.6±0.13
Nocellara messinese	4.1±2.41	38.7±12.1	30.45±5.69	15.45±3.83	16.43±4.95	7.64±3.43	2.94±1.19	6.76±2.46	4.45±1.64	2.24±1.82	4.47±1.46	1.1±0.8	29.58±6.76	3.28±2.67	6.52±1.9	0.46±0.35
Nociara	0.000001	50.18±21.83	46.74±12.17	30.08±6.12	24.91±8.16	9.99±4.84	15.37±2.47	32.54±17.29	1.8±1.73	16.02±5.85	13.26±3.99	14.37±7.22	49.14±9.71	24.7±18.56	19.47±9.43	0.000001
Ogliarola	5.15±2.52	25.93±7.27	19.67±5.75	14.66±4.85	10.22±3.89	4.17±2.49	8.46±2.11	7.11±2.51	0.000001	8.43±3.01	6.91±1.99	0.26±nan	36.36±9.11	7.12±3.73	5.67±1.77	0.000001
Pendolino	0.000001	31.54±6.21	46.36±21.93	43.49±19.9	25.32±12.2	21.3±12.76	15.79±8.03	45.62±23.88	6.86±0.62	22.86±8.69	18.33±9.13	21.94±9.18	58.32±6.12	44.33±9.75	13.31±2.57	2.95±2.16
Picholine	0.000001	68.91±26.2	34.4±10.81	36.12±13.31	20.9±9.88	10.96±2.91	11.92±4.96	29.49±10.07	1.35±0.97	15.06±6.29	11.69±5.06	10.26±4.15	58.43±10.43	25.46±10.91	11.54±4.36	0.95±0.87

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CHAPTER IV

Olive germplasm resistance to spittlebugs: an alternative approach to curb *Xylella fastidiosa* spread

5. Olive germplasm resistance to spittlebugs: an alternative approach to curb *Xylella fastidiosa* spread

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Under review

Abstract

The deployment of resistant plant varieties represents a durable and effective strategy to reduce economic losses caused by vector-borne plant pathogens. Since the emergence of the bacterium *Xylella fastidiosa* in Europe, research has largely focused on identifying plant resistance to the pathogen, overlooking plant traits underlying vector-plant interaction, thus shaping the transmission dynamic. Yet, plant genotypes possessing anatomical and/or chemical traits that reduce their suitability for insect vectors can hinder pathogen transmission by decreasing vector abundance and permanence on the host. Here, using the Electrical Penetration Graph (EPG), we evaluated host suitability for the spittlebug *Philaenus spumarius* in olive varieties widespread in Apulia Region, Southern Italy (Arbequina, Bella di Spagna, Cima di Melfi, Coratina, Frantoio, FS-17, Lecciana, Leccino, Leccio del Corno, Nocellara Messinese, Nociara, Ogliarola Salentina, Pendolino and Picholine). We then developed a plant suitability index integrating multiple EPG variables (escape events frequency, time to probe and reach xylem vessels, total probing time, and total duration of xylem ingestion) reflecting the likelihood of each olive genotype to serve as favorable host for spittlebugs. Analysis revealed that some of the varieties tested as Leccino, Leccio del Corno, Pendolino, and Arbequina present traits of resistance to spittlebugs reducing plant suitability, and their contribution to pathogen spread as source/recipient hosts could be limited. Overall, our index provides an integrative framework for assessing host resistance to xylem feeders and predicting disease epidemiology based on vectors behavior. Additionally, our study emphasizes the need to incorporate vector-plant interaction studies into breeding and management strategies to curb *X. fastidiosa* spread.

Keywords: Electrical Penetration Graph (EPG), Xylem index, Plant suitability index, xylem feeders

Introduction

Vector-borne plant pathogens, *i.e.,* bacteria, viruses and phytoplasmas, pose a major threat for agriculture worldwide (Huang et al. 2020). Their establishment and spread within the environment are intimately linked to ecology and behavior of their insect vectors, namely how vectors interact with their surroundings, including plants (Purcell 1981; Cornara et al. 2018). In particular, host plant location and acceptance behaviors are pivotal components of the transmission cycle of vector-borne plant pathogens (Daugherty et al. 2011). Insect vectors locate putative host plants within an environment through a multimodal process involving the integration of multiple cues, mainly visual cues and volatile organic compounds (VOCs). Upon landing, physical and chemical traits are assessed before the insect proceed with trial ingestions. This latter step permits the insect to evaluate whether the plant represents a suitable food source; namely, the presence/absence of the chemical components matching vector requirements will determine host acceptance and settling or, vice versa, rejection and search for a new host (Fereres and Moreno 2009; Döring 2014). Therefore, plant traits facilitating location and acceptance/settling will positively contribute to insect vectors abundance and permanence on the plant. For the transmission of some vector-borne plant pathogens such as the bacterium *Xylella fastidiosa* Wells, abundance and time vectors are on plants are positively associated with the likelihood of transmission to occur. Indeed, the higher the number of insect vectors on a plant and the longer they remain on it, the greater the number of probing events and the chances to engage with probing behavioral activities leading to pathogen acquisition and inoculation by the vector (Jackson et al. 2008; Daugherty and Almeida 2009). Therefore, a plant exhibiting traits of resistance to insect vectors, *i.e.,* anatomical and/or chemical traits reducing plant suitability, would hypothetically play a less relevant role in pathogen epidemiology compared to a suitable host (Mitchell et al. 2016; Ripamonti et al. 2022).

The meadow spittlebug *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) is the main European insect vector of *X. fastidiosa* subsp. *pauca* ST53, the causal agent of the Olive Quick Decline Syndrome in Southern Italy (Cornara et al. 2017). *Xylella fastidiosa* is a propagative non-circulative vector-borne bacterium, solely transmitted by xylem sap feeders. Transmission requires relatively short vector-plant interaction (few minutes), either for acquisition from source or inoculation to recipient host plants (Purcell and Finlay, 1979; Cornara et al. 2024a; Bodino et al. 2023). Due to the pathogen transmission dynamic, traditional control strategies relying on pesticides to curb insect vectors density usually yield inconsistent results in terms of containing pathogen spread (Dongiovanni et al. 2020; Lago et al. 2022). An alternative and

potentially more effective approach involves disrupting the vector's ability to locate and accept host plants, thereby reducing both the frequency and duration of vector–plant interactions and, consequently, the likelihood of pathogen transmission (Avosani et al. 2024). In addition to behavioral deterrents that interfere with host location and settling prior to transmission events, the use of plant genotypes that exhibits resistance traits to insect vectors offers a promising strategy to limit the spread of *X. fastidiosa*. So far, research has mainly focused on the search of sources of tolerance or resistance in plants against the bacterium (Wallis et al. 2013; Saponari et al. 2017; Montilon et al. 2023; Morales-Cruz et al. 2023), almost overlooking vector-plant interaction. To the best of our knowledge, traits of resistance to spittlebugs have solely been investigated in sugarcane varieties, whose key pest in Brazil is the spittlebug *Mahanarva fimbriolata* Stål (Hemiptera: Cercopidae) (Dinardo-Miranda et al. 2016). Greenhouse trials on different sugarcane genotypes showed limited *M. fimbriolata* development and survival on wild sugarcane accessions exhibiting potential antibioc and/or antixenotic traits (Orozco-Restrepo et al. 2017). Furthermore, traits of resistance to spittlebugs were reported in commercial sugarcane cultivars on which insects showed reduced nymphal survival, along with reduced feeding and oviposition (García et al. 2011; Dinardo-Miranda et al. 2014, 2016). Regarding traits of resistance to insect vectors of *X. fastidiosa* in plants of agricultural importance such as grapevine, olive and citrus, Kleina et al. (2020) correlated reduced *X. fastidiosa* prevalence in some plum genotypes to reduced suitability for the sharpshooter vector *Bucephalogonia xanthophis* Berg (Hemiptera: Cicadellidae). Additionally, Cornara et al. (2024b) observed a reduced suitability for *P. spumarius* of olive plants of the variety Leccino compared to Ogliarola Salentina (we refer to as "Ogliarola" therein) variety associated with a less favorable xylem sap chemical profile in the former compared to the latter. The reduced plant suitability for the insect vector, combined with Leccino's tolerance to *X. fastidiosa*, could explain the reduced pathogen prevalence in this genotype in the Southern Italian (Apulia Region) bacterium outbreak (Saponari et al. 2017; Cornara et al. 2024b). Leccino's plants less favorable chemical profile can be interpreted as a trait of resistance, given this chemical characteristic would make the plant less palatable, triggering the abandonment and the search for a new host. The study by Cornara et al. (2024b) focused exclusively on three olive varieties—specifically those most studied as hosts of *X. fastidiosa* ST53—where the authors observed differences primarily related to xylem and xylem-associated activities (i.e. ingestion of xylem sap). However, other olive varieties may exhibit different resistance traits against the insect vectors, including anatomical or chemical characteristics not directly linked to the xylem, which could hinder plant suitability for the insect vectors. Characterizing the suitability

of different other olive varieties for the spittlebug could open new venues for controlling bacterium spread through the deployment of plant genotypes less suitable for the vector, thus less likely to serve as source/recipient of *X. fastidiosa*.

Therefore, here, using the Electrical Penetration Graph (EPG), we characterized host plant suitability for the meadow spittlebug in fourteen olive varieties widespread in the Apulia region, Southern Italy, namely Arbequina, Bella di Spagna, Cima di Melfi, Coratina, Frantoio, FS-17, Lecciana, Leccino, Leccio del Corno, Nocellara Messinese, Nociara, Ogliarola, Pendolino and Picholine (Boscia et al. 2017; EFSA 2017; Saponari et al. 2017; Pavan et al. 2021; Camposeo et al. 2021, 2022). Thereafter, building EPG-indexes developed for phloem-sap feeding insects (see for example Girma et al. 1992 and 1994), we developed a plant suitability index for xylem feeders incorporating sequential and non-sequential EPG variables. The index expresses the likelihood of a plant to be accepted and served as food source for the spittlebug, and the possible presence of traits of resistance to *X. fastidiosa* vectors.

Materials and Methods

Insects and plants rearing

Third and fourth instar nymphs of *Philaenus spumarius* were collected in an *X. fastidiosa*-free area (41.018037°N, 16.902046°E) in Apulia region (Southern Italy). Nymphs were reared on ten-day-old host plants, including *Vicia sativa* L. (vetch) and *Vicia faba* L. (fava bean) plants until adulthood with plants replaced weekly. Newly molted individuals were collected and caged on a mix of *Vitis vinifera* L. (grapevine), *Amaranthus retroflexus* L. (common amaranth), fava bean, vetch and periwinkle (*Catharanthus roseus*) (sex ratio 1:1, ca. 200 individuals per cage). Both nymphs and adults were reared in Bugdorms inside an insect-proof shaded screenhouse at the DiSSPA facilities at University of Bari Aldo Moro, (Bari, South-Eastern Italy) (ca. 23 ± 6°C and 50 ± 10% RH, no artificial light). Herbaceous plants were replaced weekly; grapevines were pruned monthly. Plants were watered every other day. Periwinkle plants were replaced every two weeks and tested for *X. fastidiosa* one and three months after spittlebugs access to the plants by qPCR following the EPPO diagnostic standard PM 7/24 (5) (EPPO 2023) with the primer and TAQ probe described by Harper et al. (2010), to ensure that all individuals used in the trials were free of the bacterium (Spadavecchia-Hamouche et al. 2024).

For EPG experiments, one-year-old olive plants grown from cuttings in 1 L pots filled with a 2:1 mixture of Typical Brill substrate (Brill® Substrate 70 L, Italia) and perlite (PERALIT25—Perlite Italiana srl, MI, Italia) were used. Plants were kept inside an insect proof greenhouse, at temperature 22 ± 1°C and relative humidity 60 ± 5% (no photoperiod control), watered every other day and fertilized weekly with Plantafol 20:20:20 (NPK, Valagro) 1 g L^{-1}. To avoid interference with spittlebug probing behavior, no pesticides were applied.

EPG-assisted analysis of *Philaenus spumarius* probing and feeding behavior

The probing and feeding behavior of adult *P. spumarius* (both males and females) on fourteen olive varieties (Arbequina, Bella di Spagna, Cima di Melfi, Coratina, Frantoio, FS-17, Lecciana, Leccino, Leccio del Corno, Nocellara Messinese, Nociara, Ogliarola Salentina "hereafter Ogliarola", Pendolino and Picholine), was assessed by means of EPG technique, following the protocol described by Markheiser et al. (2023). Recordings were conducted during May-June 2025.

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 (EPG Systems, Wageningen, Netherlands (epgsystems.eu)). For the connection to the EPG, one to two months old spittlebug adults were anesthetized through brief exposure to -20°C in Eppendorf tubes for 1.5 minutes, then immobilized with a vacuum device and tethered to 18 µm gold wire (EPG-Systems, Wageningen, Netherlands) with a double layer of silver conductive paint (Pelco®, 16062, Ted Pella, USA). Following wiring, insects were transferred to aerated Petri-dishes for one hour starvation period, allowing them to acclimate to the attached wires. Before EPG connection, the wire-electrode connection was reinforced with additional conductive silver paint. The probing behavior of each spittlebug was recorded for 6 h. The recordings were performed inside a Faraday cage, under controlled conditions (23 ± 1°C, 60 ± 5% RH, supplemented with artificial lights 1,000 ± 200 lx). The insects were offered the apical 5 cm portion of olive plants, providing access to both stem tissue and at least one petiole or leaf. A minimum of ten replicates/recordings per sex per variety were carried out, with single combinations of insect/olive plant (one plant per spittlebug). To avoid positional effects, the position of each variety was switched between replicates.

EPG waveforms were manually marked using 'Stylet+a' software (EPG Systems, Wageningen, Netherlands) and then analyzed by "XylFeed" MS Excel macro (Markheiser *et al.,* 2023). Following the protocol described by Spadavecchia-Hamouche et al. (2024), EPG waveforms considered were 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) N, non-pathway interruption of xylem activity; vii) N2, second type of non-pathway xylem interruption; viii) R, resting; ix) Xe, behavior associated with *X. fastidiosa* inoculation; x) W, stylets withdrawal; xi) Esc, escaping of the individual from the host plant. EPG variables considered were: i) Total probing time - TPT (time spent by the spittlebugs with the stylets inserted in plant tissues over the 6 h); ii) Total residence time - TRT (time spent by the spittlebugs on the plant without escaping over the 6 h); iii) Probes: number of probes without (unsuccessful) and with xylem ingestion (successful), number of probes with only xylem vessel contact (initial); number of probes with a xylem ingestion event longer than 5 min (sustained); iv) NWEI: number of waveform events per insect; v) WDEI: median duration of the single waveform event per insect; vi) WDI/TPT: total duration of the waveform per insect over the total probing time; vii) WDI/TRT: total duration of the waveform per insect over the total residence time; viii) Sequential: time required to perform a certain waveform calculated either

from the beginning of the EPG recording or from the first probe. To account for "escape" events, the ratios WDI/TPT and WDI/TRT were analyzed instead of considering WDI alone (*i.e.*, total time spent by spittlebugs engaging in a specific waveform).

Statistical analysis of the EPG data was carried out using R software (Version 4. 3. 1). Variety-associated probing behavioral differences (*i.e.,* total probing time, total residence time, probes, non-sequential and sequential variables) were investigated with Generalised Linear Mixed Models using Template Model Builder (GLMM.TMB). Olive variety and sex were used as explanatory variables, with the day of the recording as random factor. The data obtained from the EPG were transformed, when necessary, with ln (x+1) or √ (x) to reduce heteroscedasticity and improve normal distribution. However, for variables where these transformations proved insufficient and showed distributional misfit, lognormal family was implemented to better model their distributions (Brooks et al. 2017). Models were run using the "glmmTMB" package (Venables & Ripley, 2002), while residual distribution was checked using the "DHARMa" package (Fox & Weisberg, 2019). An analysis of variance (ANOVA) was performed using the "car" package to assess the overall significance of fixed effects in the model. For statistically significant effects (p<0.05), pairwise comparisons among olive varieties were conducted using the "emmeans" package (Lenth, 2021). A summary of the raw datasets was prepared by "psych" package (Revelle, 2021). Plots were generated using visreg package (Breheny & Burchett, 2017).

Plant suitability index:

We generated a simplified plant suitability index based on EPG variables expressing the likelihood for a plant to be accepted and serve as food source for the spittlebug, *i.e.,* to host larger vector populations for longer periods. The EPG variables considered are:

- **Frequency of escape events (Esc):** Spittlebugs abandon the host plant when sensing cues not matching their requirements or when disturbed by exogenous stressors. A higher frequency of escapes might therefore indicate the plant the insect is given access to presents chemical and/or anatomical traits not matching spittlebugs requirements.
- **Time to first probe:** indicate the time needed for the spittlebug upon landing to insert the stylets within the plant tissues. High values might indicate the presence of chemical and/or anatomical traits reducing the host attractiveness, or the absence of the necessary cues triggering the probe;

- **Time to the first xylem contact (Xc):** indicate the time required by the spittlebug to reach xylem vessels upon landing. High values might suggest difficulties in reaching the vessels and initiate feeding;
- **Time to the first xylem ingestion (Xi):** indicate the time required by the spittlebug to start feeding (xylem sap uptake) upon landing. High values might reflect either difficulties in reaching the vessels and initiating feeding, or the absence of chemical cues promoting rapid sap intake;
- **Time to the first low frequency xylem ingestion (LF):** indicate the time for the onset of a low frequency xylem ingestion (discontinuous ingestion driven by scattered contraction of the cibarial pump) upon spittlebug landing on the plant. This behavior reflects difficulties in maintaining a constant sap intake powered by steady cibarial muscles contraction, resulting in a scattered activity (LF) while still keeping the stylets inserted into the xylem vessel. Therefore, the sooner LF occurs upon landing and initiation of probing, the higher the likelihood of plant presenting traits possibly affecting feeding. The chronological order of sequential EPG variables used in the plant suitability index is illustrated in Online Resource 1;
- **Total probing time:** indicates the time spent by spittlebugs with stylets inserted into host plant tissues during the EPG recording. The shorter the probing time, the higher the likelihood the plant exhibits anatomical and/or chemical traits impairing probing and reducing the plant suitability;
- **Xylem ingestion index, expressed as overall duration of the ingestion (Xi+LF) period over the total probing time (TPT):** the variable expresses the percentage of time spent with stylets inserted into host plant tissues (probe) during which the spittlebug is actively feeding, (ingesting xylem sap). The higher this percentage, the shorter the time the insect is engaged in behaviors different from feeding. Pie charts representing the proportion of total residence time spent in non-probing (np), xylem ingestion (Xi+LF), and other probing activities for each olive variety are presented in Online Resource 2. Each pie chart represents the relative duration of the three EPG phases, averaged across all recordings.

To generate the plant suitability index, for each of the EPG variables considered, olive varieties were grouped according to their similarities through hierarchical clustering. Adjusted EPG variables means were estimated for each olive variety using the “emmeans” package. The

estimated marginal means were extracted and used to compute a Euclidean distance matrix. Hierarchical clustering was then performed using Ward's minimum variance method (ward.D2) as implemented in the hclust function (Murtagh & Legendre, 2014). The resulting dendrogram was generated with the "dendextend" package.

Thereafter, we generated a simplified matrix attributing to each olive variety a score depending on the cluster; the score indicates spittlebug performance on each variety in relation to a specific probing and feeding pattern. For example, hierarchical clustering for the xylem ingestion index grouped the varieties into eight clusters, ranging from cluster 1 grouping the varieties with the lowest index, to cluster 8 grouping the varieties with the highest index. Thus, for the xylem ingestion index, varieties have been attributed a score ranging from 1, *i.e.,* varieties with the lowest duration of the xylem ingestion in relation to the total probing time, to 8, varieties with the longest ingestion in relation to their total probing time. Since the number of clusters varied across EPG variables (ranging from 8 to 10 depending on the variable), raw scores were converted into percentages (with highest cluster corresponding to 100%) to standardize their contribution and prevent bias due to variables with more clusters. Afterwards, the scores were scaled by dividing by 100, ensuring comparability and yielding smaller, more manageable final values.

The final score in the matrix represents the overall value obtained, summing all the relative values for each of the EPG variables considered. The score for each EPG variable depended on the number of clusters generated through hierarchical clustering; however, the scores always ranged from a minimum value (worst performance) to a maximum value (best performance). When EPG variables selected for the plant suitability index did not significantly differ among the olive varieties according to the results of the GLMM model, we attributed to all the varieties a score of zero.

Results

Philaenus spumarius probing and feeding behavior on olive varieties

Here, only statistically significant differences in spittlebug probing and feeding behavior on the fourteen olive varieties are reported, along with the corresponding raw mean values ± standard error. Detailed data including ANOVA results and significant pairwise comparisons for relevant EPG variables are summarized in Online Resource 3. Descriptive statistics of EPG variables with non-significant results are provided in Online Resource 4.

The total probing time was significantly shorter on Arbequina (236.43 ± 27.79 min) compared to Picholine (306.99 ± 17.37 min), FS-17 (312.01 ± 15.28 min), and Bella di Spagna (325.16 ± 10.73 min), and longer on FS-17 (312.01 ± 15.28 min) compared to Pendolino (252.62 ± 25.85 min), and on Bella di Spagna (325.16 ± 10.73 min) compared to Cima di Melfi (245.33 ± 27.27 min), Pendolino (252.62 ± 25.85 min), Leccio del Corno (288.54 ± 24.02 min), and Nociara (305.01 ± 19.95 min). Similarly, the total residence time was significantly longer on Bella di Spagna (360 min) compared to Cima di Melfi (272.48 ± 28.2 min), Leccio del Corno (319.95 ± 20.01 min), Pendolino (322.53 ± 21.24 min), and Nociara (329.83 ± 19.51 min). Spittlebugs on Cima di Melfi (272.48 ± 28.2 min) exhibited shorter total residence time compared to Picholine (339.17 ± 14.95 min) and FS-17 (347.94 ± 11.68 min).

The total number of probes was significantly greater on FS-17 (4.41 ± 0.75) compared to Lecciana (2.65 ± 0.78).

The average duration of single pathway events (C WDEImd) was significantly longer on Bella di Spagna (3.72 ± 1.28 min) compared to FS-17 (0.91 ± 0.1 min).

The total number of xylem contact events (Xc NWEI) was significantly lower on Arbequina (3.9 ± 0.65), Cima di Melfi (4.20 ± 0.88) and Leccio del Corno (4.4 ± 0.91) compared to Coratina (9.5 ± 1.16), FS-17 (9.59 ± 1.11), and Ogliarola (11.53 ± 1.69). On Ogliarola, spittlebugs exhibited more xylem contact events (Xc NWEI) (11.53 ± 1.69) compared to Nociara (5.05 ± 0.85) and Pendolino (5.4 ±1.16). Moreover, the total duration of xylem contact with respect to the total residence time (Xc WDI/TRT) was significantly longer on Ogliarola (0.58 ± 0.1) compared to Cima di Melfi (0.36 ± 0.1) and Leccio del Corno (0.38 ± 0.13).

Additionally, insects on Arbequina showed significantly fewer xylem ingestion events (Xi NWEI) (11.5 ± 1.85) compared to Picholine (15.95 ± 1.59), Coratina (19.19 ± 2.12), Frantoio (19.65 ±3.01), FS17 (18.64 ± 1.7), Lecciana (22.04 ± 5.34), Ogliarola (21.05 ± 2.77). Similarly,

spittlebugs on Cima di Melfi performed significantly fewer Xi NWEI (13.9 ± 1.87) than Coratina, FS17, and Ogliarola. However, the number of xylem ingestion events was higher on Coratina than Leccio del Corno (12.05 ± 1.51), Nocellara Messinese (12.38 ± 1.63), and Pendolino (13.8 ± 1.99). Spittlebugs on Frantoio exhibited higher Xi NWEI than Leccio del Corno, and Nocellara Messinese. Moreover, insects displayed higher Xi NWEI on FS17 compared to Leccio del Corno, Nocellara Messinese, and Pendolino, and on Lecciana compared to Leccio del Corno and Nocellara Messinese. Nonetheless, Xi NWEI recorded on Ogliarola was significantly reduced compared to Leccio del Corno, Nocellara Messinese, and Nociara, while remaining higher than on Pendolino.

The total duration of xylem ingestion with respect to the total probing time (Xi WDI/TPT) was significantly longer on Bella di Spagna (79.85 ± 4.68) compared to Leccino (54.84 ± 5.65), Pendolino (56.07 ± 6.81), Picholine (57.2 ± 5.52), Arbequina (59.68 ± 5.6), and Nocellara Messinese (63.66 ± 6.42). Conversely, spittlebugs given access to Leccino and Pendolino plants displayed significantly shorter durations of Xi WDI/TPT compared to FS-17 (68.09 ± 5.26), Coratina (69.41 ± 4.4), Frantoio (72.06 ± 5.63), Leccio del Corno (72.09 ± 5.28), Ogliarola (74.05 ± 4.79), Nociara (77.76 ± 6.09). Moreover, the total duration of xylem ingestion with respect to the total residence time (Xi WDI/TRT) was significantly shorter on Arbequina (43.43 ± 6.12) compared to Ogliarola (67.11 ± 4.87) Nociara (70.71 ± 6.11), and Bella di Spagna (72.84 ± 5.08). Additionally, spittlebugs on Pendolino exhibited shorter Xi WDI/TRT (43.76 ± 6.96) compared to Nociara (70.71 ± 6.11) and Bella di Spagna (72.84 ± 5.08). Conversely, Ogliarola displayed longer Xi WDI/TRT (67.11 ± 4.87) compared to Leccino (46.48 ± 5.23).

The shortest xylem ingestion index, *i.e.,* the overall duration of the total xylem ingestion (Xi + LF) relative to the total probing time (Xi + LF WDI/TPT) was observed for spittlebugs given access to Leccino, Pendolino, and Picholine. Specifically, the xylem index was significantly shorter on Leccino (69.14 ±5.08) compared to Leccio del Corno (79.04 ± 4.4), FS-17 (79.65 ± 3.91), Frantoio (81.65 ± 3.56), Coratina (82.65 ± 3.04), Ogliarola (83.28 ± 2.74), Bella di Spagna (84.28 ± 4.28), and Nociara (85.38 ± 4.35). Similarly, insects on Pendolino exhibited shorter Xi + LF WDI/TPT (67.15 ± 5.56) than on Coratina, Frantoio, FS-17, Leccio del Corno, Nociara and Ogliarola. Moreover, the xylem index for *P. spumarius* on Picholine (70.74 ± 5.31) was significantly lower compared to Bella di Spagna, Coratina, Frantoio, Nociara and Ogliarola.

Furthermore, the overall duration of the total xylem ingestion (Xi + LF) relative to the total residence time (Xi + LF WDI/TRT) was significantly shorter on Arbequina (54.74 ± 6.75) compared to Frantoio (67.91 ± 4.11), Cima di Melfi (68.59 ± 3.68), FS-17 (70.96 ± 4.32), Coratina (71.19 ± 4.12), Nocellara Messinese (72.53 ± 4.91), Ogliarola (75.91 ± 3.55), Bella di Spagna (77.04 ± 4.98), and Nociara (78.19 ± 4.96).

Spittlebugs on Frantoio exhibited shorter average duration of single xylem ingestion bout (Xi WDEImd) (3.92 ± 0.44 min) compared to Nocellara Messinese (13.78 ± 2.61 min) and Leccio del Corno (15.38 ± 3.82 min).

The total number of non-pathway events (N NWEI) was significantly lower on Arbequina (5.9 ± 0.96) compared to Coratina (13.54 ± 2.06) and Ogliarola (15.68 ± 2.43).

Significant differences were also observed among the olive varieties in EPG sequential variables. Indeed, the time required for the stylets to reach xylem vessels and for the onset of xylem sap ingestion, calculated either from the start of EPG recording or from the first probe, was significantly shorter on Leccino (np to 1st Xc: 5.29 ± 0.72 min; np to 1st Xi: 5.76 ± 0.72 min; 1st C to 1st Xc: 3.31 ± 0.61 min; 1st C to 1st Xi: 3.79 ± 0.63 min) compared to Leccio del Corno (np to 1st Xc: 24.43 ±5.32 min; np to 1st Xi: 24.8 ± 5.33 min; 1st C to 1st Xc: 15.84 ± 4.18 min; 1st C to 1st Xi: 16.24 ± 4.2 min) and Bella di Spagna (np to 1st Xc: 25.78 ± 7.54 min; np to 1st Xi: 26.2 ± 7.52 min; 1st C to 1st Xc: 21.86 ± 6.67 min; 1st C to 1st Xi: 22.27 ± 6.65 min). Moreover, the time from the start of EPG to first xylem ingestion (np to 1st Xi) was significantly shorter on Leccino (5.76 ± 0.72 min) compared to Nocellara Messinese (23.49 ± 6.45 min).

The time required to perform the first sustained xylem ingestion calculated from the start of EPG recording (np to 1st sustained Xi) was significantly shorter on Leccino (17.78 ± 7.96 min) compared to Frantoio (39.92 ± 9.64 min). Similarly, when calculated from the onset of the first probe (1st C to 1st sustained Xi), spittlebugs on Leccino showed a significantly shorter time to engage with xylem sap ingestion (3.79 ± 0.63 min) than on both Coratina (8.26 ± 2.4 min) and Frantoio (19.16 ± 8.63 min).

The time needed to initiate the first low-frequency xylem ingestion (LF) calculated from the start of the EPG recordings (np to 1st LF) was significantly shorter on Coratina (157.22 ± 29.67 min) compared to Ogliarola (225.38 ± 30.89 min) and Frantoio (264.92 ± 28.16 min), and also shorter on Leccino (156.99 ± 27.29 min) compared to Frantoio (264.92 ± 28.16 min). When calculated from the first probe (1st C to 1st LF), insects on Frantoio exhibited a significantly

longer time to first LF (260.04 ± 27.71 min) compared to Coratina (154.2 ± 29.37 min), Leccino (155.03 ± 27.14 min), Lecciana (155.08 ± 28.3 min), Pendolino (170.38 ± 32.41 min) and Cima di Melfi (190.73 ± 34.91 min). Additionally, spittlebugs on Coratina required a shorter time to perform the first LF (154.2 ± 29.37 min) compared to Ogliarola (222.01 ± 31.07 min).

Independently of varieties, the average duration of single xylem ingestion bouts (Xi WDEImd) was significantly shorter in males (7.83 ± 0.75 min) compared to females (9.7 ± 0.91 min). Moreover, the total duration of resting event with respect to the total probing and residence times (R WDI/TPT and R WDI/TRT) was significantly shorter in males (22.23 ± 2.13; 19.51 ± 1.93, respectively) compared to females (31.68 ± 2.28; 27.61 ± 2.15, respectively).

The waveform Xe (pattern leading to *X. fastidiosa* inoculation) was observed only in three EPG recordings: one on Coratina and one on Leccino, both performed by female spittlebugs, and a third on Pendolino by a male spittlebug.

Plant suitability index

The plant suitability index was calculated based on seven EPG variables considered relevant to spittlebug host plant acceptance, namely: i) frequency of Escape events; ii) Time to first probe; iii) Time to first Xc; iv) Time to first Xi; v) Time to first LF; vi) Total probing time; vii) Xylem ingestion index (Xi+LF WDI/TPT). Among these, Time to first probe and Escape events did not differ significantly among the varieties and were therefore assigned a score of zero. The other variables were grouped into clusters through hierarchical clustering (Fig. 1) with Time to first Xc, Time to first Xi and Xylem ingestion index grouping the varieties into eight clusters, Time to first LF into nine clusters, and Total probing time into ten clusters. The cluster-based scores attributed to each variety along with the final scores are shown in Table 1.

Overall, the plant suitability index revealed a gradient of host suitability among the 14 olive varieties tested. Ogliarola ranked as the most suitable variety, achieving the highest score (4.26), followed in decreasing order by Coratina (3.44), Frantoio (3.40), FS-17 (3.34), Leccino (3.05), Nociara (3.04), Lecciana (2.95), Bella di Spagna (2.68), Nocellara Messinese (2.34), Picholine (2.23), Cima di Melfi (2.16), Leccio del Corno (2.06), Pendolino (2.06), and Arbequina (2.00) (Fig. 2).

Figure 1. Hierarchical clustering of olive varieties for each EPG variable that differed significantly according to the GLMM model; (a) Time to first Xc, (b) Time to first Xi, (c) Time to first LF, (d) Total probing time, (e) Xylem ingestion index (Xi+LF WDI/TPT). Numbers above the branches indicate cluster groups of olive varieties for each variable. For time to first Xc and time to first Xi (a-b), lower cluster numbers correspond to longer times required to reach xylem vessels, whereas higher cluster numbers indicate shorter times and earlier xylem access. Conversely, for the time to first LF (c), lower cluster numbers indicate an earlier onset of low-frequency ingestion, while higher numbers correspond to longer delays before LF activity begins. For total probing time and xylem ingestion index (d-e), increasing cluster numbers represent higher values, indicating longer probing duration and greater proportions of time devoted to xylem ingestion, respectively.

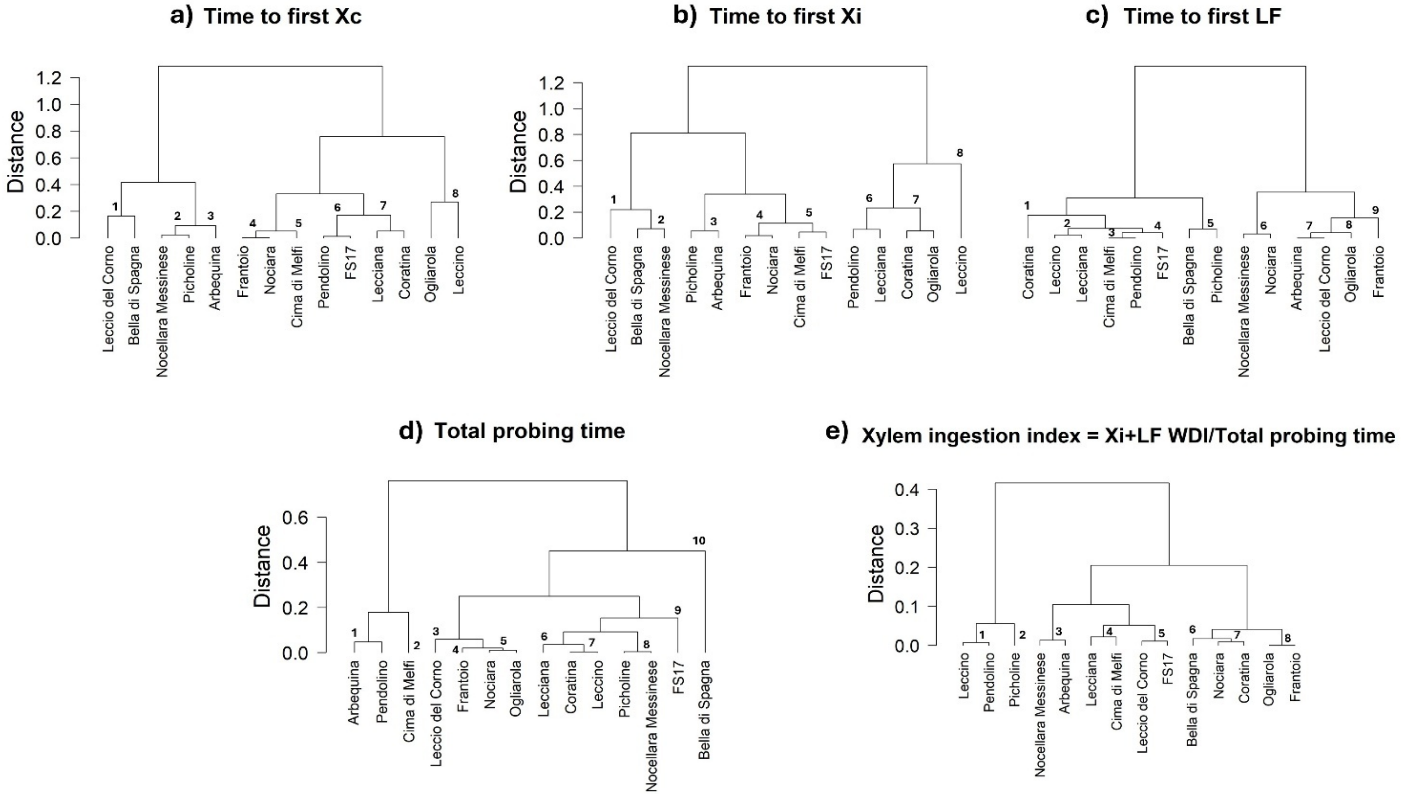


Figure 2. Contribution of each EPG variable (expressed as a percentage) to the plant suitability index for each olive variety. Olive varieties are arranged from the highest to the lowest plant suitability index.

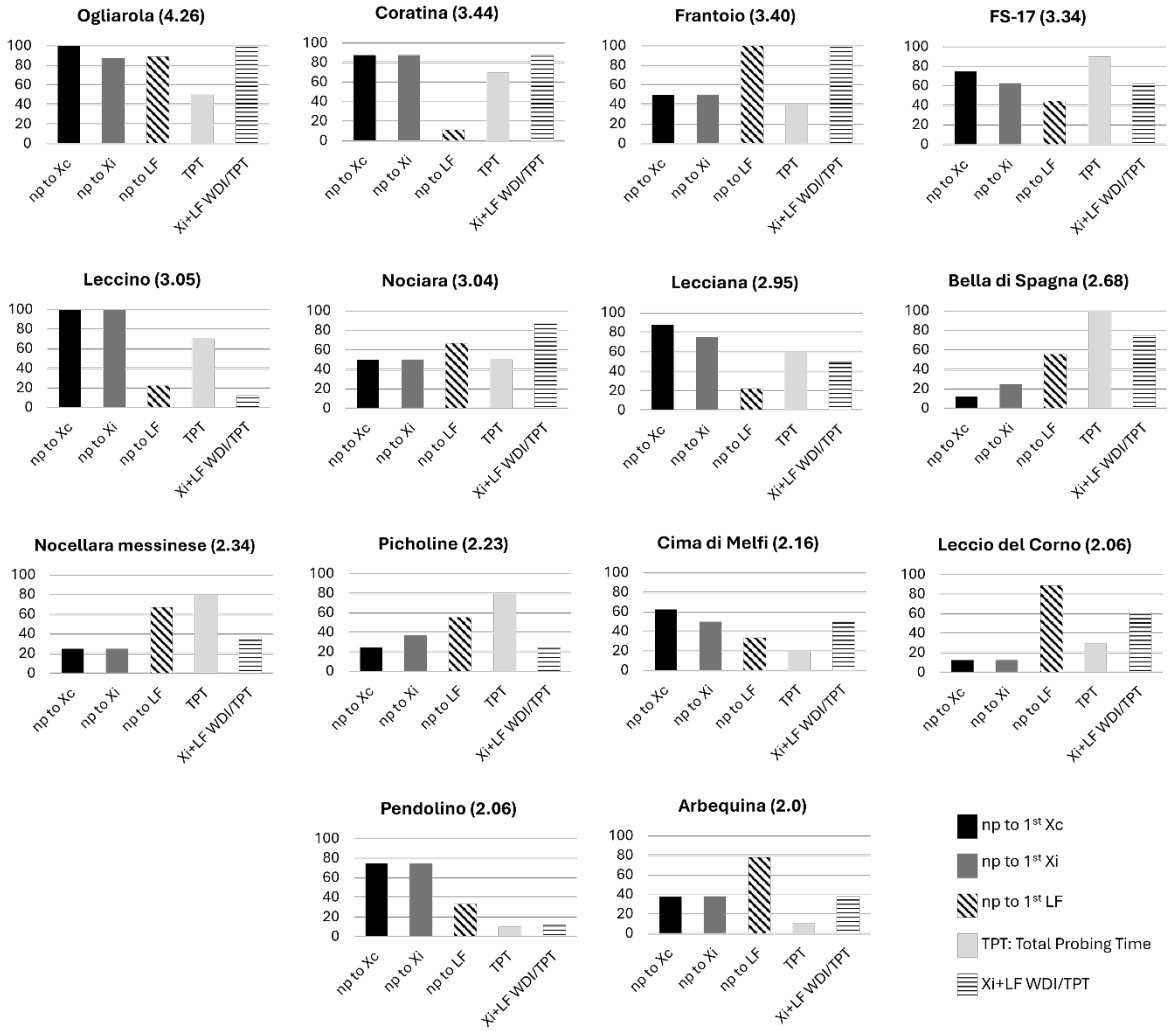


Table 1. Plant suitability index scores for olive varieties based on different EPG variables. The varieties are ordered from the highest to the lowest index.

EPG Variable	Escape	Time to 1st probe	Time to 1st Xc	Time to 1st Xi	Time to 1st LF	Total probing time	Xi+LF WDI/TPT	Final Score
Number of clusters	-	-	8	8	9	10	8	
Ogliarola	0	0	1	0.875	0.889	0.5	1	4.26
Coratina	0	0	0.875	0.875	0.111	0.7	0.875	3.44
Frantoio	0	0	0.5	0.5	1	0.4	1	3.40
FS-17	0	0	0.75	0.625	0.444	0.9	0.625	3.34
Leccino	0	0	1	1	0.222	0.7	0.125	3.05
Nociara	0	0	0.5	0.5	0.667	0.5	0.875	3.04
Lecciana	0	0	0.875	0.75	0.222	0.6	0.5	2.95
Bella di Spagna	0	0	0.125	0.25	0.556	1	0.75	2.68
Nocellara messinese	0	0	0.25	0.25	0.667	0.8	0.375	2.34
Picholine	0	0	0.25	0.375	0.556	0.8	0.25	2.23
Cima di Melfi	0	0	0.625	0.5	0.333	0.2	0.5	2.16
Leccio del Corno	0	0	0.125	0.125	0.889	0.3	0.625	2.06
Pendolino	0	0	0.75	0.75	0.333	0.1	0.125	2.06
Arbequina	0	0	0.375	0.375	0.778	0.1	0.375	2.00

Discussion

In the present study, we characterized *P. spumarius* probing and feeding behavior on 14 olive varieties, namely Arbequina, Bella di Spagna, Cima di Melfi, Coratina, Frantoio, FS-17, Lecciana, Leccino, Leccio del Corno, Nocellara messinese, Nociara, Ogliarola, Pendolino and Picholine widespread in the Apulia Region (Southern Italy) through Electropenetrography (EPG). Thereafter, using some of the EPG sequential (*e.g.,* time to perform a certain behavior) and non-sequential (*e.g.,* duration or frequency of a specific behavior) variables, we developed a plant suitability index reflecting the likelihood for a certain genotype to support large spittlebug populations for longer intervals, and the possible presence of chemical/anatomical traits ultimately leading to host rejection.

Traits of resistance to insect vectors, *i.e.,* unfavorable plant traits reducing abundance and permanence of the insect on the plant, might significantly influence the transmission of a vector-borne plant pathogen (Ripamonti et al. 2022). Indeed, a plant genotype with traits not matching insect requirements for location and/or acceptance could hypothetically be less relevant as source/recipient host for the pathogen, namely its contribution to pathogen spread would be less relevant than plants with suitable traits for the insect vector. The index that we developed accounts for the process of host acceptance, *i.e.,* upon insect landing on the host, without considering traits mediating host location as visual cues (Lazar et al. 2025). Conclusive data on the epidemiological role of each genotype would therefore require additional trials encompassing choice tests and transmission trials coupled with field data, the latter being so far scattered. Nevertheless, here we discuss our data on host plant acceptance and their possible reflection on how the olive varieties tested could support *X. fastidiosa* spread considering plant suitability upon landing.

Host plant acceptance by xylem feeders (upon landing) largely depends on the chemical profile of the xylem sap (Cornara et al. 2024b; Tinelli et al. 2025). The xylem ingestion index, intended as the proportion of the probing time spent in ingesting the xylem sap, is therefore a variable of paramount importance for host plant acceptance. A high index reflects a xylem sap metabolome favorable for the insect and promoting sap intake and insect permanence on the plant. On the other hand, low xylem index is expected on plants whose xylem sap does not match spittlebugs requirements, and that would be abandoned after xylem tasting/testing. Considering only the xylem index, the most and least suitable varieties for *P. spumarius* among those tested are Ogliarola and Frantoio, and Leccino and Picholine, respectively. Independently

of the xylem index, the absence of anatomical and/or chemical traits impairing the stylets insertion and the rapid finding of a xylem vessel, along with the rapid onset of a continuous (EPG waveform Xi) rather than scattered (EPG waveform LF) xylem sap intake, are all factors that might potentially concur with the xylem index in determining plant suitable for the spittlebugs. Therefore, the plant suitability index we developed incorporates all the EPG variables reflecting the time needed from the access to the plant to the first stylets insertion, access to xylem vessels, initiation of sap intake, and occurrence and duration of scattered/discontinuous ingestion.

The variety with the highest plant suitability index, *i.e.,* the variety on which *P. spumarius* fed most effectively, was Ogliarola. Indeed, access to xylem vessels was relatively rapid, xylem ingestion was the prevalent behavior exhibited during probing (ca. 80-85% of the total probing time), with scattered/discontinuous ingestion (LF) occurring occasionally and later during the probe than in other varieties. Therefore, the high suitability of this genotype for the insect vector, combined with the high susceptibility for the bacterium (Saponari et al. 2017), likely explains the high pathogen prevalence in olive groves dominated by Ogliarola in the Apulia Region.

Coratina, Frantoio and FS-17 also displayed high plant suitability index, permitting rapid access to xylem vessels and relatively long xylem sap ingestion. Along with Ogliarola, these varieties provided a highly palatable xylem profile, combined with the lack of structural obstacles or deterrent chemical cues in non-xylematic tissues, which facilitated rapid stylets penetration and access to xylem vessels. Coratina and Frantoio are varieties susceptible to *X. fastidiosa* ST53, although hosting lower bacterial population and exhibiting milder symptoms compared to Ogliarola (Saponari et al. 2017). On the other hand, FS-17 is a tolerant variety (*sensu* Cooper and Jones 1983), *i.e.,* a genotype that can be systemically and chronically infected by the bacterium without causing severe symptoms. The combination of bacterial tolerance and high suitability for the insect vector presents an epidemiological conundrum. Vector preference varies dynamically as disease progresses (Daugherty et al. 2017); when symptoms appear at the end of the incubation period, insect vectors are expected to abandon symptomatic hosts searching for asymptomatic green shoots (Marucci et al. 2004; Rashed et al. 2011). However, if an infected plant, besides being suitable for the insect vector, never or seldomly develop symptoms, it could theoretically support spillover into pathogen-susceptible populations over the long term compared to susceptible/symptomatic genotypes (Sisterson and Stenger 2018; Zeilinger et al. 2021). Moreover, both plant infection status and vector infectivity

can modulate vector probing and feeding behavior and host acceptance (Marucci et al. 2004; Cornara et al. 2020), potentially altering the suitability patterns observed under non-infected conditions. Further studies combining field surveys and transmission tests should carefully investigate the FS-17 role in *X. fastidiosa* spread, given this variety is being vastly planted in the Apulia Region for replacing susceptible olive varieties (Pavan et al. 2021; Camposeo et al. 2022; Regione Puglia 2024). Similar investigations are dramatically needed for other two bacterium-tolerant olive varieties that Apulian authorities have recently approved for planting in *X. fastidiosa* ST53-infected areas, namely Leccino and Lecciana (Regione Puglia 2024). The two varieties have similar plant suitability index, although Leccino scores are higher than Lecciana for variables related to the easy and rapid access to xylem vessels and onset of xylem sap ingestion, while displaying one of the lowest xylem index among all the varieties tested due to an unfavorable xylem sap chemical profile (Cornara et al. 2024b). On the other hand, access to xylem and ingestion onset required longer time in Lecciana compared to Leccino, while xylem index was significantly greater than Leccino and overall similar to values observed for FS-17, suggesting a more suitable xylem metabolome. Overall, given the importance for xylem sap feeders of xylem sap chemical profile for determining host plant acceptance, we speculate the variety Lecciana could be more relevant than Leccino for supporting bacterium spread, although further data are needed to verify this hypothesis.

Leccio del Corno, Pendolino and Arbequina olive varieties displayed the lowest plant suitability indexes among the 14 genotypes tested. On Leccio del Corno and Arbequina, spittlebugs required relatively long time to reach xylem vessels and initiate the ingestion. However, xylem index in Leccio del Corno was similar to values observed for FS-17 and Lecciana, and higher than in Arbequina. Conversely, Pendolino showed rapid xylem accessibility but stood out for its short total probing time and the lowest xylem index (*i.e.,* proportion of probing time devoted to xylem sap ingestion) along with Leccino. This indicates that Pendolino and Leccino offer the least suitable xylem chemical profiles for *P. spumarius*. Overall, these olive genotypes could possibly be the poorest contributor to bacterium spread among the ones tested, given abundance and permanence of insect vectors on these plants, thus the likelihood of pathogen transmission, are expected to be limited.

Eventually, our study on *P. spumarius* probing and feeding behavior on 14 olive varieties widespread in the Apulia Region reveals substantial variation in host suitability for spittlebugs and potential different contribution to *X. fastidiosa* spread. Our findings also emphasize the need to incorporate vector-plant interaction studies into breeding and management strategies

aimed at mitigating the spread of *X. fastidiosa* through the deployment of genotypes with traits of resistance to insect vectors.

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Competing Interests

The authors declare no competing interests.

Author Contributions

Conceptualization: DC and ZH; Methodology: DC and AF; Formal analysis and investigation: ZH, GS; Writing - original draft preparation: ZH and DC; Writing - review and editing: all the authors; Funding acquisition: DC; Supervision: DC

Data Availability

Additional data are available from the corresponding author (daniele.cornara@uniba.it) upon request.

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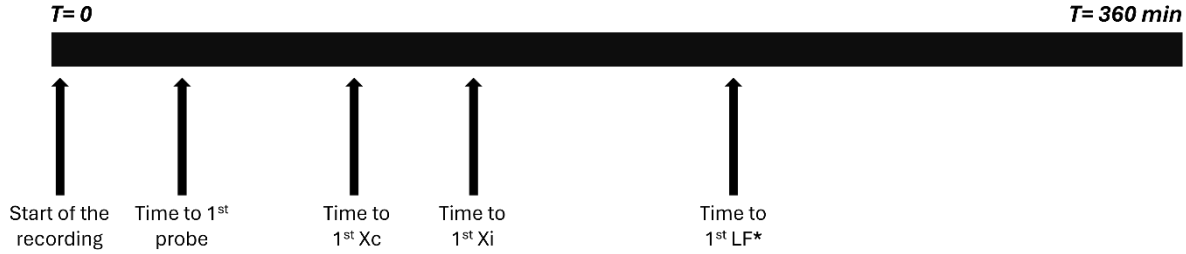
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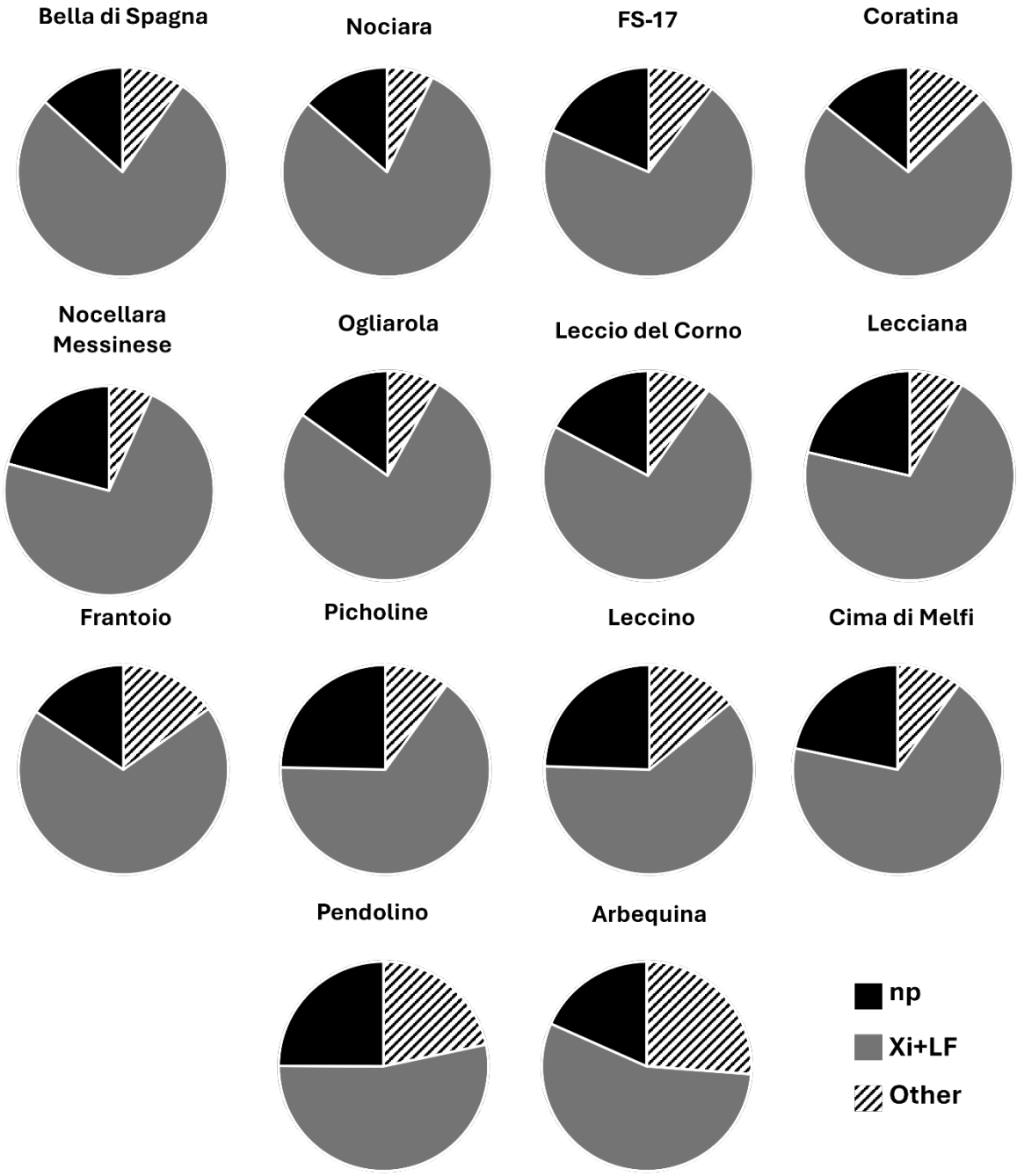
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Supporting Information

Online Resource 1. Chronological order of sequential EPG variables used in the plant suitability index; (*****: Time to first LF may precede the time to first Xi on less suitable varieties).



Online Resource 2. Proportion of total residence time spent in non-probing (np), xylem ingestion (Xi+LF), and other probing activities for each olive variety. Each pie chart represents the relative duration of the three EPG phases, averaged across all recordings. The values are based on raw data. Pie charts are arranged in decreasing order of total duration of xylem ingestion (Xi+LF).



Online Resource 3. Anova results and pairwise comparisons of the statistically significant EPG variables (*p*-values: * < 0.05; ** < 0.01; *** < 0.001).

EPG Variables	ANOVA					Tukey test				
		Chisq	Df	Pr(>Chisq)		contrast	estimate	SE	z ratio	*p* value
Total probing time	**Variety**	57.764	13	<.0001	***	Arbequina - Bella di Spagna	-0.723	0.1387	-5.213	<.0001
						Arbequina - FS17	-0.538	0.1375	-3.916	0.0069
						Arbequina - Picholine	-0.456	0.123	-3.71	0.015
						Bella di Spagna - Cima di Melfi	0.547	0.1279	4.276	0.0016
	Sex	0.094	1	0.759		Bella di Spagna - Leccio del Corno	0.436	0.119	3.667	0.0174
						Bella di Spagna - Nociara	0.400	0.1127	3.548	0.0265
						Bella di Spagna - Pendolino	0.678	0.1255	5.403	<.0001
						FS17 - Pendolino	0.494	0.138	3.577	0.024
Total residence time	**Variety**	50.092	13	2.88E-06	***	Bella di Spagna - Cima di Melfi	0.594	0.117	5.073	<.0001
						Bella di Spagna - Leccio del Corno	0.321	0.0946	3.394	0.044
						Bella di Spagna - Nociara	0.385	0.0956	4.03	0.0044
	Sex	0.021	1	0.886		Bella di Spagna - Pendolino	0.438	0.1002	4.376	0.001
						Cima di Melfi - FS17	-0.464	0.1223	-3.792	0.011
						Cima di Melfi - Picholine	-0.380	0.1115	-3.406	0.0423
Total probes	**Variety**	26.317	13	0.015	*	FS17 - Lecciana	0.463	0.13	3.559	0.0255
	Sex	0.000	1	0.995						
C WDEImd	**Variety**	26.961	13	0.013	*	Bella di Spagna - FS17	0.177	0.048	3.682	0.0191
	Sex	0.245	1	0.620						
Xc NWEI	**Variety**	63.031	13	1.50E-08	***	Arbequina - Coratina	-0.755	0.181	-4.164	0.0033
						Arbequina - FS17	-0.774	0.188	-4.108	0.0041
						Arbequina - Ogliarola	-0.929	0.195	-4.756	0.0003
						Cima di Melfi - Coratina	-0.733	0.181	-4.046	0.0051
						Cima di Melfi - FS17	-0.752	0.188	-3.995	0.0062
						Cima di Melfi - Ogliarola	-0.907	0.195	-4.647	0.0004
	Sex	0.341	1	0.559		Coratina - Leccio del Corno	0.700	0.181	3.861	0.0102
						FS17 - Leccio del Corno	0.719	0.188	3.816	0.012
						Leccio del Corno - Ogliarola	-0.874	0.195	-4.475	0.0009
						Nociara - Ogliarola	-0.759	0.198	-3.833	0.0113
						Ogliarola - Pendolino	0.740	0.195	3.788	0.0133
Xi NWEI						Arbequina - Coratina	-0.500	0.178	-2.817	0.0052
						Arbequina - Frantoio	-0.483	0.184	-2.621	0.0093

						Arbequina - FS17	-0.499	0.182	-2.737	0.0066
						Arbequina - Lecciana	-0.447	0.181	-2.477	0.0139
						Arbequina - Ogliarola	-0.570	0.189	-3.015	0.0028
						Arbequina - Picholine	-0.369	0.186	-1.981	0.0485
						Cima di Melfi - Coratina	-0.373	0.179	-2.086	0.0379
						Cima di Melfi - FS17	-0.373	0.183	-2.035	0.0427
						Cima di Melfi - Ogliarola	-0.443	0.19	-2.335	0.0203
		Chisq	Df	Pr(>Chisq)		Coratina - Leccio del Corno	0.438	0.178	2.459	0.0145
	Variety	30.4741	13	0.004022	**	Coratina - Nocellara Messinese	0.487	0.175	2.787	0.0057
	Sex	2.6047	1	0.106548		Coratina - Pendolino	0.368	0.176	2.091	0.0374
						Frantoio - Leccio del Corno	0.421	0.186	2.257	0.0248
						Frantoio - Nocellara Messinese	0.470	0.186	2.533	0.0118
						Frantoio - Nociara	0.330	0.189	1.751	0.0811
						FS17 - Leccio del Corno	0.437	0.183	2.39	0.0175
						FS17 - Nocellara Messinese	0.487	0.181	2.684	0.0077
						FS17 - Pendolino	0.368	0.182	2.015	0.0449
						Lecciana - Leccio del Corno	0.385	0.181	2.127	0.0343
						Lecciana - Nocellara Messinese	0.435	0.177	2.454	0.0147
						Leccio del Corno - Ogliarola	-0.508	0.19	-2.68	0.0078
						Nocellara Messinese - Ogliarola	-0.558	0.188	-2.971	0.0032
						Nociara - Ogliarola	-0.418	0.193	-2.17	0.0308
						Ogliarola - Pendolino	0.438	0.189	2.323	0.0209
Xc WDI/TRT	**Variety**	31.995	13	0.002	**	Cima di Melfi - Ogliarola	-0.512	0.152	-3.362	0.0487
	Sex	0.221	1	0.638		Leccio del Corno - Ogliarola	-0.544	0.154	-3.542	0.027
Xi WDI/TPT						Arbequina - Bella di Spagna	-0.251	0.115	-2.183	0.029
						Bella di Spagna - Leccino	0.423	0.137	3.076	0.0021
						Bella di Spagna - Nocellara Messinese	0.304	0.148	2.052	0.0402
						Bella di Spagna - Pendolino	0.433	0.13	3.327	0.0009
						Bella di Spagna - Picholine	0.295	0.13	2.269	0.0233
						Coratina - Leccino	0.300	0.11	2.716	0.0066
						Coratina - Pendolino	0.311	0.136	2.28	0.0226
						Frantoio - Leccino	0.312	0.138	2.258	0.0239
	Variety	30.584	13	0.004	**	Frantoio - Pendolino	0.323	0.132	2.442	0.0146
	Sex	2.142	1	0.143		FS17 - Leccino	0.277	0.121	2.289	0.0221
						FS17 - Pendolino	0.288	0.147	1.965	0.0494
						Leccino - Leccio del Corno	-0.354	0.137	-2.589	0.0096

						Leccino - Nociara	-0.353	0.139	-2.545	0.0109
						Leccino - Ogliarola	-0.377	0.113	-3.33	0.0009
						Leccio del Corno - Pendolino	0.365	0.128	2.84	0.0045
						Nociara - Pendolino	0.364	0.133	2.744	0.0061
						Ogliarola - Pendolino	0.388	0.139	2.797	0.0052
Xi WDI/TRT						Arbequina - Bella di Spagna	-0.573	0.137	-4.184	0.0023
						Arbequina - Nociara	-0.524	0.137	-3.832	0.0095
	Variety	48.137	13	0.000	***	Arbequina - Ogliarola	-0.541	0.146	-3.706	0.0151
	Sex	3.519	1	0.061	.	Bella di Spagna - Pendolino	0.569	0.14	4.054	0.004
						Leccino - Ogliarola	-0.413	0.12	-3.456	0.0359
						Nociara - Pendolino	0.520	0.138	3.782	0.0115
Xi+LF WDI/TPT						Bella di Spagna - Leccino	0.244	0.0991	2.461	0.0139
						Bella di Spagna - Picholine	0.200	0.0979	2.042	0.0411
						Coratina - Leccino	0.258	0.0757	3.415	0.0006
						Coratina - Pendolino	0.251	0.0914	2.746	0.006
						Coratina - Picholine	0.214	0.0922	2.325	0.02
						Frantoio - Leccino	0.273	0.0915	2.986	0.0028
	Chisq		**Df**	**Pr(>Chisq)**		Frantoio - Pendolino	0.266	0.0843	3.154	0.0016
	Variety	34.001	13	0.001	**	Frantoio - Picholine	0.229	0.0848	2.704	0.0069
	Sex	1.250	1	0.264		FS17 - Leccino	0.217	0.0828	2.618	0.0088
						FS17 - Pendolino	0.210	0.0954	2.196	0.0281
						Leccino - Leccio del Corno	-0.208	0.0968	-2.147	0.0318
						Leccino - Nociara	-0.278	0.0938	-2.966	0.003
						Leccino - Ogliarola	-0.274	0.0839	-3.268	0.0011
						Leccio del Corno - Pendolino	0.201	0.0902	2.223	0.0262
						Nociara - Pendolino	0.271	0.086	3.149	0.0016
						Nociara - Picholine	0.234	0.0909	2.576	0.01
						Ogliarola - Pendolino	0.267	0.0954	2.797	0.0052
						Ogliarola - Picholine	0.230	0.0959	2.4	0.0164
Xi+LF WDI/TRT						Arbequina - Bella di Spagna	-0.553	0.128	-4.322	0.0013
						Arbequina - Cima di Melfi	-0.535	0.1219	-4.39	0.0009
						Arbequina - Coratina	-0.502	0.1252	-4.004	0.0048
						Arbequina - Frantoio	-0.499	0.1166	-4.28	0.0015
						Arbequina - FS17	-0.506	0.1282	-3.945	0.0061
	Chisq		**Df**	**Pr(>Chisq)**		Arbequina - Nocellara Messinese	-0.488	0.1348	-3.622	0.0204
	Variety	56.560	13	2.15E-07	***	Arbequina - Nociara	-0.596	0.1179	-5.06	<.0001
	Sex	0.905	1	0.341		Arbequina - Ogliarola	-0.581	0.1284	-4.524	0.0005
						Bella di Spagna - Pendolino	0.412	0.1194	3.448	0.037
						Cima di Melfi - Pendolino	0.394	0.1122	3.507	0.0304

						Leccino - Nociara	-0.373	0.113	-3.301	0.0589
						Leccino - Ogliarola	-0.357	0.1048	-3.412	0.0415
						Nociara - Pendolino	0.455	0.1087	4.181	0.0023
						Ogliarola - Pendolino	0.439	0.1229	3.572	0.0244
Xi WDEImd	**Variety**	35.677	13	0.001	***	Frantoio - Leccio del Corno	-0.855	0.234	-3.661	0.0206
						Frantoio - Nocellara Messinese	-0.874	0.229	-3.812	0.0122
	Sex	4.025	1	0.045	*	Female - Male	0.173	0.0863	2.006	0.0458
N NWEI	**Variety**	31.965	13	0.002	**	Arbequina - Coratina	-0.698	0.201	-3.467	0.0388
	Sex	1.712	1	0.191		Arbequina - Ogliarola	-0.890	0.216	-4.121	0.0039
R WDI/TPT	**Variety**	9.595	13	0.727		Female - Male	0.332	0.0946	3.512	0.0006
	Sex	12.335	1	0.000	***					
R WDI/TRT	**Variety**	10.952	13	0.615		Female - Male	0.294	0.093	3.165	0.0019
	Sex	10.017	1	0.002	**					
np to 1st Xc	**Variety**	29.960	13	0.005	**	Bella di Spagna - Leccino	1.060	0.282	3.758	0.0147
	Sex	1.042	1	0.307		Leccino - Leccio del Corno	-1.220	0.275	-4.441	0.0011
np to 1st Xi	**Variety**	30.587	13	0.004	**	Bella di Spagna - Leccino	1.133	0.301	3.765	0.0143
	Sex	0.769	1	0.381		Leccino - Leccio del Corno	-1.283	0.294	-4.358	0.0015
						Leccino - Nocellara Messinese	-1.057	0.292	-3.616	0.0239
np to 1st sustained Xi	**Variety**	30.923	13	0.003	**	Frantoio - Leccino	0.953	0.272	3.501	0.0348
	Sex	0.725	1	0.395						
np to 1st LF	**Variety**	48.837	13	4.71E-06	***	Coratina - Frantoio	-0.710	0.183	-3.878	0.008
	Sex	1.346	1	0.246		Coratina - Ogliarola	-0.600	0.172	-3.483	0.0329
						Frantoio - Leccino	0.630	0.177	3.556	0.0258
1st C to 1st Xc	**Variety**	29.248	13	0.006	**	Bella di Spagna - Leccino	1.373	0.336	4.086	0.0044
	Sex	0.479	1	0.489		Leccino - Leccio del Corno	-1.287	0.334	-3.855	0.0104
1st C to 1st Xi	**Variety**	29.341	13	0.006	**	Bella di Spagna - Leccino	1.290	0.32	4.026	0.0055
	Sex	0.522	1	0.470		Leccino - Leccio del Corno	-1.181	0.32	-3.687	0.0188
1st C to first sustained Xi	**Variety**	31.575	13	0.003	**	Coratina - Frantoio	-0.981	0.285	-3.444	0.0415
	Sex	0.773	1	0.379		Frantoio - Leccino	0.986	0.283	3.49	0.036
1st C to 1st LF	**Variety**	50.879	13	2.11E-06	***	Cima di Melfi - Frantoio	-0.667	0.189	-3.536	0.0275
						Coratina - Frantoio	-0.730	0.186	-3.935	0.0064
						Coratina - Ogliarola	-0.609	0.175	-3.473	0.034
	Sex	0.735	1	0.391		Frantoio - Lecciana	0.631	0.185	3.4	0.0431
						Frantoio - Leccino	0.641	0.179	3.572	0.0244
						Frantoio - Pendolino	0.637	0.187	3.413	0.0414

Online Resource 4. Summary statistics for EPG parameters with non-significant results.

	Variety	n	mean	sd	median	min	max	se
np NWEI	Arbequina	20	3	1.84	2	1	6	0.41
	Bella di Spagna	20	2.85	1.57	2.5	1	6	0.35
	Cima di Melfi	20	2.85	2.13	2	1	8	0.48
	Coratina	26	3.69	2.05	3.5	1	9	0.4
	Frantoio	20	3.3	1.81	3	1	7	0.4
	FS17	22	4.5	3.56	4	1	17	0.76
	Lecciana	23	2.83	3.76	2	1	18	0.78
	Leccino	27	3.85	2.61	3	1	11	0.5
	Leccio del Corno	20	3	2.13	2.5	1	7	0.48
	Nocellara Messinese	21	2.33	1.74	2	1	8	0.38
	Nociara	20	3.35	2.48	3	1	11	0.55
	Ogliarola	19	4.11	3.73	3	1	15	0.85
	Pendolino	20	3.45	1.82	3.5	1	7	0.41
	Picholine	19	3.74	3.03	2	1	11	0.7
C NWEI	Arbequina	20	5.4	3.1	5	2	15	0.69
	Bella di Spagna	20	7.75	7.11	5.5	1	29	1.59
	Cima di Melfi	20	5.45	4.61	3	1	18	1.03
	Coratina	26	7.08	3.38	7	2	13	0.66
	Frantoio	20	7.9	5.06	8	2	25	1.13
	FS17	22	9.55	5.06	8	3	23	1.08
	Lecciana	23	8.17	7.33	7	2	38	1.53
	Leccino	27	6.67	3.99	6	1	13	0.77
	Leccio del Corno	20	6.1	4.42	4.5	2	19	0.99
	Nocellara Messinese	21	7.76	4.24	8	2	19	0.93
	Nociara	20	6.6	4.64	5.5	2	19	1.04
	Ogliarola	19	8.53	5.98	7	3	27	1.37
	Pendolino	20	6.95	4.77	6.5	1	19	1.07
	Picholine	19	7.42	4.65	6	2	18	1.07
LF NWEI	Arbequina	11	6.91	8.88	5	1	33	2.68
	Bella di Spagna	11	3.82	4.02	2	1	14	1.21
	Cima di Melfi	11	5.09	5.22	3	1	16	1.58
	Coratina	18	5.56	3.94	4.5	1	16	0.93
	Frantoio	10	5.5	6.02	2.5	1	16	1.9
	FS17	17	4.82	3.76	4	1	15	0.91
	Lecciana	17	5.06	3.78	3	1	14	0.92
	Leccino	20	5.25	3.67	4	1	12	0.82
	Leccio del Corno	8	6.62	4.75	5	1	15	1.68
	Nocellara Messinese	12	5	3.36	4	1	10	0.97
	Nociara	8	3.88	3.48	2	1	9	1.23
	Ogliarola	11	4.45	3.93	3	1	12	1.19
	Pendolino	14	4.36	4.14	2.5	1	15	1.11
	Picholine	16	4.88	4.6	3.5	1	19	1.15
R NWEI	Arbequina	12	1.92	1.38	1	1	5	0.4

	Bella di Spagna	7	1.43	0.53	1	1	2	0.2
	Cima di Melfi	9	4.33	3.04	3	2	10	1.01
	Coratina	10	2.2	1.23	2	1	4	0.39
	Frantoio	8	4.25	4.86	1.5	1	14	1.72
	FS17	12	2.08	1.56	1.5	1	6	0.45
	Lecciana	14	2.36	1.34	2	1	5	0.36
	Leccino	17	2.24	1.09	2	1	5	0.26
	Leccio del Corno	7	2.57	1.27	2	1	5	0.48
	Nocellara Messinese	10	2.4	1.26	2.5	1	5	0.4
	Nociara	6	1.83	0.75	2	1	3	0.31
	Ogliarola	7	2.14	1.07	2	1	4	0.4
	Pendolino	14	2.14	1.41	2	1	6	0.38
	Picholine	13	5.08	4.73	3	1	17	1.31
np WDI/TRT	Arbequina	20	27.88	29.35	14.64	0.3	84.18	6.56
	Bella di Spagna	20	9.68	13.33	2.46	0.26	44.56	2.98
	Cima di Melfi	20	10.98	11.01	8.55	0.19	38.39	2.46
	Coratina	26	14.62	15.42	7.77	0.15	50.41	3.02
	Frantoio	20	16.74	15.56	14.63	0.37	43.16	3.48
	FS17	22	10.83	13.93	3.84	0.35	43.41	2.97
	Lecciana	23	10.14	18.26	0.63	0.26	62.57	3.81
	Leccino	27	14.75	20.4	2.81	0.13	73.49	3.93
	Leccio del Corno	20	12.81	21.93	2.83	0.21	85.72	4.9
	Nocellara Messinese	21	6.42	9.3	2.1	0.15	38.01	2.03
	Nociara	20	8.69	11.04	4.33	0.16	41.08	2.47
	Ogliarola	19	9.13	10.5	1.98	0.18	29.24	2.41
	Pendolino	20	22.71	25.26	12.72	0.08	82.07	5.65
	Picholine	19	11.18	11.63	5.21	0.32	30.09	2.67
C WDI/TPT	Arbequina	20	9.89	10.35	4.46	0.8	37.45	2.31
	Bella di Spagna	20	6.35	6.28	3.92	0.97	26.1	1.4
	Cima di Melfi	20	7.86	8.71	5.02	0.59	33.63	1.95
	Coratina	26	7.15	6.92	4.08	0.36	23.62	1.36
	Frantoio	20	8.9	12.27	4	1.04	53.22	2.74
	FS17	22	5.78	3.52	5.02	0.68	13.17	0.75
	Lecciana	23	9.02	13.73	4.27	0.82	51.94	2.86
	Leccino	27	9.54	18.96	2.91	0.27	73.4	3.65
	Leccio del Corno	20	8.46	11.85	4.36	0.66	45.75	2.65
	Nocellara Messinese	21	8.17	8.85	4.73	0.66	34.04	1.93
	Nociara	20	5.28	6.42	2.82	0.81	22.96	1.43
	Ogliarola	19	6.82	6.2	3.16	1.38	21.61	1.42
	Pendolino	20	10.28	14.21	4.16	0.88	61.44	3.18
	Picholine	19	4.94	4.14	2.27	0.91	12.22	0.95
C WDI/TRT	Arbequina	20	5.67	7.27	3.57	0.67	32.1	1.62
	Bella di Spagna	20	5.29	4.57	3.66	0.92	21.46	1.02
	Cima di Melfi	20	6.75	7.6	4.11	0.59	29.74	1.7
	Coratina	26	5.56	5.15	3.54	0.36	18.31	1.01
	Frantoio	20	6.87	9.05	2.92	1.03	36.21	2.02

	FS17	22	4.94	2.83	4.65	0.68	10.92	0.6
	Lecciana	23	6.85	10.14	4.25	0.81	47.94	2.11
	Leccino	27	6.66	14.29	2.67	0.27	68.8	2.75
	Leccio del Corno	20	5.76	6.66	4.07	0.66	27.94	1.49
	Nocellara Messinese	21	7.52	8.48	4.1	0.66	33.89	1.85
	Nociara	20	4.57	5.59	2.65	0.81	21.06	1.25
	Ogliarola	19	5.84	5.21	3.09	1.33	19.46	1.2
	Pendolino	20	7.31	11.56	3.21	0.88	49.37	2.59
	Picholine	19	4.21	3.52	2.08	0.89	11.65	0.81
Xc WDI/TPT	Arbequina	20	0.59	0.51	0.44	0.04	1.74	0.11
	Bella di Spagna	20	0.4	0.31	0.33	0.08	1.32	0.07
	Cima di Melfi	20	0.43	0.55	0.24	0.06	2.33	0.12
	Coratina	26	0.97	1.91	0.46	0.1	10.06	0.37
	Frantoio	20	0.46	0.7	0.26	0.04	3.29	0.16
	FS17	22	0.56	0.51	0.46	0.09	2.34	0.11
	Lecciana	23	0.45	0.39	0.32	0.06	1.85	0.08
	Leccino	27	0.44	0.42	0.34	0.03	1.97	0.08
	Leccio del Corno	20	0.55	1.05	0.25	0.03	4.87	0.23
	Nocellara Messinese	21	0.44	0.37	0.37	0.04	1.75	0.08
	Nociara	20	0.31	0.25	0.24	0.04	0.93	0.06
	Ogliarola	19	0.66	0.48	0.46	0.12	1.6	0.11
	Pendolino	20	0.44	0.47	0.26	0.1	1.76	0.11
	Picholine	19	0.4	0.27	0.37	0.08	1.12	0.06
LF WDI/TPT	Arbequina	11	23.42	18.87	15.26	3.62	57.38	5.69
	Bella di Spagna	11	8.05	6.86	5.29	0.42	18.9	2.07
	Cima di Melfi	11	16.48	14.64	13.03	0.51	42.28	4.41
	Coratina	18	19.13	21.13	12.19	0.34	74.91	4.98
	Frantoio	10	19.18	26.26	8.02	0.69	70.91	8.3
	FS17	17	14.96	13.18	9.56	2.76	47.57	3.2
	Lecciana	17	17.04	20.83	9.24	0.67	79.37	5.05
	Leccino	20	19.31	18.54	14.61	0.37	78.5	4.15
	Leccio del Corno	8	17.37	8.38	14.31	8.71	29.26	2.96
	Nocellara Messinese	12	23.82	17.32	20.4	2.39	58.38	5
	Nociara	8	18.16	30.68	5.42	0.58	91.55	10.85
	Ogliarola	11	15.95	17.53	5.51	1.71	47.66	5.28
	Pendolino	14	15.83	17.23	11.89	0.25	55.84	4.6
	Picholine	16	16.89	16.89	11.38	0.54	62.59	4.22
LF WDI/TRT	Arbequina	11	20.56	19.16	9.57	2.24	57.19	5.78
	Bella di Spagna	11	7.64	6.86	5.06	0.34	18.83	2.07
	Cima di Melfi	11	15.89	14.54	11.85	0.51	41.55	4.38
	Coratina	18	17.77	20.69	11.94	0.34	74.79	4.88
	Frantoio	10	14.06	16.23	7.97	0.59	44.74	5.13
	FS17	17	14.1	13.03	9.47	2	45.26	3.16
	Lecciana	17	16.6	20.75	9.21	0.66	79.09	5.03
	Leccino	20	18.44	19	13.6	0.29	78.38	4.25
	Leccio del Corno	8	16.72	8.84	14.16	7.2	28.75	3.12

	Nocellara Messinese	12	23.05	17.43	17.52	2.03	57.73	5.03
	Nociara	8	17.84	30.78	4.82	0.58	91.41	10.88
	Ogliarola	11	15.2	17.37	5.43	1.68	47.24	5.24
	Pendolino	14	13.4	15.75	8.88	0.17	48.46	4.21
	Picholine	16	14.96	15.15	8.41	0.54	54.66	3.79
N WDI/TPT	Arbequina	20	0.73	0.5	0.56	0.15	1.82	0.11
	Bella di Spagna	19	0.53	0.35	0.52	0.06	1.38	0.08
	Cima di Melfi	19	1.12	1.58	0.6	0.06	6.94	0.36
	Coratina	26	1.03	1.23	0.66	0.06	6.43	0.24
	Frantoio	20	0.94	0.55	0.7	0.07	2.3	0.12
	FS17	22	0.73	0.39	0.68	0.21	1.98	0.08
	Lecciana	22	1.67	3.54	0.72	0.12	16.87	0.76
	Leccino	27	0.68	0.52	0.53	0.08	2.1	0.1
	Leccio del Corno	19	0.71	0.65	0.6	0.08	2.92	0.15
	Nocellara Messinese	19	0.49	0.34	0.34	0.11	1.35	0.08
	Nociara	19	0.64	0.39	0.63	0.04	1.52	0.09
	Ogliarola	19	1.07	0.67	0.9	0.3	2.99	0.15
	Pendolino	20	0.82	0.54	0.76	0.14	1.9	0.12
	Picholine	19	0.71	0.5	0.57	0.11	2.12	0.11
N WDI/TRT	Arbequina	20	0.46	0.37	0.33	0.13	1.58	0.08
	Bella di Spagna	19	0.49	0.33	0.51	0.05	1.34	0.08
	Cima di Melfi	19	0.99	1.43	0.52	0.05	6.31	0.33
	Coratina	26	0.8	0.77	0.58	0.06	3.98	0.15
	Frantoio	20	0.77	0.48	0.62	0.07	2.28	0.11
	FS17	22	0.64	0.33	0.57	0.21	1.47	0.07
	Lecciana	22	1.42	2.93	0.58	0.12	13.92	0.63
	Leccino	27	0.54	0.4	0.47	0.08	1.89	0.08
	Leccio del Corno	19	0.53	0.38	0.53	0.08	1.62	0.09
	Nocellara Messinese	19	0.44	0.3	0.33	0.11	1.27	0.07
	Nociara	19	0.57	0.32	0.5	0.04	1.1	0.07
	Ogliarola	19	0.94	0.51	0.82	0.3	2.25	0.12
	Pendolino	20	0.56	0.39	0.43	0.13	1.67	0.09
	Picholine	19	0.6	0.36	0.51	0.1	1.48	0.08
Unsuccessful probes	Arbequina	20	1.45	1.54	1	0	5	0.34
	Bella di Spagna	20	1.45	1.47	1	0	4	0.33
	Cima di Melfi	20	1.1	1.71	0	0	5	0.38
	Coratina	26	1.65	1.83	1	0	7	0.36
	Frantoio	20	1.45	1.73	1	0	6	0.39
	FS17	22	2.45	3.36	1	0	15	0.72
	Lecciana	23	0.96	1.61	0	0	6	0.34
	Leccino	27	2	2.15	1	0	8	0.41
	Leccio del Corno	20	1.55	1.82	1	0	5	0.41
	Nocellara Messinese	21	0.81	1.33	0	0	4	0.29
	Nociara	20	1.7	2.05	1	0	8	0.46
	Ogliarola	19	1.74	2.28	1	0	8	0.52
	Pendolino	20	1.6	1.64	1.5	0	5	0.37

	Picholine	19	1.95	2.39	1	0	8	0.55
Successful probes	Arbequina	20	1.35	0.67	1	1	3	0.15
	Bella di Spagna	20	1.4	0.6	1	1	3	0.13
	Cima di Melfi	20	1.35	0.67	1	1	3	0.15
	Coratina	26	1.85	0.92	2	1	4	0.18
	Frantoio	20	1.65	0.81	1	1	3	0.18
	FS17	22	1.86	0.89	2	1	4	0.19
	Lecciana	23	1.7	2.7	1	1	14	0.56
	Leccino	27	1.63	0.93	1	1	5	0.18
	Leccio del Corno	20	1.25	0.55	1	1	3	0.12
	Nocellara Messinese	21	1.24	0.44	1	1	2	0.1
	Nociara	20	1.5	0.89	1	1	4	0.2
	Ogliarola	19	2.05	2.07	1	1	9	0.47
	Pendolino	20	1.7	0.92	1	1	4	0.21
	Picholine	19	1.58	0.9	1	1	4	0.21
Sustained probes	Arbequina	20	1.25	0.55	1	1	3	0.12
	Bella di Spagna	20	1.4	0.6	1	1	3	0.13
	Cima di Melfi	20	1.15	0.37	1	1	2	0.08
	Coratina	26	1.65	0.85	1	1	4	0.17
	Frantoio	20	1.35	0.67	1	1	3	0.15
	FS17	22	1.5	0.51	1.5	1	2	0.11
	Lecciana	23	1.35	1.27	1	1	7	0.26
	Leccino	27	1.37	0.63	1	1	3	0.12
	Leccio del Corno	20	1.1	0.45	1	1	3	0.1
	Nocellara Messinese	21	1.19	0.4	1	1	2	0.09
	Nociara	20	1.2	0.52	1	1	3	0.12
	Ogliarola	19	1.63	1.12	1	1	5	0.26
	Pendolino	20	1.4	0.68	1	1	3	0.15
	Picholine	19	1.37	0.6	1	1	3	0.14
np WDEImd	Arbequina	20	24.92	36.26	7.04	0.24	151.52	8.11
	Bella di Spagna	20	8.97	16.16	2.22	0.25	70.97	3.61
	Cima di Melfi	20	7.38	8.38	4.56	0.68	36.07	1.87
	Coratina	26	5.64	8.71	2.44	0.26	39.43	1.71
	Frantoio	20	8.93	12.18	3.45	0.22	45.06	2.72
	FS17	22	7.87	17.29	1.35	0.13	78.13	3.69
	Lecciana	23	6.32	13.61	1.54	0.63	64.39	2.84
	Leccino	27	3.68	6.6	1.51	0.17	31.92	1.27
	Leccio del Corno	20	12.16	21.18	4.62	0.62	78.62	4.74
	Nocellara Messinese	21	6.69	11.85	1.75	0.38	49.99	2.59
	Nociara	20	6.02	10.92	2.33	0.37	44.34	2.44
	Ogliarola	19	5.76	10.12	1.93	0.1	39.9	2.32
	Pendolino	20	8.76	13.64	2.67	0.28	56.99	3.05
	Picholine	19	9.04	13.7	4.09	0.35	46.78	3.14
Xc WDEImd	Arbequina	20	0.22	0.11	0.21	0.05	0.56	0.02
	Bella di Spagna	20	0.15	0.08	0.12	0.06	0.34	0.02
	Cima di Melfi	20	0.17	0.08	0.15	0.06	0.37	0.02

	Coratina	26	0.13	0.06	0.11	0.07	0.29	0.01
	Frantoio	20	0.14	0.06	0.12	0.06	0.28	0.01
	FS17	22	0.14	0.08	0.11	0.07	0.42	0.02
	Lecciana	23	0.13	0.07	0.11	0.06	0.34	0.01
	Leccino	27	0.13	0.05	0.11	0.06	0.23	0.01
	Leccio del Corno	20	0.21	0.17	0.16	0.07	0.72	0.04
	Nocellara Messinese	21	0.15	0.09	0.13	0.05	0.41	0.02
	Nociara	20	0.17	0.12	0.13	0.06	0.59	0.03
	Ogliarola	19	0.13	0.07	0.11	0.03	0.3	0.02
	Pendolino	20	0.17	0.09	0.15	0.08	0.37	0.02
	Picholine	19	0.15	0.05	0.14	0.08	0.28	0.01
LF WDEImd	Arbequina	11	10.32	7.77	8.06	4.06	25.66	2.34
	Bella di Spagna	11	6.71	6.18	4.97	1.24	18.92	1.86
	Cima di Melfi	11	7.79	5.71	7.46	1.49	22.04	1.72
	Coratina	18	7.86	5.64	6.58	1.24	20.79	1.33
	Frantoio	10	12.18	21.17	5.1	2.14	71.64	6.69
	FS17	17	8.83	6.42	6.4	2.08	22.28	1.56
	Lecciana	17	8.56	9.49	5.57	1.2	35.48	2.3
	Leccino	20	8.88	6.43	7.91	1.06	21.12	1.44
	Leccio del Corno	8	10.11	7.24	7.76	2.11	25.93	2.56
	Nocellara Messinese	12	12.02	7.6	9.16	3.65	28.36	2.19
	Nociara	8	11.49	14.8	6.14	2.08	46.93	5.23
	Ogliarola	11	7.93	6.31	5.56	0.97	19.54	1.9
	Pendolino	14	12.65	21.46	5.52	0.63	71.72	5.74
	Picholine	16	10.18	11.58	5.39	1.93	36.4	2.89
W WDEImd	Arbequina	11	0.62	1.16	0.26	0.03	4.05	0.35
	Bella di Spagna	9	1.24	2.93	0.25	0.04	9.02	0.98
	Cima di Melfi	11	0.59	0.83	0.27	0.03	2.77	0.25
	Coratina	17	0.21	0.18	0.18	0.03	0.8	0.04
	Frantoio	11	0.85	2.09	0.19	0.04	7.11	0.63
	FS17	15	0.33	0.31	0.17	0.04	1.06	0.08
	Lecciana	9	1.45	2.07	0.58	0.07	6.34	0.69
	Leccino	16	0.8	1.3	0.16	0.02	5.11	0.33
	Leccio del Corno	9	0.69	0.56	0.67	0.13	1.56	0.19
	Nocellara Messinese	8	0.94	0.95	0.52	0.04	2.39	0.34
	Nociara	6	0.73	0.46	0.7	0.1	1.42	0.19
	Ogliarola	11	0.39	0.46	0.18	0.02	1.55	0.14
	Pendolino	14	0.33	0.24	0.28	0.04	0.81	0.06
	Picholine	8	0.76	0.66	0.5	0.12	1.62	0.23
Time to 1st probe	Arbequina	20	7.08	13.61	1.45	0.1	44.6	3.04
	Bella di Spagna	20	3.91	6.42	1.65	0.3	26.3	1.44
	Cima di Melfi	20	5.58	8.4	2.1	0.3	36.1	1.88
	Coratina	26	3.03	4.6	1.35	0.3	21.9	0.9
	Frantoio	20	4.9	9.86	1.55	0.3	45.1	2.21
	FS17	22	4.55	11.68	1.2	0.5	55.3	2.49
	Lecciana	23	5.08	8.88	2	0.5	42.1	1.85

	Leccino	27	1.98	2.33	1.5	0.4	10.1	0.45
	Leccio del Corno	20	8.57	17.61	2.5	0.6	78.6	3.94
	Nocellara Messinese	21	6.9	12.13	1.4	0.5	50	2.65
	Nociara	20	6.77	13.34	1.8	0.4	46.5	2.98
	Ogliarola	19	3.37	8.86	1.4	0.5	39.9	2.03
	Pendolino	20	4	6.65	1.55	0.3	23.5	1.49
	Picholine	19	7.08	10.53	3.1	0.3	45.6	2.42
Time to 1st probe with Xi	Arbequina	20	19.72	32.79	4.95	0.4	136	7.33
	Bella di Spagna	20	19.31	28.82	6.85	0.3	106.3	6.44
	Cima di Melfi	20	10.67	12	5.2	0.3	36.1	2.68
	Coratina	26	8.2	12.49	2.75	0.3	56.7	2.45
	Frantoio	20	21.09	36.3	6.2	0.3	146.2	8.12
	FS17	22	13.95	30.21	6.25	0.5	145.4	6.44
	Lecciana	23	7.23	12.05	2.1	0.5	57	2.51
	Leccino	27	3.48	3.68	2	0.4	15	0.71
	Leccio del Corno	20	15.75	19.75	10.3	0.6	86.2	4.42
	Nocellara Messinese	21	21.5	30	3.1	0.5	109.8	6.55
	Nociara	20	13.2	16.22	5.8	0.6	60.8	3.63
	Ogliarola	19	7.46	12.77	1.9	0.6	42.8	2.93
	Pendolino	20	7.2	7.96	3.05	0.3	23.5	1.78
	Picholine	19	15.01	16.91	9.5	1.1	57.3	3.88
Time to 1st probe with LF	Arbequina	20	194.68	156.78	163.65	0.4	359.9	35.06
	Bella di Spagna	20	168.4	177.21	61.45	0.3	359.2	39.62
	Cima di Melfi	20	169.64	173.11	66.9	0.3	359.4	38.71
	Coratina	26	118.07	161.82	11.75	0.5	359.7	31.73
	Frantoio	20	217.22	152.91	287.65	1.3	359.7	34.19
	FS17	22	87.03	145.57	14.25	0.5	359.2	31.04
	Lecciana	23	96.97	155.7	2.2	0.5	359.1	32.47
	Leccino	27	106.93	155.4	9.2	0.4	359.5	29.91
	Leccio del Corno	20	210.94	172.04	338.65	0.7	359.4	38.47
	Nocellara Messinese	21	169.63	162.75	109.8	0.5	359.5	35.51
	Nociara	20	217.57	168.34	335	0.6	359.6	37.64
	Ogliarola	19	173.82	169.61	163.7	0.6	359.3	38.91
	Pendolino	20	143.88	159.85	38.9	0.3	359.5	35.74
	Picholine	19	80.91	125.08	42.9	1.6	359.7	28.69
Time to 1st probe with sustained Xi	Arbequina	20	27.91	45.89	7.2	0.4	165.5	10.26
	Bella di Spagna	20	19.31	28.82	6.85	0.3	106.3	6.44
	Cima di Melfi	20	39.35	82.3	8.2	0.3	354.3	18.4
	Coratina	26	8.2	12.49	2.75	0.3	56.7	2.45
	Frantoio	20	28.38	41.78	10	0.3	146.2	9.34
	FS17	22	33.25	78.56	11.35	0.5	358.6	16.75
	Lecciana	23	7.23	12.05	2.1	0.5	57	2.51
	Leccino	27	13.43	40.34	2	0.4	211.2	7.76
	Leccio del Corno	20	19.61	23.81	13.85	0.6	86.2	5.33
	Nocellara Messinese	21	22.15	29.71	7.4	0.5	109.8	6.48
	Nociara	20	17.77	18.92	8.15	0.6	60.8	4.23

	Ogliarola	19	7.46	12.77	1.9	0.6	42.8	2.93
	Pendolino	20	11.29	14.08	5.1	0.3	56.4	3.15
	Picholine	19	21.31	23.7	11.2	1.6	86.9	5.44

CHAPTER V

Water stress alters the spittlebug–olive interaction in a variety-dependent manner

6. Water stress modulates spittlebug–olive interaction in a variety-dependent manner

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Abstract

As climate change intensifies, water stress events are expected to be more frequent, potentially altering the ecological balance between herbivores, including insect vectors of plant pathogens and plants. These changes may in turn influence the transmission dynamics of vector-borne plant pathogens. Here, we investigated how plant water status influences vector-plant interactions in olive varieties that differ in both drought responses and vector preference, *i.e.,* Leccino and Ogliarola. Influence of water status on host plant acceptance by the meadow spittlebug *Philaenus spumarius* was characterized by Electropenetrography (EPG). The impact of water status on xylem sap chemical profile, *i.e.,* the pivotal plant trait underlying host plant acceptance, was evaluated by untargeted HR-MS/MS metabolomics and multivariate analysis. Results revealed that water status modulates spittlebug-plant interaction and plant acceptance in a variety-dependent manner. Metabolomic profiling showed that Ogliarola plants maintained a stable xylem-sap composition across water regimes, whereas Leccino plants exhibited a pronounced, water status-dependent reprogramming of its xylem-sap metabolome. This in turn affected spittlebug-plant interaction, with similar probing behavior across water regimes in Ogliarola, while water-stress increased Leccino plants suitability for *P. spumarius*. The increased suitability was possibly associated with a decrease in the overall organic acids content in stressed plants, consistently with previous observations. Our preliminary results are consistent with the hypothesis that plant water status may modulate host-plant acceptability for spittlebugs in a variety-dependent manner. Furthermore, our data suggests that agronomic practices including irrigation might exert an underappreciated but important influence on vector-mediated *Xylella fastidiosa* spread.

Introduction

Water availability is one of the key components of agricultural productivity, influencing plant growth, nutrient uptake, and physiological functions (Jamieson *et al.,* 2012). Irrigated agriculture consumes approximately 70% of the world's freshwater resources, reflecting the great dependence of agriculture on consistent water inputs (FAO, 2021). With the intensification of climate change and the disruption of rainfall regimes, drought events are expected to become more frequent and intense (IPCC, 2022). These shifts pose a critical threat to global food security, particularly in regions where agriculture is constrained by limited water supply (Farooq *et al.,* 2009). Indeed, drought stress impairs plant water relations, disrupts photosynthesis, affects nutrient mobility, and alters metabolic processes, ultimately reducing growth and yield (Chaves *et al.,* 2003).

While the direct physiological effects of drought on plants are well documented, its broader ecological impacts, as plants interaction with other organisms including herbivores, are relatively unexplored and often overlooked in agroecosystem management. Insights into the influence of water stress on insect-plant interactions are critical for predicting pest dynamics and disease outbreaks in agroecosystems. Water deficit can affect plant secondary metabolism, alter volatile organic compound emissions, and change tissue nutrient profiles, all of which can impact herbivore host selection, feeding behavior, and fitness (Boyer, 1995; Huberty & Denno, 2004; Jamieson *et al.,* 2012). These responses, however, are highly variable: in some systems, drought may reduce pest pressure by lowering plant palatability or insect performance (Mattson & Haack, 1987); in others, stressed plants may be more vulnerable due to compromised defenses or altered growth patterns (White, 1984). The outcome depends on the intensity and duration of the drought, the crop species, and the biology of the herbivore involved.

Xylem-sap feeding insects are deemed to be highly vulnerable to host water status, owing to their dependence on a continuous flow of xylem fluid which is very diluted and hard to extract (Redak *et al.,* 2004). Previous research studies on the glassy-winged sharpshooter *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae) have shown that water stress negatively affects both insect population dynamic and feeding efficiency (Andersen *et al.,* 1992; Krugner *et al.,* 2009; Krugner & Backus, 2014), while the impact on the transmission of *Xylella fastidiosa*, a xylem limited bacterium solely transmitted by xylem feeders, is largely uncertain (Del Cid *et al.,* 2018).

Water status-dependent changes in xylem sap chemical profile might affect host plant acceptability for the insect vectors, thus vector abundance and time they spent on the plant (Andersen *et al.,* 1995). As a response to drought, plants might modify their metabolic profile by storing osmoprotective compounds such as soluble sugars and certain amino acids to maintain cell turgor (Ahkami *et al.,* 2019; Sharma *et al.,* 2019; Wallis & Gorman, 2024). These substances not only lessen water loss but also affect herbivores interactions, sensed by the insect during xylem sap tasting/testing upon landing and acting as positive or negative contributor to host plant acceptance (Brodbeck *et al.*, 1990; Andersen *et al.,* 1992, 1995; Chapman, 2003; Rogers & Newland, 2003; Krugner *et al.,* 2009; Mitchell *et al.,* 2016; De Pascali *et al.,* 2022; Lin *et al.,* 2023).

Olive (*Olea europaea* L.) trees are well adapted to dry Mediterranean climates (Sofo *et al.,* 2008). However, a considerable genotypic variation in tolerance to drought among olive varieties has been reported (Vitagliano & Sebastiani, 2002; Karimi *et al.,* 2018). For instance, differences in plant physiology, *e.g.,* stomatal control, hydraulic efficiency, and osmotic adjustment capacity, induce different variety-dependent responses to drought conditions. Hypothetically, drought-sensitive and drought-resistant varieties are expected to differentially respond to water stress also in terms of changes in the xylem sap chemical profile. What is unknown so far is how these differential responses might shape the insect vector-plant interactions.

The meadow spittlebug *Philaenus spumarius* L. (Hempitera: Aphrophoridae) is the main European vector of *X. fastidiosa* subsp. *pauca* (ST53), *i.e.* the causal agent of the Olive Quick Decline Syndrome, causing significant agricultural losses (Cornara *et al.,* 2017; Saponari *et al.,* 2019). Knowledge on insect vector-plant interaction, including how agriculture practices as irrigation might shape such interaction, is currently partial and fragmented.

In the present study, we aimed to assess how water status influences vector-plant interactions in olive varieties that differ in both susceptibility to water stress and suitability for spittlebugs, namely Leccino and Ogliarola (De Pascali *et al.,* 2019; Cornara *et al.,* 2024; Marchioni *et al.,* 2024). Additionally, we characterized the xylem sap chemical profile of plants under different water regimes, and the role of metabolome reprogramming in shaping insect-plant interaction. Our preliminary findings indicate that plant water status may be a key, variety-dependent driver of insect-plant interactions, and that agronomic practices as irrigation might exert an underappreciated but important influence on vector-mediated *X. fastidiosa* spread.

Materials and methods

1) Insects and plants rearing

Third and fourth instar nymphs of *Philaenus spumarius* were collected in an *X. fastidiosa*-free area (41.018037°N, 16.902046°E) in Apulia region (Southern Italy). Nymphs were reared on ten-day-old host plants, including *Vicia sativa* L. (vetch) and *Vicia faba* L. (fava bean) plants until adulthood with plants replaced weekly. Newly molted individuals were collected and caged on a mix of grapevine (*Vitis vinifera* L.), common amaranth (*Amaranthus retroflexus* L.), fava bean, vetch and periwinkle (*Catharanthus roseus*) (sex ratio 1:1, ca. 200 individuals per cage). Both nymphs and adults were reared in Bugdorms inside an insect-proof shaded screenhouse (ca. 23 ± 6°C and 50 ± 10% RH, no artificial light). Herbaceous plants were replaced weekly; grapevines were pruned monthly. Plants were watered every other day. Periwinkle plants were replaced every two weeks and tested for *X. fastidiosa* one and three months after spittlebugs access to the plants by qPCR following the EPPO diagnostic standard PM 7/24 (5) (EPPO 2023) with the primer and TAQ probe described by Harper et al. (2010), to ensure that all individuals used in the trials were free of the bacterium (Spadavecchia-Hamouche *et al.,* 2024).

For EPG experiments, one-year-old olive plants grown from cuttings vars. Leccino and Ogliarola, approximately 40 cm tall, grown in 1 L pots filled with a 2:1 mixture of Typical Brill substrate (Brill® Substrate 70 L, Italia) and perlite (PERALIT25—Perlite Italiana srl, MI, Italia) were used. Plants were kept inside an insect proof greenhouse, at temperature 22 ± 2°C and relative humidity 60 ± 5% (no photoperiod control), watered every other day and fertilized weekly with Plantafol 20:20:20 (NPK, Valagro) 1 g L^{-1}. To avoid interference with spittlebug probing behavior, no pesticides were applied.

2) Water regimes

Three soil moisture levels were imposed to simulate different water stress scenarios in the two olive varieties, *i.e.* the drought-resistant Ogliarola and the drought-sensitive Leccino.

To define the stress thresholds, a substrate calibration curve using soil moisture sensors (Gravity: Analog Waterproof Capacitive Soil Moisture Sensor, DFRobot, China) was developed. The same sensors were specifically adjusted to match the physical characteristics of the growth substrate. Based on volumetric water content (VWC), three stress levels were

set: i) fully irrigated (15–22% VWC), corresponding to field capacity; ii) moderately stressed (10–14% VWC); iii) severely stressed (5–9% VWC). In total, 60 plants were used (30 per variety), with 10 replicates for each treatment/water regime.

To maintain precise control of soil moisture, we implemented a custom irrigation system. Each irrigation strategy was monitored by a dedicated moisture sensor connected to an industrial Internet of Things (IoT) micro–Programmable Logic Controller (PLC) (Arduino Opta Wi-Fi model with expansion capability, Qualcomm, San Diego, California, USA). The PLC processed the humidity signals and was programmed to turn on and off irrigation whenever thresholds were reached. Irrigation was also automated through six 2-way solenoid valves (Baccara G75 1"CCF, 24 VAC, Irritec, Italy), which allowed each treatment group to receive irrigation independently, based on real-time sensor feedback.

The entire system was installed inside a screenhouse under uncontrolled conditions, at the Department of Soil, Plant and Food Science, University of Bari Aldo Moro. After a 15-day acclimation period, plants were subjected to the assigned water regime (fully irrigated, moderately stressed, severely stressed) for 30-day before the EPG recordings (see details below) to ensure the development of clearly defined stress responses (Fig. 1).

To monitor plants response to the water regime, Stem Water Potential (SWP; MPa) was measured using a Scholander chamber (Model 3000F01 plant water status console, Soil moisture Equipment Corp., Santa Barbara, California, USA) on three sprouts per treatment per date, after two weeks from the start of stress, every 7 days (three times: 23/06, 30/06 and 07/07/2025). On the 7th of July, plants were taken for EPG experiment.

Statistical analysis of SWP was performed using two-way ANOVA. When significant differences were detected, Tukey’s post-hoc test was applied. All analyses were conducted using R software (version 4.5.1).



Figure 1. Olive plants irrigated with a custom irrigation system.

3) Water stress impact on host plant acceptance by *Philaenus spumarius*

3.1) EPG-assisted analysis of probing and feeding behavior

The host acceptance of plants subjected to the different water regimes has been characterized through Electropenetrography (EPG), namely characterizing possible variation of probing and feeding behaviors underlying the acceptance of the host plant by adults of *P. spumarius* (both males and females). Recordings were conducted during July 2025 following the protocol described by Markheiser *et al.* (2023).

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 (EPG Systems, Wageningen, Netherlands (epgsystems.eu)). For the connection to the EPG, two months old spittlebug adults were anesthetized through brief exposure to -20°C in Eppendorf tubes for two minutes, then immobilized with a vacuum device and tethered to 18 µm gold wire (EPG-Systems, Wageningen, Netherlands) with a double layer of silver conductive paint (Pelco®, 16062, Ted Pella, USA). Following wiring, insects were transferred to aerated Petri-dishes for one hour starvation period, allowing them to acclimate to the

attached wires. Before EPG connection, the wire-electrode connection was reinforced with additional conductive silver paint. The probing behavior of each spittlebug was recorded for four hours. The recordings were performed inside a Faraday cage, under controlled conditions (23 ± 1°C, 60 ± 5% RH, supplemented with artificial lights 1,000 ± 200 lx). The insects were offered the apical 5 cm portion of olive plants, providing access to both stem tissue and at least one petiole/leaf. A minimum of eight replicates/recordings per sex per variety per water regime were carried out, with single combinations of insect/olive plant (one plant per spittlebug). To avoid positional effects, the position of each variety was switched between replicates.

EPG waveforms were manually marked using 'Stylet+a' software (EPG Systems, Wageningen, Netherlands) and then analyzed by "XylFeed" MS Excel macro (Markheiser *et al.,* 2023). Following the protocol described by Spadavecchia-Hamouche *et al.* (2024), EPG waveforms considered were 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) N, non-pathway interruption of xylem activity; vii) N2, second type of non-pathway xylem interruption; viii) R, resting; ix) Xe, behavior associated with *X. fastidiosa* inoculation; x) W, stylets withdrawal; xi) Esc, escaping of the individual from the host plant. EPG variables considered were: i) Total probing time - TPT (time spent by the spittlebugs with the stylets inserted in plant tissues over the 4 h); ii) Total residence time - TRT (time spent by the spittlebugs on the plant without escaping over the 4 h); iii) Probes: number of probes without (unsuccessful) and with xylem ingestion (successful), number of probes with only xylem vessel contact (initial); number of probes with a xylem ingestion event longer than 5 min (sustained); iv) NWEI: number of waveform events per insect; v) WDEI: median duration of the single waveform event per insect; vi) WDI/TPT: total duration of the waveform per insect over the total probing time; vii) WDI/TRT: total duration of the waveform per insect over the total residence time; viii) Sequential: time required to perform a certain waveform calculated either from the beginning of the EPG recording or from the first probe. To account for "escape" events, the ratios WDI/TPT and WDI/TRT were analyzed instead of considering WDI alone (*i.e.*, total time spent by spittlebugs engaging in a specific waveform).

Statistical analysis of EPG data was carried out using R software (Version 4. 3. 1). To assess the effects of water treatments, sex, and their interaction on spittlebugs probing behavior, generalized linear mixed-effects models (GLMMs) were fitted using Template Model Builder (glmmTMB). The model included treatment, sex, and their interaction as fixed effects, and date of the recording as a random effect. Datasets for the two varieties, *i.e* Leccino and Ogliarola,

were analyzed separately. The data obtained from the EPG were transformed, when necessary, with ln (x+1) or √ (x) to reduce heteroscedasticity and improve normal distribution. However, for variables where these transformations proved insufficient and showed distributional misfit, lognormal family was implemented to better model their distributions (Brooks *et al.,* 2017). Models were run using the "glmmTMB" package (Venables & Ripley, 2002), while residual distribution was checked using the "DHARMa" package (Fox & Weisberg, 2019). Pairwise comparisons were conducted by Tukey's Honest Significant Differences (HSD) test, using the "emmeans" package (Lenth, 2021). A summary of the raw datasets was prepared by "psych" package (Revelle, 2021). Plots were generated using "ggplot2" package (Wickham, 2016).

To explore similarities among water treatments based on spittlebug probing and feeding behavior, hierarchical clustering analysis was performed on the adjusted means of EPG variables. Estimated marginal means for each water regime were obtained using the emmeans package and used to compute a Euclidean distance matrix. Clustering was then conducted using Ward's minimum variance method (ward.D2) as implemented in the hclust function (Murtagh & Legendre, 2014). The resulting dendrogram, visualized with the dendextend package, allowed the identification of groups of water regimes according to their similarities.

3.2) Analysis of xylem sap chemical profile under different water regimes

Following the 4-hour EPG recordings, xylem sap was extracted from four randomly selected olive plants for each combination variety/water regime, using a Scholander pressure chamber, following the protocols described by Hamouche-Crescenzi *et al.* (2025, manuscript submitted). Briefly, at the end of the EPG recording, plants were excised at the base; two basal cm of the main stem was debarked and disinfected with sterile paper moistened in absolute ethanol. Thereafter, the cutting was inserted upside down into the chamber. Ethanol was left to evaporate before sap extraction. The applied pressure was gradually increased up to a maximum of 25 bars to prevent cellular damage and contamination. Xylem sap was collected in 5 mL sterile Eppendorf tubes containing 100 µL of absolute ethanol, maintaining samples on ice throughout the process. An average of 1-2 mL of xylem sap was collected from each plant. Samples were stored at -80°C until the chemical analysis.

Xylem sap samples from Leccino and Ogliarola varieties were prepared according to the protocol established for metabolomic analysis by Hamouche-Crescenzi *et al.* (2025, manuscript submitted). The chemical profile was characterized using the UHPLC-Q-Exactive

instrument (Thermo Fisher Scientific, Bremen/Germany). Chemometric analysis was performed on the UHPLC-Q-Exactive data to classify samples and investigate differential metabolite expression in xylem sap. Following data processing in MZmine (http://mzmine.sourceforge.net/), which included retention time alignment and noise removal (threshold: 5.0×10^6), a pseudo-targeted approach was adopted. The resultant data set was Pareto-scaled and subjected to multivariate analysis in SIMCA-P+ software 12.0 (Umetrix AB). A Principal Component Analysis (PCA) was employed to group samples based on metabolic similarity. The model was validated using cross-validation and permutation tests to ensure statistical robustness.

For Leccino, the data matrix (24 samples × 54 variables) was further analyzed using a PCAA model. The model exhibited strong predictive power, with a cumulative Q^2 of 70%. The first principal component captured 80% of the variance, while the fourth explained 2%. Moreover, analysis of Ogliarola xylem sap samples generated a separate data matrix (24 samples × 54 variables). The corresponding PCA model yielded a cumulative Q^2 of 68%. In this model, the first component explained 61% of the variance, and the third component accounted for 8%. The variables correspond to the identified compounds, which constitute the columns of the matrix, while the rows represent the different samples analyzed.

Following the PCA, it was not possible to identify specific marker metabolites clearly distinguishing all sample classes (*e.g.,* fully irrigated and moderately stressed clusters). Therefore, a one-way ANOVA was applied to assess whether significant differences in metabolite expression existed between the fully irrigated and moderately stressed samples using the MetaboAnalyst 5.0 platform (https://www.metaboanalyst.ca/). The normalized metabolomic dataset was analyzed using a non-parametric Kruskal-Wallis test with a false discovery rate (FDR) cutoff of 0.05. Metabolites showing significant variation (FDR < 0.05) were subsequently subjected to a post-hoc analysis to identify specific pairwise differences between the water regimes.

Results

1) Stem water potential of olive varieties

The SWP of the two varieties was monitored until 7th of July, the starting date of EPG trials (Fig. 2). Leccino showed statistical difference among treatments (Fig. 2a). Severely stressed plants (-1.6 MPa) presented a 60% lower SWP than fully irrigated and moderately stressed ones (-1 and -1.1 MPa, respectively). On the other hand, Ogliarola displayed a different pattern (Fig. 2b): moderately stressed plants (-0.97 MPa) occupied an intermediate position, while a statistically significant difference was observed between fully irrigated (-0.81 MPa) and severely stressed plants (-1.1 MPa).

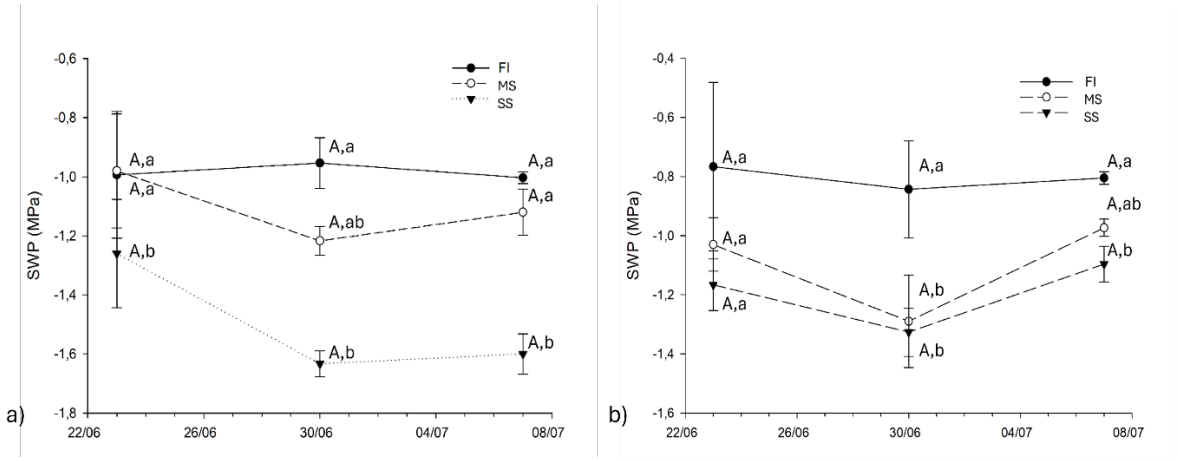


Figure 2. Stem Water Potential (SWP; MPa) measured on olive plants of Leccino (a) and Ogliarola (b). Uppercase Letters denote statistical difference among data for the same treatment; lowercase letters denote statistical difference (p<0.05) among treatments for the same date (FI: Fully Irrigated; MS: Moderately Stressed; SS: Severely Stressed).

2) Water stress impact on host plant acceptance by *Philaenus spumarius*

2.1) EPG-assisted analysis of *Philaenus spumarius* probing behavior

Here, we describe only EPG variables that were statistically significantly different among the three water regimes, reporting mean values ± standard error, and outcomes of pairwise comparisons. Analyses were conducted separately for the two olive varieties, Leccino and Ogliarola, comparing the behavior of spittlebugs under different water conditions (*i.e.,* fully irrigated, moderately stressed and severely stressed plants).

On Leccino, the overall duration of xylem ingestion with respect to the total probing time Xi+LF WDI/TPT was significantly longer on severely stressed plants compared to the fully irrigated plants (88.38 ± 1.82 and 76.62 ± 4.55, respectively; t= 1.73, p<0.001) (Fig. 3a). Conversely, the total duration of resting with respect to the total probing time R WDI/TPT was significantly shorter on severely stressed plants compared to the moderately stressed plants (7.54 ± 1.59 and 21.94 ± 5.77, respectively; t= 2.75, p= 0.027) (Fig. 3b).

The time needed to perform the first low frequency xylem ingestion event, either calculated from the start of EPG recording or from the first probe, was significantly longer on moderately stressed plants (np to 1st LF: 141.31 ± 24.69 min; 1st C to 1st LF: 138.68 ± 24.63 min) compared to fully irrigated plants (np to 1st LF: 59.02 ± 16.3 min, t= 2.61, p= 0.031; 1st C to 1st LF: 52.14 ± 16.56 min, t= 3.14, p= 0.006) (Fig. 3c).

Furthermore, the time needed for the first probe with sustained Xi was significantly shorter on severely stressed plants (6.92 ± 1.47 min) compared to fully irrigated plants (28.78 ± 12.86 min, t= 2.57, p= 0.034) (Fig. 3d).

Hierarchical clustering of water regimes for each EPG variable that differed significantly in the GLMM model is shown in Figure 4.

In contrast to Leccino, we did not observe significant changes associated with the water regime in Ogliarola plants. The only significant differences were sex-associated (Supplementary Material S1).

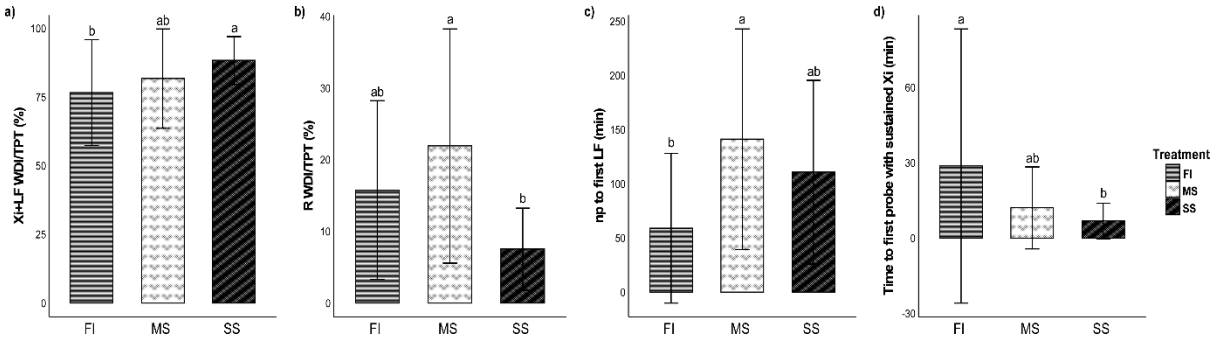


Figure 3. Overall duration of xylem ingestion Xi+LF WDI/TPT (a) and Total duration of resting R WDI/TPT (c) with respect to the total probing time; The time needed from the start of EPG recording to first low frequency xylem ingestion LF (c); and The time needed to the first probe accompanied with a sustained xylem ingestion Xi (d) on Leccino plants under different water treatments (FI: Fully Irrigated; MS: Moderately Stressed; SS: Severely Stressed). Treatments indicated with different letters are statistically different between each other (p < 0.05).

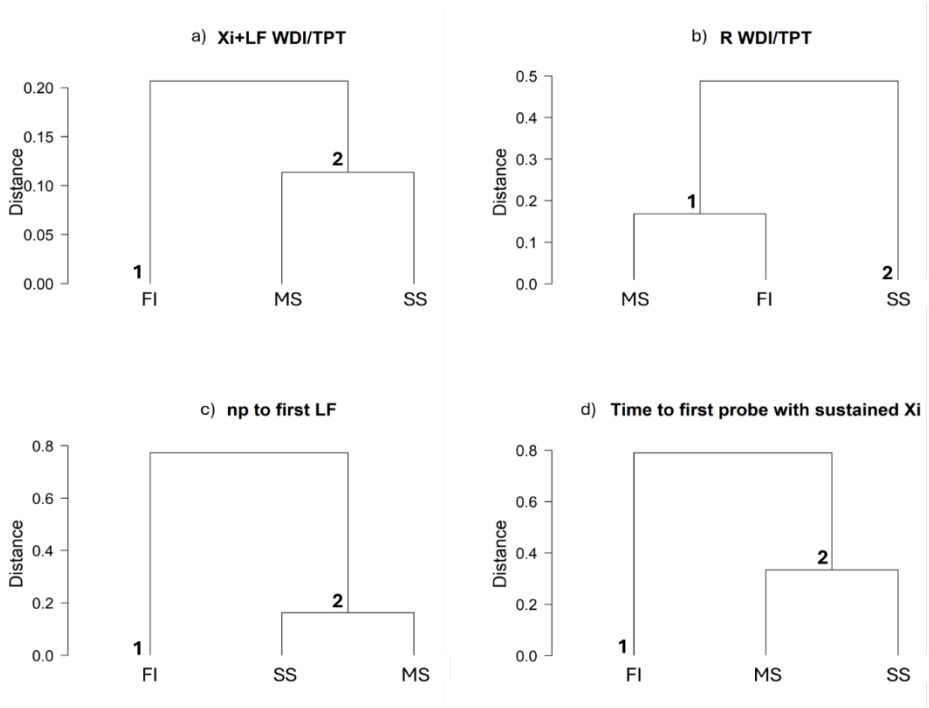


Figure 4. Hierarchical clustering of water regimes for each EPG variable that differed significantly according to the GLMM model; a) Xylem ingestion index (Xi+LF WDI/TPT), b) R WDI/TPT, c) Time to first LF, d) Time to first probe with sustained Xi. Numbers above the branches indicate cluster groups of water regimes for each variable. For xylem ingestion index (a), cluster number **2** represents higher values, indicating longer proportions of time devoted to xylem ingestion, compared to cluster number **1**; For total duration of resting event R (b), cluster number **2** represents shorter periods of resting, compared to cluster number **1**. For the time to first LF (c), cluster number **1** indicates an earlier onset of low-frequency ingestion, while number **2** corresponds to longer delays before LF activity begins; For the time to first probe with sustained Xi (d), cluster number **2** represents shorter time to initiate a probe with sustained ingestion whereas cluster number **1** corresponds to longer times.

2.2) Untargeted metabolomics reveal contrasting xylem sap responses to water stress in olive

The application of an untargeted metabolomics workflow (HR-MS/MS analysis), combined with chemometric analysis (PCA), revealed no significant changes in the xylem sap metabolome independently of the water regime in the drought-resistant Ogliarola, in contrast with a dramatic reprogramming of the xylem chemical profile in the drought-sensitive Leccino. Specifically, Ogliarola metabolic profile remained largely stable across all water regimes, indicating a limited biochemical responsiveness to increasing stress levels (Fig. 5b). Conversely, Leccino displayed a marked metabolic plasticity, with clear differences emerging between irrigation levels. The distinct separation of the severe stress group from the fully

irrigated and moderately stressed samples highlights a threshold beyond which substantial metabolic adjustments occur (Fig. 5a). Several metabolites were more abundant in the xylem of severely stressed Leccino plants, while others were predominant or evenly distributed under full irrigation and moderate stress conditions (Fig. 5c-d).

Specifically, the fully irrigated and moderately stressed plants formed a single metabolic cluster characterized by elevated levels of amino acids, *i.e.,* oxoproline, asparagine, glutamine, and organic acids, *i.e.,* succinic acid, together with higher expression of several sugars (*e.g.,* sucrose, D-manno-2-heptulose), terpenes (*e.g.,* barlerin, oleuropein), and phenolic compounds (*e.g.,* vanillin, verbascoside). Within this cluster, post-hoc comparisons revealed that some metabolites (Fig. 6), particularly citric acid, malic acid, fumaric acid, 2-furoic acid (organic acids), and L-aspartic acid (amino acids), were more abundant in fully irrigated plants, indicating a fine-scale differentiation within the broader shared profile. In contrast, the severely stressed Leccino plants displayed a distinct metabolic signature marked by strong accumulation of specific secoiridoid glucosides, particularly ligustroside, various isomers of elenolic acid glucoside, and oleoside, as well as lignans such as (–)-olivil 4″-O-glucoside and 8-acetoxypinoresinol 4-glucoside. Additionally, several phenolic compounds, including salidroside and hydroxytyrosol 4-O-glucoside and sugars, *e.g.,* 2-O-methylxylose, mannitol, and gluconic acid were notably upregulated under severe stress.

Details on the chemical markers detected by PCA in the xylem of Leccino under different water regimes are reported in Table 1.

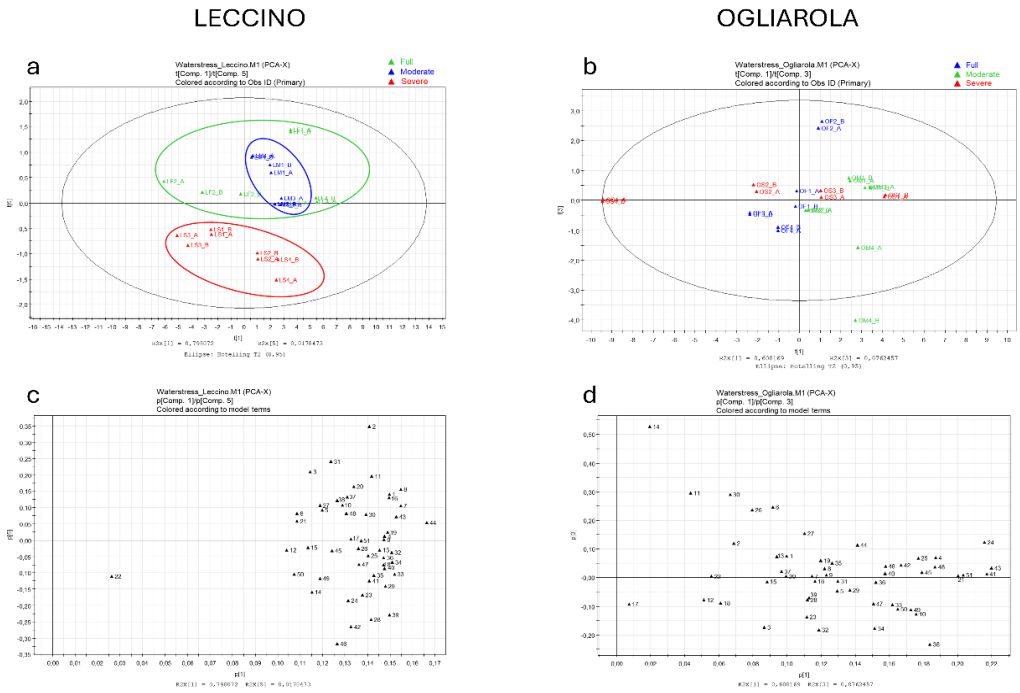


Figure 5. Principal Component Analysis (PCA) of Leccino and Ogliarola xylem sap samples under different water stress conditions (fully irrigated, moderately stressed, and severely stressed) obtained using a pseudo-targeted approach. (a–b) Score scatter plots showing sample clustering by irrigation regime, and (c–d) loading scatter plots. In the loading plots, numbers correspond to the identified metabolites contributing to PCA separation; metabolite identities are reported in detail in Supplementary Material Table S2.

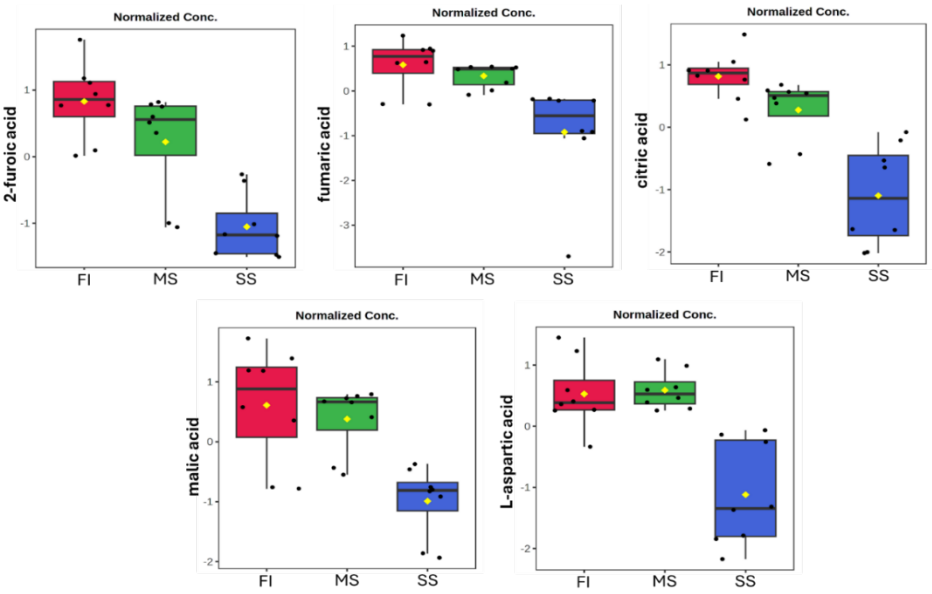


Figure 6. Box plots showing the normalized abundance of key metabolites across different water stress conditions in Leccino variety (FI: Fully Irrigated; MS: Moderately Stressed; SS: Severely Stressed). Post-hoc analysis, following a one-way ANOVA, confirmed a statistically significant decrease (FDR < 0.05).

Table 1. Treatment-associated changes in xylem sap metabolites of the olive variety Leccino under moderate and severe water stress relative to fully irrigated conditions. Metabolites are grouped by chemical class. Symbols indicate qualitative changes inferred from PCA-based discrimination: ↑, higher association with the stress condition; ↓, lower association relative to fully irrigated plants; =, similar contribution across treatments.

Class	Metabolite	Moderately stressed vs Fully irrigated	Severely stressed vs Fully irrigated
Organic acids	L-threonic acid	=	=
	Quinic acid	=	=
	Daucic acid	=	=
	Succinic acid	=	↓
	2-furoic acid	↓	↓
	Fumaric acid	↓	↓
	Malic acid	↓	↓
	Citric acid	↓	↓
	Ribonic acid	=	↑
Amino acids	Oxoproline	=	↓
	Asparagine	=	↓
	Glutamine	=	↓
	L-aspartic acid	↓	↓
Sugars	D-manno-2-heptulose	=	↓
	Sucrose	=	↓
	α-L-Galactopyranose, 6-deoxy-3-O-(2,6-dideoxy-4-O-methyl-α-D-arabino-hexopyranosyl)-2-O-methyl-, 4-acetate (9CI, ACI)	=	↓
	Gluconic acid	=	↑
	dimethyl-4-(α-D-mannopyranosyloxy)-5-methoxy-1,2-cyclohexanedicarboxylate	=	↑
	D-mannose	=	↑
	Mannitol	=	↑
	2-O-methylxylose	=	↑
Phenols	Vanillin	=	↓
	2-hydroxyflavanone naringenin	=	↓
	Eriodictyol-7-O-glucoside	=	↓
	Verbascoside	=	↓
	koaburaside	=	↑

	decaffeoylverbascoside	=	↑
	8-hydroxypinoresinol 4′-O-β-D-glucopyranoside	=	↑
	naringenin dihydrochalcone	=	↑
	4'-hydroxy-5,7,2'-trimethoxyflavanone 4'-rhamnosyl-(1->6) -glucoside	=	↑
	β-hydroxyacteoside	=	↑
	Gingerol	=	↑
	Salidroside	=	↑
	Nigrescin	=	↑
	hydroxytyrosol 4-O-glucoside	=	↑
Terpenes	Oleuropein diglucoside	=	=
	Plantarenaloside	=	↓
	Barlerin	=	↓
	Oleuropein	=	↓
	10-hydroxyoleuropein	=	↓
	Ligustroside	=	↑
	oleoside isomer 1	=	↑
	oleoside isomer 2	=	↑
	elenolic acid glucoside isomer 1	=	↑
	elenolic acid glucoside isomer 2	=	↑
	adinoside A	=	↑
	Eucommioside	=	↑
	Lamiol	=	↑
	Aucubin	=	↑
Lignans	8-acetoxypinoresinol 4-glucoside	=	↑
	(-)-olivil 4″-O-glucoside	=	↑
Phenilpropanoids	Gein	=	↑
Benzenoids	3-hydroxybenzyl alcohol	=	=

Discussion

The present study provides the first preliminary experimental evidence that water stress might shape spittlebug-olive interaction in drought-sensitive olive varieties, by inducing a significant reprogramming of the xylem sap chemical profile.

Overall, severely stressed plants were more suitable than fully irrigated ones for *P. spumarius*, as evidenced by the faster onset and longer duration of continuous xylem sap ingestion in the former compared to the latter. These findings suggest that, at least under no-choice conditions (for EPG recordings, insects are forced to feed on a limited plant portion), severe water stress increases the suitability of Leccino plants for spittlebugs, making them more favorable hosts than fully irrigated plants. Conversely, we observed no significant variations of spittlebugs probing behavior independently of the water regime on Ogliarola plants.

This differential response to water stress in Leccino and Ogliarola plants could have been primed by water-availability driven changes in two plant traits: i) xylem sap tension; ii) xylem sap chemical profile. Although water stress increased xylem sap tension, the SWP values recorded before EPG trials (up to ~ –1.6 in severely stressed Leccino and ~ –1.0 MPa in Ogliarola, compared with ~ –1.0 MPa and ~ –0.8 MPa in fully irrigated plants) remained within the range allowing xylem sap uptake by the meadow spittlebug (Bergman *et al.,* 2021). Thus, sap tension seems not to be the factor underlying the probing behavioral differences observed in Leccino plants in response to water stress. In contrast, we hypothesize the changes in plant suitability for the spittlebug were mainly driven by dramatic alterations in xylem sap chemical profile that occurred in the drought-sensitive variety Leccino.

As the duration of xylem ingestion is the key indicator of host suitability (Markheiser *et al.,* 2020), the longer ingestion periods observed on severely stressed Leccino plants point towards a response to water stress encompassing a shift toward a more favorable xylem sap chemical profile compared to fully irrigated plants. On the other hand, *P. spumarius* behavior was not influenced by the water regime in Ogliarola plants.

Untargeted metabolomic analysis revealed differences in the way the two olive varieties responded to water stress. Ogliarola displayed a relatively stable metabolomic profile across all water treatments. In contrast, Leccino showed a stress-dependent metabolic shift, with severely stressed plants clearly distinct from both fully irrigated and moderately stressed plants.

The fully irrigated Leccino plants had a profile rich in organic acids (*i.e.,* 2-furoic acid, fumaric acid, succinic acid, malic acid, and citric acid), amino acids (*i.e.,* oxoproline, asparagine, L-aspartic acid, and glutamine), sugars (*e.g.,* D-manno-heptulose and sucrose), some phenols (*e.g.,* vanillin and verbascoside) and terpenes (*e.g.,* barlerin and oleuropein). Moderately stressed plants exhibited a similar chemical profile to that of fully irrigated ones, although relatively poorer in organic acids. On the other hand, severely stressed Leccino plants were relatively poor in organic acids but showed a remarkable higher expression of terpenes (*e.g.,* aucubin and elenolic acid glucoside) and phenols (*e.g.,* salidroside and gingerol) along with some sugars (*e.g.,* 2-*O*-methylxylose, mannitol and gluconic acid). These results align with previous studies demonstrating that drought stress triggers the activation of secondary metabolism, leading to increased production of terpenes and phenols (de Abreu & Mazzafera, 2005; de Matos Nunes *et al.,* 2014; Piasecka *et al.,* 2016). For instance, the notable accumulation of secoiridoid glucosides (terpenes), particularly ligustroside and various isomers of elenolic acid glucoside and oleoside, suggests activation of the iridoid pathway, possibly reflecting a metabolic shift toward compounds with enhanced stress-related functions. Moreover, the upregulation of lignans like (-)-olivil 4″-*O*-glucoside and 8-acetoxypinoresinol 4-glucoside highlight their potential defensive role, as these phenylpropanoid dimers may fortify xylem cell walls and protect against collapse under extreme tension (El Sayed *et al.,* 2019; Šamec *et al.,* 2021).

In our previous work (Chapter III), we characterized the main xylem sap metabolites potentially promoting or deterring host plant acceptance and feeding by *P. spumarius*. Specifically, metabolites such as glutamine, malic acid, and daucic acid seem to act as phagostimulants, while gluconic acid, salidroside, oleuropein diglucoside, and D-manno-2-heptulose discourage feeding. Additionally, independently of the effect of single organic acids on plant suitability for spittlebugs, least suitable olive varieties displayed higher concentrations of all the selected biomarkers belonging to this chemical class.

In the present work, either fully irrigated or severely stressed plants displayed a characteristic combination of phagodeterrents and phagostimulants xylem sap metabolites whose impact on spittlebug behavior is hard to predict. Fully irrigated plants showed higher abundance of glutamine, malic acid, citric acid and sucrose, metabolites previously described as feeding promoters, along with elevated levels of D-manno-2-heptulose and furoic acid, which depress feeding. In contrast, severely stressed Leccino plants exhibited greater presence of salidroside and gluconic acid, metabolites previously linked with reduced plant acceptability, together with

higher levels of 2-*O*-methylxylose, mannitol and elenolic acid glucoside, compounds observed to promote feeding activity by *P. spumarius* (Chapter III). The key difference between fully irrigated and severely stressed Leccino plants, thus between poorer and better host plants, was the total content of organic acids, which was significantly greater in fully irrigated compared to severely stressed Leccino olives. The lower suitability of plants whose xylem sap is relatively rich in organic acids is consistent with our previous findings (reported in Chapters II and III) along with other studies reporting lower xylem sap pH impairs gustatory responses and adversely affect feeding efficiency and preference in sap-feeding insects (Vega *et al.,* 1995; Bernays *et al.,* 1998; Hu *et al.,* 1999). Alternatively, differences in feeding behavior may reflect a shift in the balance between phagostimulant and phagodeterrent compounds that collectively determine plant acceptability (Chapman, 2003). In fully irrigated plants, phagodeterrents might have offset phagostimulants thereby reducing plant suitability, whereas under severe stress, this balance could have been reversed. Additionally, the overexpression of metabolites specifically expressed in response to drought and whose role in host acceptance was not characterized in the previous work (Chapter III), may have further contributed to the observed behavioral changes.

Conclusions and future perspectives

Overall, this study highlights that water stress influences the interaction between olive plants and the meadow spittlebug in a variety-dependent manner. Indeed, in drought-sensitive olive varieties, water-stress might prime dramatic changes in xylem sap chemical profiles affecting spittlebug plant interaction, with potential consequences for *X. fastidiosa* epidemiology. Nevertheless, further studies are needed to better understand the phenomenon, particularly: i) choice tests assessing water regimes impact on host location and acceptance over a longer temporal interval than the one adopted for the EPG-characterization (4h), possibly integrating the measurement of plant spectral characteristics (essential for host location as demonstrated by Lazar *et al.,* 2025); ii) measuring xylem sap pH at different water regimes to prove our hypothesis regarding the correlation between plant acceptability and organic acids. In addition, extending this approach to a broader range of olive varieties with different drought tolerance and vector suitability will be essential to assess the generality of these findings. Ultimately, a comprehensive understanding of how water availability and irrigation modulate plant-vector interactions is essential for optimizing agronomic practices aimed at mitigating the spread of *X. fastidiosa* through the interference with vector activity.

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Supplementary Material

Table S1. Model outputs and summary statistics of EPG variables with significant sex effects in Leccino and Ogliarola.

	EPG Variable	contrast	estimate	SE	df	t ratio	*p* value	Sex	n	mean	sd	median	min	max	se
Leccino	C NWEI	Female - Male	0.117	0.05	50	2.329	0.024	Female	32	5.84	3.27	5	1	13	0.58
								Male	26	4.19	3.11	3.5	1	16	0.61
Ogliarola	Xi+LF WDI/TPT	Female - Male	9.55	4.45	41	2.148	0.038	Female	25	84.54	14.05	87.15	46.21	98.61	2.81
								Male	24	75.17	18.16	79.48	36.74	98.83	3.71
	Xi+LF WDI/TRT	Female - Male	12	5.1	41	2.356	0.023	Female	25	78.48	16.81	82.81	42.17	98	3.36
								Male	24	66.71	20.18	66.66	23.14	93.84	4.12
	np to 1st Xc	Female - Male	-0.34	0.125	41	-2.73	0.009	Female	25	11.2	11.3	7.4	1.5	56.5	2.26
								Male	24	20.84	17.22	16.45	2.5	62.8	3.51
	np to 1st Xi	Female - Male	-0.37	0.12	41	-3.07	0.004	Female	25	11.48	11.26	7.6	1.7	56.7	2.25
								Male	24	21.72	16.81	16.95	2.7	63	3.43
	np to 1st sustained Xi	Female - Male	-0.59	0.204	41	-2.86	0.007	Female	25	14.64	12.3	11.6	1.7	56.7	2.46
								Male	24	25.98	18.84	19.9	3.1	63	3.85
	Time to 1st probe	Female - Male	-0.9	0.316	41	-2.83	0.007	Female	25	4.36	5.36	1.6	0.4	21	1.07
								Male	24	10.67	11.69	5.15	1.1	41.6	2.39
	Time to 1st probe with Xi	Female - Male	-0.67	0.281	41	-2.37	0.023	Female	25	8.86	11.59	4.7	0.6	54.8	2.32
								Male	24	17.47	17.31	12.8	1.1	58	3.53
	Time to 1st probe with sustained Xi	Female - Male	-0.69	0.284	41	-2.44	0.019	Female	25	8.86	11.59	4.7	0.6	54.8	2.32
								Male	24	18.13	17.55	12.8	1.1	58	3.58

Table S2. List of metabolites identified in olive xylem sap and included in the PCA analysis.

N°	$[M-H]^-$ (*m/z*)	Identification
1	111.007	2-furoic acid
2	115.003	fumaric acid
3	117.015	succinic acid
4	123.044	3-hydroxybenzyl alcohol
5	128.034	oxoproline
6	131.038	asparagine
7	132.029	L-aspartic acid
8	133.013	malic acid
9	135.029	L-threonic acid
10	145.061	glutamine
11	151.039	vanillin
12	163.061	2-*O*-methylxylose
13	165.040	ribonic acid
14	179.056	D-mannose
15	181.071	mannitol
16	191.019	citric acid
17	191.056	quinic acid
18	195.050	gluconic acid
19	203.019	daucic acid
20	209.063	D-manno-2-heptulose
21	287.048	2-hydroxyflavanone naringenin
22	293.176	gingerol
23	299.114	salidroside
24	303.051	nigrescin
25	315.109	hydroxytyrosol 4-*O*-glucoside
26	331.104	koaburaside
27	341.109	sucrose
28	345.119	aucubin
29	349.151	eucommioside
30	359.135	plantarenaloside
31	363.157	*α*-L-Galactopyranose, 6-deoxy-3-*O*-(2,6-dideoxy-4-*O*-methyl-*α*-D-arabino-hexopyranosyl)-2-*O*-methyl-, 4-acetate (9CI, ACI)
32	377.145	lamiol
33	389.108	oleoside
34	403.126	elenolic acid glucoside
35	407.156	dimethyl-4-(α-D-mannopyranosyloxy)-5-methoxy-1,2-cyclohexanedicarboxylate
36	443.156	adinoside A
37	447.141	barlerin
38	449.106	eriodictyol-7-*O*-glucoside
39	457.168	gein

40	461.156	decaffeoylverbascoside
41	523.183	ligustroside
42	535.183	8-hydroxypinoresinol 4'-*O*-*β*-D-glucopyranoside
43	539.170	oleuropein
44	555.173	10-hydroxyoleuropein
45	577.180	8-acetoxypinoresinol 4-glucoside
46	581.187	naringenin dihydrochalcone
47	583.192	(-)-olivil 4''-*O*-glucoside
48	623.199	verbascoside
49	637.216	4'-hydroxy-5,7,2'-trimethoxyflavanone 4'-rhamnosyl- (1->6) -glucoside
50	639.205	β-hydroxyacteoside
51	701.230	oleuropein diglucoside

7. Conclusions

The present work provides the first insight into the plant traits that shape host plant acceptance by *Philaenus spumarius* in the olive system. It integrates vector behavior with plant metabolomics to elucidate insect-plant interactions and the cues driving spittlebug preference and permanence. The dissertation begins with identifying major knowledge gaps in spittlebugs-plant interactions, focusing on how spittlebugs locate host plants and what drives host plant acceptance. The limited understanding of sensory cues (*i.e.,* visual, olfactory and chemical) mediating host location and acceptance guided the focus of this thesis toward elucidating the plant traits that determine host suitability for the vector, with a specific focus on the mechanisms underlying host plant acceptance.

This research demonstrates that host plant acceptance by *P. spumarius* is primarily driven by plant chemical traits rather than structural or anatomical traits. Experimental studies revealed that the xylem sap metabolomic profile is the main determinant of host suitability for spittlebugs, with acceptance shaped by the overall chemical profile rather than by single compounds. While individual metabolites may act as phagostimulants or deterrents, profiles enriched in organic acids were consistently associated with reduced suitability and lower insect acceptance. A broader screening of olive germplasm identified genotypes displaying traits of resistance to spittlebugs, likely due to unfavorable xylem chemical profiles, reducing vector acceptance and permanence on the plants, thereby limiting their contribution to pathogen spread. Water availability further modulated these interactions: drought-induced shifts in xylem chemistry altered spittlebug behavior and increased host suitability in drought-sensitive genotype.

Further research is needed to validate the correlation between high organic acids content (or low xylem sap pH) with host plant acceptance by measuring and/or manipulating xylem pH under different conditions, and testing its effects on spittlebug behavior. Studies should then explore how seasonal dynamics and agronomic factors, such as irrigation, soil composition, and fertilization, affect xylem chemistry and vector performance, offering ways to reduce plant suitability. Further work should also assess the epidemiological relevance of the presented findings through transmission tests to verify whether reduced vector activity on less acceptable varieties translates into lower bacterium acquisition and inoculation. Expanding this framework to other *X. fastidiosa* vectors will help determine whether similar chemical mechanisms shape host acceptance across xylem-feeding insects.

Overall, this thesis highlights the important role of xylem sap profile in shaping host acceptance for spittlebugs and opens new opportunities for developing chemical-based or breeding-oriented strategies that manipulate xylem sap chemistry and render olive plants less suitable to the vector, thereby reducing pathogen spread while minimizing environmental impact.

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﴿ وَآخِرُ دَعْوَاهُمْ أَنِ الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ ﴾