MARCHE POLYTECHNIC UNIVERSITY
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Functional anatomy and ecology of *Philaenus spumarius* and other *Xylella fastidiosa* potential vectors

Ph.D. Thesis

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I'd love to dedicate this thesis to my girlfriend MC
At that time she was a PhD student like me
After a discussion she told me I am not tailored to work in the Uni
I'm still questioning myself whether is a bad or a good thing

Preface

SULLE MACERIE ERETTA UNA COLONNA DA CHIAMARE INFAME. LUNGI ADUNQUE DA QUI, ALLA LARGA, PROBI CITTADINI, CHE UN ESECRANDO SUOLO NON ABBIA A CONTAMINARVI!

It is what is written in a plaque stored in the museums of the Sforza's castle in Milan. Erected in 1630 by the Milan government in memory of the bubonic plague spreader Gian Giacomo Mora. This plaque was placed at the basement of a column, originally erected as a mark of shame for the plague spreader. Thanks to the novel written by Alessandro Manzoni: 'Storia della colonna infame' it is nowadays remembered in European history as a symbol of superstition and injustice of that historical period, becoming the stygma of the shame and dishonour of the government itself. What happened with *Xylella fastidiosa* disease epidemic in Italy, reminds me that novel. It was not only addressed as a critical fitosanitary threat, but also a public distrust of science and scientists that has led to conspiracy theories, social turmoil and heated debates that until now divide the society and the academic community. This debate has forced us to reconsider many paradigms about cultural components, researchers' role, scientific communication and political decisions.

Abstract

The recent outbreak of the xylem limited bacterium *Xylella fastidiosa*, the agent responsible for the Olive Quick Decline Syndrome (OQDS) in the Salento peninsula (Apulia, Italy), is considered a relevant phytosanitary problem also due to the high probability of spread from established infested area. The common meadow spittlebug *Philaenus spumarius* (Homoptera: Auchenorrhyncha), at present, is the only insect proven to be able in transmitting the pathogen in Italy but any other xylem-sap feeding species has to be considered a potential vector until contrasting evidences. In the present thesis, it is presented the outcome of 3-years work on the functional anatomy and the ecology of *Philaenus spumarius* and other potential vectors. The study of the functional morphology of *P. spumarius* antennae, labium and foregut was carried out through scanning and transmission electron microscopy techniques as well as micro computed tomography. The aim of these investigations were to understand the role of different structures involved in the host plant selection and in the pathogen transmission. The dispersal of *P. spumarius*, and other xylem-sap feeders in relationship with wild-plant abundance and olive trees, were studied over 1 year in an olive agroecosystem of the Marche region (Central Italy). Adults were sampled using yellow sticky traps at 2 different heights in a regular geo-referenced grid and wild cover vegetation was surveyed around each sampling point. Distribution maps were built based on spatial analysis by distance indices, showing patch/gap distribution and plant-associations.

Riassunto

Xylella fastidiosa e' il batterio xilematico associato al Complesso del Disseccamento Rapido dell'Olivo (CoDiRO) nella penisola salentina (Puglia, Italia). Questa patologia e' considerata un grave problema fitosanitario anche a causa della elevata probabilità di diffusione. La comune 'sputacchina' *Philaenus spumarius* (Homoptera: Auchenorrhyncha), allo stato attuale, è l'unico vettore accertato in Italia; nonostante questo, tutti gli insetti ad alimentazione xilematica sono considerati potenziali vettori. Nella presente tesi sono riportati i risultati di 3 anni di studio inerenti l'anatomia funzionale e l'ecologia di *P. spumarius* e di altri potenziali vettori. La morfologia funzionale delle antenne e dell'apparato boccale di *P. spumarius* è stata studiata attraverso tecniche di microscopia elettronica a scansione e a trasmissione e mediante micro-tomografia computerizzata. Lo scopo di queste indagini è stato quello di comprendere il ruolo di diverse strutture coinvolte nella selezione delle piante ospiti e nella trasmissione dei patogeni. Viene inoltre presentato lo studio della distribuzione spaziale e della fenologia di *P. spumarius* e altri insetti xilemomizi in relazione all'abbondanza di piante spontanee, in un oliveto della regione Marche (Italia centrale). Gli adulti sono stati campionati, in un periodo di un anno, utilizzando trappole cromotropiche posizionate a 2 diverse altezze in una griglia geo-referenziata. La vegetazione spontanea è stata analizzata intorno ad ogni punto di campionamento. Sono state costruite mappe di distribuzione basate sulle analisi spaziali e sono stati studiati i livelli di associazione con la vegetazione spontanea.

Table of contents

Introduction

The problem: *Xylella fastidiosa*

Newton B. Pierce was the first, in 1892, to study what he called 'California Vine Disease' (Pierce, 1892). He could neither isolate nor culture the causal agent, and he ended his career just suspecting that a 'minute microorganism' was involved. Later, in 1939, Prof William Hewitt, a plant pathologist at UC Davis, named the disease 'Pierce's disease' in his honor (Hewitt, 1939). At that time (and for more than three decades), researchers thought a virus was the causal agent of that pathology. The infectious was systemic, the agent was too small to be seen with light microscopy techniques and was often transmitted by insect vectors. Everything fitted with the virus hypothesis. The 'virus' paradigm slowly changed within the years, under the help of different clues among which: the recoveries of the symptoms during the winter, the transmission mediated by xylem-sap feeding insects, the wide plant host range and the responses of the plants to tetracyclines therapy (Hopkins *et al.*, 1971; Purcell, 2013). In early seventies, the causal agent was considered to be a mycoplasma-like organism first, then a ricketsia-like organism because *Rickettsia* spp. were not culturable (Goheen *et al.*, 1973; Hopkins, 1977). Probably the most important step forward in the research was made in 1978, when a selective medium for the pathogen was developed and pathogen was successfully isolated (Davis *et al.*, 1978). The disease was then associated with a xylem-limited bacterium and finally in 1987 this bacterium was described and named as *Xylella fastidiosa* (Wells *et al.*, 1987). The etimology of the name comes from 'fastidious', re-

ferring to the nutritional fastidiousness of the organism and so the difficulty occurred in the first isolation (Wells *et al.*, 1987). It was the first plant-pathogenic bacterium to have the genome completely sequenced and after years of studies, new subspecies were described based on pathogenicity, phylogeny and DNA characteristics (Purcell, 2013). Nowadays *X. fastidiosa* is included in EPPO A1 list of quarantine pathogens (EPPO/OEPP 1992). It is defined as a gram-negative, xylem-limited, slow-growing bacterium, transmitted by different xylem-feeding insects vectors and more then 300 plants are recognized as hosts in the America continent (Baker *et al.*, 2015). Most of these species support some degree of pathogen multiplication without expressing symptoms. Susceptible hosts infected with *X. fastidiosa* often show disease symptoms only after months or years, although epidemics can spread fast and be devastating (Almeida, 2016). Its properties, biology, epidemiology and disease management strategies have been widely described in many reviews (Hopkins, 1989; Purcell, 1996; Redak, 2004; Chatterjee, 2008; Janse, 2010; Purcell, 2013). In 2010, olive trees on the west coast of Salento Peninsula, a tourist destination in Italy's southern region of Apulia, began to decline and die with a condition of unknown etiology called 'Olive Quick Decline Syndrome' (OQDS). On 21st October 2013, the Italian phytosanitary service notified the European Commission (EC) that the plant pathogen *Xylella fastidiosa* had been detected in olive trees (Saponari, 2013) and it has been shown that the subspecies occurring on these plants is *X. fastidiosa* subspecies *pauca*. The unexpected outbreak of *X. fastidiosa* in Italy has created unprecedented turmoil and unrest. Local environmentalists and politicians efforts to protect olive trees by blocking mandatory EC containment strategies have, however, only exacerbated the problem (Almeida, 2016). In the early autumn of 2013, the pathogen affected a surface of more than 8000 ha of arable land. This area has extended in 2016 to a good deal of the olive-growing area of the province of Lecce (about 23,000 ha) (Martelli, 2015). *Philaenus spumarius* L. (meadow spittlebug), a widespread insect of the Aphrophoridae family, was identified in 2014 as the main vector of the pathogen in Apulia (Saponari, 2014; Cornara, 2016). The etimology of *Philaenus spumarius* comes from the greek word *philein* = 'love' and the latin *spuma* = 'foam'. The 'foam lover' meadow spittlebug

is a very common insect occurring in many habitats worldwide (Yurtsever, 2000). It has attracted the interest of scientists and naturalists from century due to the particular ecology of the larval stages, that produce foamy masses. It has gradually become one of the most studied species on various aspects of biology, since it is very suitable for genetics, ecology and other population studies (Yurtsever, 2000). It is known to be a *X. fastidiosa* vector in America since long time (Severin, 1950) but its role in the new world is considered marginal thus it was never associated with the disease epidemics (Baker *et al.*, 2015). In contrast the sharpshooters (Homoptera: Cicadellini), such as *Graphocephala atropunctata*, are considered epidemiologically relevant vectors. In Europe, sharpshooter diversity is not so large and their presence in the ecosystems is not so abundant, while *P. spumarius* is widely distributed and highly polyphagous. Despite the central epidemiological role played by this species, every insect that feeds primarily in the xylem has to be considered potential vector until further contrasting evidences (Baker *et al.*, 2015; Purcell, 1989). The hypothesis, in fact, is that competence as a *X. fastidiosa* vector is more a function of vector feeding behavior than phylogeny (Frazier, 1965). Overall, many epidemiological factors are involved in the complex *X. fastidiosa* disease epidemiology, most of which were barely investigated in *P. spumarius* and in the other potential European vectors. There are many unsolved questions that underline the importance of a capillary work to define the situation and help in the correct management. The ecology and the functional anatomy of these insect species, particularly *Philaenus spumarius*, of which the transmission ability was proven in different systems, are the object of this thesis.

Outline of the Work

CHAPTERS:

1. Fine structure of antennal sensilla of the spittlebug *Philaenus spumarius* L. (Insecta:Hemiptera:Aphrophoridae). I. Chemoreceptors and thermo-/hygroreceptors.
   Published on Arthropod Structures and Developments.
   Wherein there was used a morphological approach to understand the importance of the stimuli perception in the antennae of *P. spumarius*. The work aims to understand structures and clues related with the search of the host plant.

2. Sensory Receptors Associated with Labial Tip and Precibarium of the Meadow Spittlebug, *Philaenus spumarius*.
   Study completed - Draft paper.
   Wherein there was studied the structures involved in the contact-evaluation and the acceptance of the host plant.

3. Morphological characterization of the retention sites of *Xylella fastidiosa*. Two vectors compared: *Philaenus spumarius* and *Graphocephala atropunctata*.
   Preliminary study.
   Wherein there was used a novel morphological approach to study the morphometry of the retention site of *X. fastidiosa* in the two vectors. This work aims to understand anatomical factors related to the attachment and dislodgement of *X. fastidiosa* from insects.

4. Functional anatomy of *Philaenus spumarius* precibarial valve.
   Preliminary study.
   Wherein a new functioning mechanism of precibarial valve of *P. spumarius* is proposed.

5. Spatio-temporal distribution patterns of xylem-sap feeding insects in a central Italy olive agroecosystem.
   Study completed - Draft paper.

Wherein there was analyzed the phenology, abundance, spatial distribution and plant association of the xylem-sap sucking insects in an olive orchard of the Marche region (central Itlay) to understand the *X. fastidiosa* potential spread-risk factors linked with these species.

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Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* subsp. International Journal of Systematic Bacteriology 37, 136-143.

Chapter 1

Fine structure of antennal sensilla of the spittlebug *Philaenus spumarius* L. (Insecta: Hemiptera: Aphrophoridae). I. Chemoreceptors and thermo-/hygroreceptors

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Introduction

Philaenus spumarius L. (Hemiptera: Cercopoidea: Aphrophoridae) is an insect vector of the xylem-limited bacterium *Xylella fastidiosa*, responsible for the Olive Quick Decline Syndrome (OQDS) on olives trees in the Salento Peninsula, Italy (Saponari *et al.* 2014). At present, *P. spumarius*, is the only species proved to be able to acquire and transmit this bacterium in Italy (Saponari *et al.* 2014). *P. spumarius* is widespread, abundant and polyphagous, with hundreds of plants in its host range (Halkka *et al.* 1967; Whittaker 1968; Nickel 2003). The spread of *X. fastidiosa* in the agroecosystem is associated with many factors tightly related to the vectors such as the transmission efficiency (Daugherty *et al.* 2010) and the host plants (Redak 2004, Cornara 2016). The behaviour of many leafhoppers and planthoppers, is mainly mediated by various signals, among which semiochemicals produced by host plants, conspecifics and other associated taxa are the most important (Bourgoin and Deiss 1994; Youn 2002; Romani *et al.* 2009; Riolo *et al.* 2012). Volatile organic compounds (VOCs) emitted by plants provide important cues for insects in their search for hosts, and these VOCs are detected by olfactory sensilla that are located on insect antennae (Bruce and Pickett 2011). Besides aphids and psyllids, for which several studies were carried out to investigate the fine structure of the antennal sensilla (Bromley *et al.* 1979, 1980; Kristoffersen *et al.* 2006; Onagbola *et al.* 2008), in leafhoppers and planthoppers, the presence of antennal ofactory sensilla was reported in details only in few species (Lewis and Marshall 1970, Aljunid and Anderson 1983, Romani *et al.* 2009; Rossi Stacconi and Romani 2012). At present, there is a lack of knowledge on Aphrophoridae antennal sensory equipment, and little is known on closely related taxa despite the crucial role the antennae play in insects host location and recognition (Anderson *et al.* 2000; Isidoro *et al.* 2001; Kristoffersen *et al.* 2006), as well as mating behavior (Bartlet *et al.* 1994; Romani *et al.* 2008). Few morphological reports carried out by scanning electron microscopy (SEM) techniques are present on antennae of Cercopoidea (Liang and Fletcher 2002; Liang and Webb 2002; Paladini *et al.* 2010; Hamilton 2012; Paladini *et al.* 2015). However, these studies are focused mainly on phylogeny and taxonomy, and no ul-

trastructural analysis of the antennal sensilla is presented for an exhaustive functional study. In this paper, we present the distribution, typology and fine structure of the antennal sensilla of *P. spumarius* males and females. The study was conducted using SEM (scanning electron microscopy) and TEM (transmission electron microscopy). The results are a starting point to unveil fine structural details that may be useful for future studies in taxonomy, phylogeny and physiology on the Aphrophoridae family.

Materials and methods

Insects.

Adults of *P. spumarius* were caught on its known plant hosts (Halkka *et al.* 1967; Whittaker 1968; Nickel 2003) in some olive orchards of the Ancona district (central-eastern Italy), using a modified leaf blower (Tanaka Togyo Co., THB-2510) in which the intake port was fitted with a fine mesh organza bag. Captured specimens were positioned in a cage (Bugdorm-I, Megaview) with wet paper and fresh host plants (lemon balm and clover) shoots, until arrival at the laboratory.

Scanning Electron Microscopy (SEM).

Thirty individuals of each sex were used for the observations. Insects were anaesthetized by exposure to cold temperatures (−18 °C) for 60 sec, then they were dip in alcohol 60%. Individuals were dissected removing the antennae from the head capsule, in some cases the whole head with the antennae in their natural position was detached from the rest of the body. In most individuals, flagella were detached from pedicels to obtain better dehydration. Specimens were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min each. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma®) and the specimens were allowed to dry under a hood, at room conditions; this step was repeated twice. On each aluminum stub, 5 samples were mounted, taking care to place them with different orientations in order to obtain a clear view of the

ventral, dorsal and lateral sides. Mounted specimens were gold-sputtered using a 'Balzers Union® SCD 040' unit (Balzers, Vaduz, Liechtenstein). The observations have been carried out using a FE-SEM Zeiss® SUPRA 40 (Carl Zeiss NTS GmbH, Oberkochen, Germany) operating at 7-10 KV, WD 9-10 mm and analyzed by a SMART-SEM® software.

Transmission Electron Microscopy (TEM).

Twenty individuals of each sex were anesthetized by exposure to cold temperatures (−18 °C) for 60 sec, then immediately immersed in a solution of glutaraldehyde and paraformaldehyde 2.5% in 0.1M cacodylate buffer +5% sucrose, pH 7.2-7.3. Each antenna was detached from its base, cross-cutted in the middle to facilitate fixative penetration, and left at 4 °C for 2 h. The specimens were kept at 4 °C overnight in 0.1M cacodylate buffer +5% sucrose, pH 7.2-7.3, then the specimens were post fixed in 1% OsO_4 (osmium tetroxide) for 1 h at 4 °C and rinsed in the same buffer. Dehydration in a graded ethanol series from 60% to 99%, was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Thin sections were taken with a diamond knife on a LEICA ULTRACUT R ultramicrotome (Leica®), and mounted on formvar coated 50 mesh grids. Then, sections on grids were stained with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature). Finally, the sections were investigated with a Philips® EM 208. Digital pictures (1376 x 1032 pixels, 8b, uncompressed greyscale TIFF files) were obtained using a high resolution digital camera MegaViewIII (SIS®) connected to the TEM.

Results

General description.

In *P. spumarius*, the antennae are located in the transition zone between fronto-clypeus and the compound eyes, and they point externally (Fig. 1A). In both male and female, the antenna is about 820 µm long, and is composed of three segments: a short cone-shaped scape (SA) (length about 140

µm) connecting the antenna with the head capsule, a cylindrical pedicel (length about 120 µm) (Fig. 1B) and a long, thread-like flagellum (length about 750 µm). The pedicel (PE) it is slightly narrower at the base and it shows a single campaniform sensillum (CAS) in the concave apex (Fig. 1B). The flagellum (FL) consists of a single antennomere, and it can be divided into a proximal bulb-shaped part (ampulla) and a distal thread-like segment (arista) departing from the base (Fig. 1A-C). Ultrastructural investigations of the antennal flagellum revealed the presence of 3 types of sensilla: a scolopidium, 3 identical basiconic sensilla (BS) and 12 coeloconic sensilla (CS) belonging to 2 morphologically distinct types (Fig. 1C). The morphological data of the scolopidium are not reported here because they will be the object of an ongoing study focused on the characterization of this structure and the Johnston's organ (JO). Twelve CS are located in a semi-circular pattern around the basiconic pegs, their position may vary slightly from an individual to another and they appear like a peg arising inside a deep cavity. No sexual dimorphism has been observed in the antennal structures.

Basiconic Sensilla (BS).

Basiconic sensilla (BS) are located near to the base of the arista and they are inserted on the antennal wall through an inflexible socket (ø16 µm). The length of the peg is about 40 µm, the base diameter is about 8 µm and they are oriented toward the arista (Fig. 2A). The cuticular wall is externally smooth, multiporous and of the thin type (thickness of about 0.5 µm) (Fig 2B-C). Cross sections proximal to the sensillum socket show a number of 27 sensory neurons (SN) organized into 9 neuronal units (NU). Each NU is composed of 3 SN enclosed in a dendrite sheath (DS) (Fig. 2E-F). Cross and longitudinal sections through the medial region of BS show dendrite enlargements (DE) (ø1-3 µm), from which dendritic branching processes arise (Fig. 2C-D). Furthermore, projections from the accessory cells (AC) enter in the lumen up to the tip of the BS (Fig 2B-E), where dendrites are located close to the cuticular wall (Fig 2B).

Coeloconic Sensilla (CS)

. SEM and TEM images of the bulb of the flagellum, revealed the presence of 12 cuticular openings with a single peg inserted into the bottom of the sub-cuticular chamber (Fig 1C). The external apertures present a ring of short, not innervated cuticular projections (CPR) pointing towards the aperture lumen (Fig. 3A). Ultrastructural analysis show the presence of 2 distinct types of CS: Double Walled Coeloconic Sensilla (DW-CS) and Thick Walled Coeloconic Sensilla (TW-CS). A total number of 8 DW-CS and 4 TW-CS per antenna were observed, distributed in a semi-circular pattern around the BS.

0.0.1 Double Walled Coeloconic Sensilla (CS).

Double walled coeloconic sensilla are the most represented sensilla on the antennae. The cuticular shaft is grooved in its distal part and it ends in a blunt tip (Fig. 3A-B-C). The peg length is about 2-3 µm, the diameter taken at the basal level is about 2.2 µm, decreasing towards the distal part. Cross and longitudinal sections revealed the typical double-walled organization. Apically, 8-14 cuticular ridges (CR) are present (Fig. 3B), while the proximal half of the cuticular shaft is smooth externally (Fig. 3C). Several cuticular pores (CP) organized in spoke channels are located in between the ridges (Fig. 3B). The base of the sensillum has no socket, being inflexibly inserted at the bottom of the cavity (Fig. 3A-E). Cross and longitudinal sections show distally 3 SN entering the lumen without branching (Fig 3B-C-D). Proximally, each sensillum presents 2 cuticular chambers: the innermost chamber (IC) (øabout 1.2 µm) is occupied by the outer dendritic segments (Fig. 3C-D), and an outermost chamber (OC) that is filled with electrondense material and small electronlucid vesicles (EV) (Fig. 3B-C-D). At the basal level, and before entering the peg, there is a fourth SN, not entering the sensillum lumen. Below the basal level, the 4 dendrites innervating this sensillum are enclosed by an electrondense DS (Fig. 3F).

Thick Walled Coeloconic Sensilla (TW-CS).

Thick walled coeloconic sensilla are set inside cuticular chambers that resemble closely those described in DW-CS. The length of the peg is about 2-3 µm and it presents an aporous cuticular wall (CW) (Fig. 4A). The CW has a thickness of about 0.6 µm. Internally, the sensillum lumen shows 2 SN embedded by an electrondense DS (Fig. 4B). Cross sections taken at the base level and just below the CS chamber, revealed the presence of another SN ending at the sensillum base (Fig. 4C-D).

Discussion

The antennal gross morphology of *P. spumarius* reveal the presence of short scape and a cylindrical pedicel, while the flagellum is long and slender. This typical organization has been observed in several groups among Auchenorrhyncha Section (Hemiptera: Homoptera): Achilixiidae (Liang 2001), Fulgoridae (Lewis and Marshall 1970), Delphacidae (Aljunid and Anderson 1983), Cixiidae (Shih and Yang 1996; Romani *et al.* 2009), Kinnaridae (Liang 2002), Meenoplidae (Bourgoin and Deiss 1994), and Cicadellidae (Howse and Claridge 1970, Rossi Stacconi and Romani 2012). In particular, as regards the Cercopidae, there are numerous morphological features in common with *P. spumarius*' antennal organization (Liang 2001b; Liang and Fletcher 2002; Liang and Webb 2002). In *P. spumarius*, the pedicel bears a single campaniform sensillum while the flagellum, as expected, has the highest density of sensilla (Zacharuk 1985) showing a total of 16 sensory structures belonging to 4 different types. The 3 basiconic sensilla show the typical olfactory organization: porous sensillar wall and highly branched dendrites (Steinbrecht 1984; Romani *et al.* 2009). These sensilla moreover show 9 groups of sensory neurons. Before entering the sensillar lumen, these units are made up of 3 sensory neurons bound together, and surrounded by a dendrite sheath produced by the thecogen cell. This peculiar organization was already reported in olfactory basiconic sensilla in Homoptera species belonging to planthoppers (Riolo *et al.* 2012; Rossi Stacconi and Romani 2012) as well as in Heteroptera (Romani and Rossi

Stacconi 2009). The 12 coeloconic sensilla located on the basal bulb-like enlargement, are not equally represented, being divided in 2 different groups. The DW-CS group, is composed by 8 sensory structures belonging to the multiporous grooved peg sensilla (MPG) group (Zacharuk 1980). They can be associated with thermo-chemosensory receptors, thermo-hygro receptors (Altner and Prillinger 1980), or more often to olfactory receptors (Pophof 1997; Diehl *et al.* 2003; Pophof *et al.* 2005). The TW-CS group are aporous sensilla (Altner *et al.* 1977) and they are closely related to those described in many other insect orders (McIver 1973; Boo and McIver 1975; McIver and Siemicki 1976; Altner *et al.* 1977; Altner *et al.* 1978; Altner *et al.* 1981; Steinbrecht 1994;). The 3 sensory neurons inside the sensillum are probably associated with cold, dry and moist detection, (Yokohari 1999 and references therein) moreover these sensilla are usually present in very low number. The fine structural features we found for these sensilla in *P. spumarius* are consistent with a thermo-hygroreceptive function (Tichy and Loftus 1996; Yokohari 1999). *P. spumarius* shows a strong reduction in antennal sensory equipment if compared to other leafhopper and planthopper species. Despite that, this characteristic was already observed in the leafhopper species *Scaphoideus titanus*, the vector of the phytoplasma responsible of Flavescence Dorée, one of the most important Grapevine Yellows in Europe (Rossi Stacconi and Romani 2012). Observations on *S. titanus*, that is monophagous on *Vitis* sp. suggest that the plant host range could be connected with the number of sensilla dedicated to olfactory host detection, so a few number of olfactory sensilla could be related with a little host range (Rossi Stacconi and Romani 2012). Therefore the strong reduction in *S. titanus* olfactory sensilla (and sensory neurons) could be explained in terms of specialization toward a few specific odorants emitted by a single host plant (Rossi Stacconi and Romani 2012). Other morphological observations performed in the leafhopper species *Hyalesthes obsoletus* Signoret, revealed a strong reduction in flagellar putative olfactory structures that are mainly located in the pedicel (Romani *et al.* 2009), this could be consistent with its little (but not monophagous) host plant range. *P. spumarius* seems not to confirm this general rule deducted by previous morphological and biological clues. This insect shows a low number of antennal olfactory

structure (both in pedicel and in flagellum) and an extremely wide plant host range (Halkka *et al.* 1967; Whittaker 1968; Nickel 2003). All these insights led us to hypothesize that in *P. spumarius* olfactory cues could not be as important as visual and vibrational stimuli during host plant location, compared to Cicadellidae and Ciixidae (Patt and Sétamou 2010; Riolo *et al.* 2012). It is also possible that the few olfactory receptor and sensory neurons could have a very low specificity. It is suggested that xylem sap-feeding insects tend to be polyphagous because, due to the low nutritional value of their food, a narrow host plant range would limit the choice of the momentarily most nutritional host (Nickel 2003). In this context, the presence of few olfactory structures could be explained in terms of a pronounced low selectivity for potential host plants. Moreover, at present there are no evidences for pheromonal communication in any Aphrophoridae or Cercopidae species, except for a self-regulatory pheromone controlling the aggregation in the spittlebug nymphal stages of *Callitettix versicolor* (Hemiptera: Cercopidae) (Chen and Liang 2015). Specific electrophysiological studies will be necessary to evaluate the sensitivity of the antennal olfactory sensilla to host-derived chemical volatiles. In many Auchenorrhyncha species, acoustic signals in vibrational communication are fundamental (Hunt *et al.* 1992; Hunt 1994; Cocroft 1998; Machado *et al.* 2001; Mazzoni *et al.* 2010; Virant-Doberlet and Zezlina 2007) and they can drive the detection of predators, mate and hosts through intraspecific communication. The presence of a single scolopidium located in the antennal flagellum of *P. spumarius*, as well as of the Johnston's organ inside the pedicel (Ranieri *et al*, unpublished data) strongly support a key role played by the vibratory stimuli. A similar organization was observed in other Homoptera species, such as *S. titanus* and *H. obsoletus* (Romani *et al.* 2009; Rossi Stacconi and Romani 2012). The presence of a large Johnston's organ (JO) located within the pedicel and tightly connected with the flagellum socket was reported for three Homoptera species in a comparative study (Rossi Stacconi and Romani, 2013). The combined presence of the single scolopidium, the Johnston's organ, the flexible socket of the flagellum and its thread-like structure led to the hypothesis that the entire flagellum could act as a mechanoreceptor possibly involved in the detection of air or substrate-born vibrations. The

characterization of the JO and the flagellar scolopidium in *P. spumarius* will be the subject of an ongoing study aimed to unveil the importance of vibratory stimuli in the biology of this species. In conclusion, *P. spumarius* antennae show a strong reduction as regards the number of sensilla, particularly olfactory sensilla, while the general structure of the antenna follows the general bauplan already reported in other Cicadomorpha and Fulgoromorpha species, for which a key role of vibratory signals was demonstrated. These findings will be a valuable starting point to better understand the eco-physiology of this harmful species in order to develop effective and sustainable control strategies.

Figures

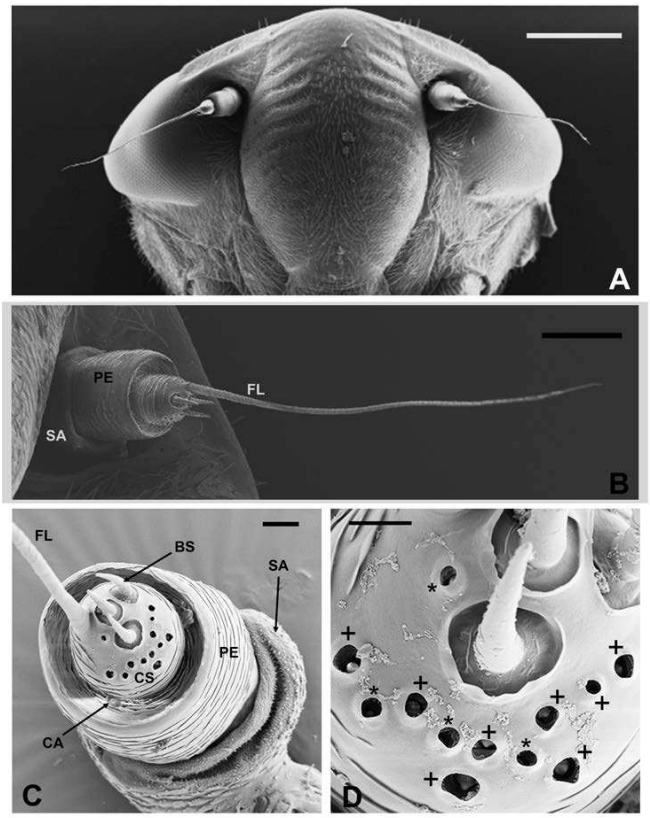

Figure 1: SEM images of *Philaenus spumarius* female antenna A) Insertion of the antenna in the head capsule showing its relative position. B) General view of a right antenna from the top showing the 3 segments: the scape (SA), the pedicel (PE) whith a single campaniform sensillum (CA) and the base of the flagellum (FL) showing the position of the basiconic sensilla (BS) and the coeloconic sensilla (CS). C) Basal bulb-like enlargement of the flagellum with the distribution of the coeloconic sensilla: '+' Double Walled Coeloconic Sensilla (DW-CS) and '*' Thick Walled Coeloconic Sensilla (TW-CS). Scale bar: A: 100 µm; B: 20 µm; C: 10 µm.

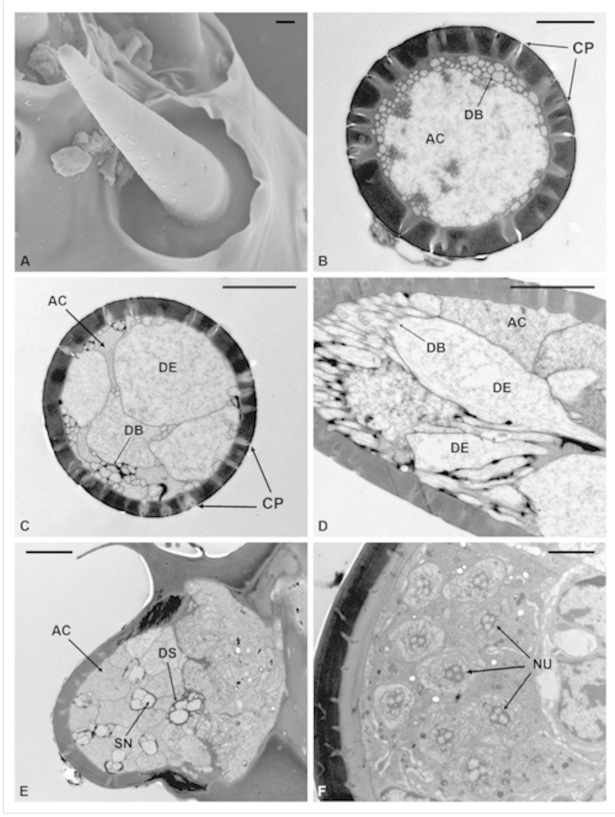

Figure 2: SEM and TEM images of Basiconic Sensilla (BS) of *Philaenus spumarius* female antenna. A) SEM image of the BS in his socket. B) Cross section of the tip of the BS showing cuticular pores (CP), dendrites branches (DB) located close to the internal cuticular wall and an accessory cell (AC) projection inside the sensillar lumen. C) Cross section of the BS in a proximal point showing CP, DB, AC projections and dendrite enlargments (DE). D) Oblique section of the BS showing the sensory neurons entering the sensillar lumen toghether with the AC, some DE and the starting of DB. E) Cross section of the BS at the socket insertion level showing groups of 3 sensory neurons (SN) embedded by dendrite sheath (DS) and separated by AC. F) Cross section of the bulb below BS socket showing the 9 groups of neuronal units (NU) (3 sensory neurons each) innervating the BS. Scale bar: A-C-D-E-F: 2 µm; B: 1 µm.

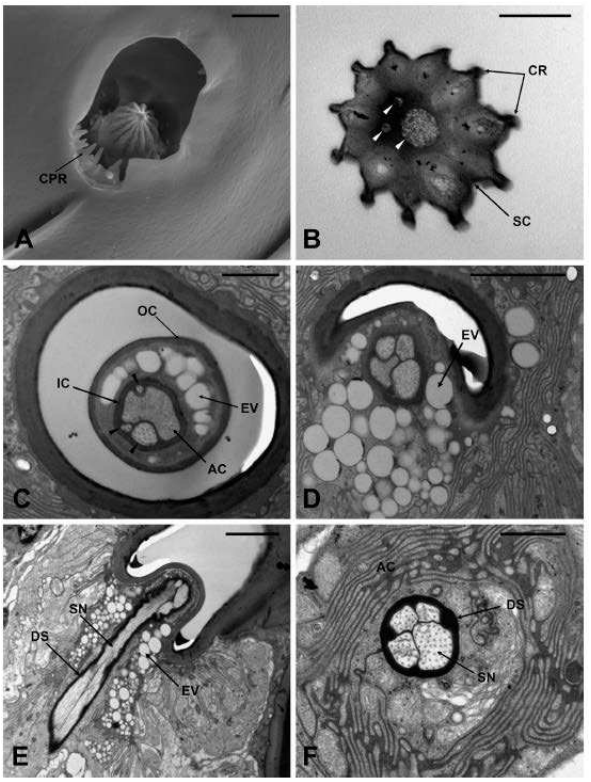

Figure 3: SEM and TEM images of Double Walled Coeloconic Sensillum (DW-CS) of *Philaenus spumarius* female antenna. A) SEM Image of DW-CS showing the peg set in the pit, the grooved distal part and the cuticular projections (CPR). B) Cross section of the distal part showing cuticular pores (CP) organized in spoke channels between the cuticular ridges (CR) and the 3 sensory neurons (SN) filling the sensillar lumen. C) Cross section of the basal part of the DW-CS showing the 2 chambers. The inner chamber (IC) presents 3 SN and a single accessory cell (AC), the external chamber (OC) shows electronlucid vescicles (EV) dispersed in an electrondense matrix. D-E) Cross and longitudinal sections of the sensillum at the socket level. Neurons embedded by a dense dendrite sheath (DS) and numerous EV can be observed. F) Cross section of the bulb below the DW-CS showing 4 SN embedded by the DS, surrounded by AC. Scale bar: A-D-E-F: 2 µm; B: 0.5 µm; C: 1 µm.

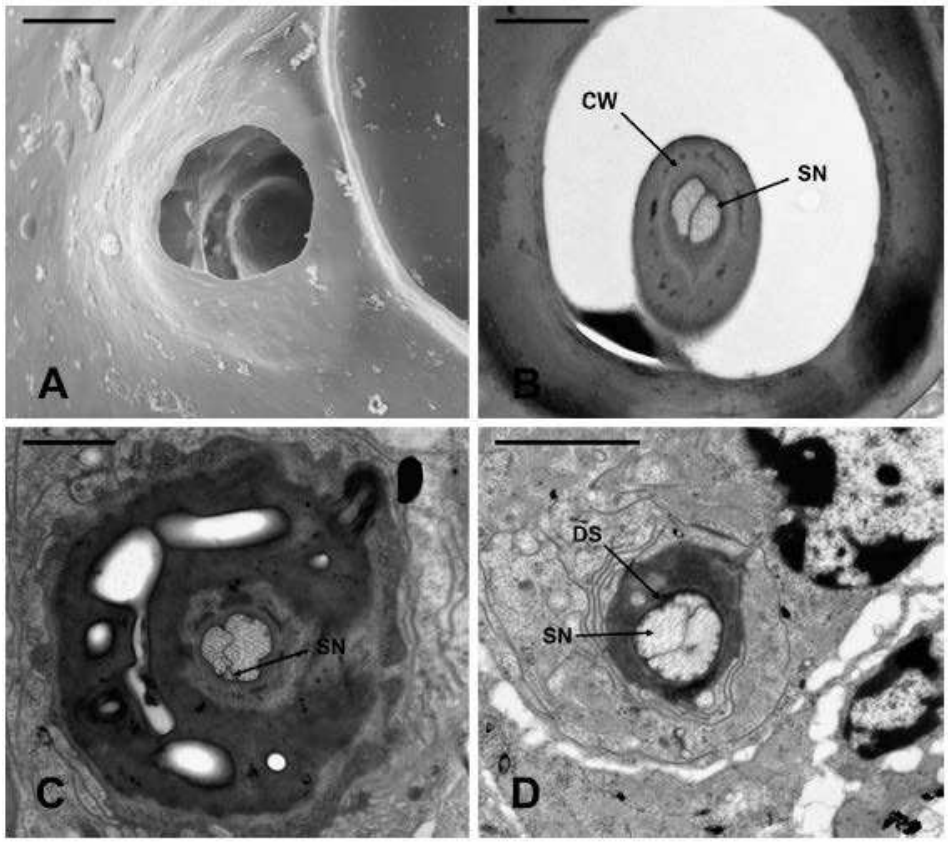

Figure 4: SEM and TEM images of Thick Walled Coeloconic Sensillum (TW-CS) of *Philaenus spumarius* female antenna. A) SEM image of TW-CS showing the aporous peg in pit. B) Cross section of the distal part of the peg showing the 2 sensory neurons (SN) filling the sensillar lumen and the thick cuticular wall (CW). C) Cross section through the socket of the TW-CS, the tip of the third SN is present. D) Cross section of the bulb below the TW-CS showing 3 SN embedded by the dendrite sheath (DS). Scale bar: A-D: 2 µm; B-C: 1 µm.

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Chapter 2

Sensory Receptors Associated with Labial Tip and Precibarium of the Meadow Spittlebug, *Philaenus spumarius*.

DRAFT

Introduction

Homoptera (Insecta: Hemiptera) are very important phytophagous insects in many agricultural systems. Phytophagous insects feeding behavior includes different steps, that play a critical role to localize and accept/reject the host plant. From the dispersal in the environment to the feeding, an insect has to go through a catenary process generally made of three steps: i) searching, ii) contact-evaluation and iii) acceptance (Schoonhoven *et al.*, 2005). The first step of plant searching includes a series of behaviors ranging from random search in the environment to very specific patterns, mainly mediated by visual and olfactory stimuli (Bernays and Chapman, 1994). Indeed, Homoptera, generally rely on these stimuli during orientation to host plant. Visual attraction has been studied in Aphidoidea (Moericke, 1969), Alerodidea (Coombe, 1982) and Psyllidae (Wenninger *et al.*, 2009). The role of olfaction was also described (Campbell *et al.*, 1993; Nottingham *et al.*, 1991), in particular, specialized sensory receptors for volatile cues have been extensively studied in the antennae of different species (Bourgoin and Deiss, 1994; Lewis and Marshall, 1970; Ranieri *et al.*, 2016; Riolo *et al.*, 2012; Romani *et al.*, 2009). The second step of contact-evaluation of the host includes tactile and gustatory information. The perception of these stimuli is achieved by specialized sensory structures, located mainly on the mouthparts (Backus, 1985; Backus, 1988). As regards the mouthparts, several studies were conducted to investigate their functional morphology in several Homoptera, i.e. Aphidoidea (Forbes, 1969), Psyllidae (Garzo *et al.*, 2012; Liang *et al.*, 2013), Aleyrodidae (Rosell *et al.*, 1995; Walker and Gordh, 1989), Cicadellidae (Leopold *et al.*, 2003; Zhao *et al.*, 2010), Pseudococcidae (Le Ru *et al.*, 1995), Aphrophoridae (Wang *et al.*, 2015), Delphacidae (Dai *et al.*, 2014; Foster *et al.* 1983) and many other Fulgoromorpha (Brozek and Bourgoin, 2013). Host acceptance is the last fundamental step, that preceeds feeding. Homoptera base the final decisions to accept or reject the host on chemical cues at the level of individual plant cell types (Tjallingii, 1992; 1995; Tjallingii and Esch, 1993; Pompon *et al.*, 2011). Thanks to their highly specialized mouthparts, Homoptera are tissue-specialists piercing-sucking insects, able to discern between dif-

ferent feeding substrates (phloem, xylem and mesophyll). The perception of these stimuli involves the contribution of an internal chemosensory system, mainly located in the precibarium (foreguts) (Backus and McLean, 1985). These sensilla, located both in epypharinx and hypopharynx, were also described in different species such as Aphidideae (Mclean and Kinsey, 1984), Psyllideae (Ullman and McLean, 1986) and Cicadellidae (Backus, 1985; Backus and McLean, 1983, 1982). Overall, this apparatus warn the insect on food availability, quality and composition. Whenever the insect's physiological requirements are fulfilled, feeding may start. A complete morpho-histological study on sensory structures located in the labial tip and precibarium of Aphrophoridae (Insect: Homoptera) is missing. *Philaenus spumarius* L. is the most widespread species belonging to this latter group. Recently, the interest for this insect has rapidly grown since it was recognized as the main Italian vector of *Xylella fastidiosa*, the bacterial strain responsible for the 'Olive Quick Decline Syndrome' in the Salento peninsula (South-east Italy) (Saponari *et al.*, 2014). *X. fastidiosa* has a unique transmission biology: it attaches and multiplies in the vectors' cibarium and precibarium without being circulative in the hemolymph (Hill and Purcell, 1995). A cyclical change of the abundance of *X. fastidiosa* cells over time in the area of distal precibarial sensilla was also observed in the American sharpshooter vector *Graphocephala atropunctata* (Backus and Morgan, 2011). Given the behavioral importance of these sensory structures (Backus and McLean, 1985), this peculiar bacterial colonization/dislodgement, happening in the foregut of competent vectors, underlines the importance of this area in the biological cycle of the pathogen. In the present work, the functional morphology of labial tip and precibarial sensilla of *P. spumarius* was described through scanning and transmission electron microscopy. The presented results are important to better understand *P. spumarius* host plant selection and feeding behavior, as well as the transmission mechanisms of *X. fastidiosa* by this vector.

Materials and methods

Insects.

Adults of *P. spumarius* were caught from hosts plant (Halkka *et al.*, 1967; Whittaker, 1968; Nickel, 2003) in fallow fields of the Ancona district (central-eastern Italy), from May till June, using a modified leaf blower (Tanaka Togyo Co., THB-2510). Captured specimens were collected in a cage (Bugdorm-I, Megaview) with wet paper and fresh host plant shoots, until arrival at the laboratory.

Scanning Electron Microscopy (SEM).

Twenty individuals of each sex were used for the observations. Insects were anaesthetized by exposure to cold temperatures (−18 °C) for 60 s, then they were dipped in 60% alcohol. Individuals were prepared removing the labium tip and dissecting hypopharynx from epypharynx. Specimens were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min each. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma®) and the specimens were allowed to dry under a hood, at room conditions; this step was repeated twice. Five samples were mounted on each aluminum stub, taking care to place them with different orientations in order to obtain a clear view of the ventral, dorsal and lateral sides. Mounted specimens were gold-sputtered using a 'Balzers Union® SCD 040' unit (Balzers, Vaduz, Liechtenstein). The observations were carried out using a FE-SEM Zeiss® SUPRA 40 (Carl Zeiss NTS GmbH, Oberkochen, Germany), operating at 10 KV and analyzed by a SMART-SEM® software.

Transmission Electron Microscopy (TEM).

Twenty individuals of each sex were anesthetized by exposure to cold temperatures (−18 °C) for 60 s, then immediately immersed into a solution of glutaraldehyde and paraformaldehyde 2.5% in 0.1M cacodylate buffer ∼5% sucrose, pH 7.2-7.3. Each labium tip was detached from the rest of the body. The samples were kept at 4 °C overnight in 0.1 M cacodylate buffer

~5% sucrose, pH 7.2-7.3, then the samples were post-fixed in 1% OsO_4 (osmium tetroxide) for 1 h at 4 °C and rinsed in the same buffer. Dehydration in a graded ethanol series from 60% to 99% was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Thin sections were taken with a diamond knife on a LEICA ULTRACUT R ultramicrotome (Leica®), and mounted on formvar coated 50 mesh grids. Then, sections on grids were stained with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature). Finally, the sections were investigated with a Philips® EM 208. Digital pictures (1376 X 1032 pixels, 8b, uncompressed greyscale TIFF files) were obtained using a high resolution digital camera MegaViewIII (SIS®) connected to the TEM.

Results

Labium Tip.

Philaenus spumarius shows typical piercing-sucking mouthparts composed by a labrum (LR), a three-segmented tube-like labium (LB) and a stylet fascicle (SF) consisting of two mandibular and two maxillary stylets (Fig. 5A). The labium ventral surface is bisected by a deep labial groove that extends to its entire length, and forms two lateral lobes at the tip where 5 different sensilla are present: sensilla trichodea type 1 (T1) and type 2 (T2), sensilla chaetica type 1 (C1) and type 2 (C2) and sensilla basiconica (B) (Fig. 5B-C). Sensillum T1 is remarkably bigger than T2, ~100µm and ~40µm in length respectively. Both T1 and T2 are aporous, characterised by an elongated and slightly grooved cuticular shaft decreasing in diameter from the base to the tip (Fig. 6A). TEM images show a thick-walled sensillum innervated by a single sensory neuron. The outer dendritic segment of the neuron, enclosed in a dendrite sheath, ends at the base of the sensillum in a tubular body attached to the joint membrane (Fig. 6B). T1 are 4 structures positioned laterally on the labium tip and coupled with 2 T2 ventrally positioned (Fig. 6A); T2 are also present more proximally to the labial groove and dorsally on the sensory complex (Fig. 5B-C). On the tip of the labium, near the groove, there are two sensory complexes (SC), one

per each side of the labium. These area present several sensory structures, arranged according to the following sensilla formula: 4+4+1 (C1,C1,C2,B + C1,C1,C1,C2 + T2) (Fig. 5C-7D). C1 and C2 are characterised by an elongated cuticular shaft that is inserted into the labium through a flexible socket (Fig. 5C; 7A). The hair shaft diameter decreases towards its rounded tip. At this level, there is a single apical pore. These sensilla are about 30 Îijm in length, and they have a thick cuticular wall. Each C1 sensillum is innervated by three sensory neurons. Two of these sensory neurons enter the peg lumen as unbranched outer dendritic segments enclosed in a common dendrite sheath, reaching the tip of the shaft. The outer dendritic segment of the third sensory neuron ends at the base of the sensillum in a tubular body attached to the joint membrane (Fig. 7B-D-E-F). TEM images of C2 show that the cellular components consist of five sensory neurons. Four of these sensory neurons enter the peg lumen as outer dendritic segments enclosed in a common dendrite sheath, and these reach the tip of the shaft without branching. The outer dendritic segment of the fifth sensory neuron ends at the base of the sensilla in a tubular body at the joint membrane level (Fig. 7C-D-E-F). Sensilla B are characterised by a thin elongated cuticular shaft, which is about 15 Îijm long, covered by numerous pores distributed over its entire surface (Fig. 5C-8A). TEM images show the thin cuticular wall, pierced by numerous minute pores, and the dendritic branches of three sensory neurons (Fig. 8B-C). Proximally, the outer dendritic segments of the sensory neurons are enclosed in a common dendrite sheath and a forth neuron ends at the base of the sensillum (Fig. 7F-8D).

Precibarium.

The precibarium of *P. spumarius* is a narrow canal starting distally from the hypofaringeal extension to the cibarial chamber. It is formed ventrally by the epipharinx (Fig. 9A) and dorsally by the hypopharynx (Fig. 9B). At the level of epipharinx 18 sensilla are present separated in two groups, the precibarial valve lays between them. The first group of sensilla, distally located, is composed by 10 oval papillae sensilla (PS), about 2µm diameter, crossed by a long slit (Fig. 9C). The second group of sensilla, proxymally

located, is composed by 6 PS and two large bulbous sensilla (BS) about 2 µm in length (Fig. 9D). On hypopharynx two PS are present, inserted in two small cuticular rise proximal to the cibarium (Fig. 9B-E).

Discussion

Philaenus spumarius sensory apparatus in the mouthparts is intimately linked with the feeding behavior of the insect. There are a number of morphological similarities in the mouthparts of this insect with those of Auchenorrhyncha species previously described (Dai *et al.*, 2014; Leopold *et al.*, 2003; Wang *et al.*, 2015; Zhao *et al.*, 2010), such as the gross morphology of the mouthparts and the type of sensilla arranged at the tip of the labium. Despite that, so far, *Philagra albinotata* is the only Aphrophoridae species studied (Wang *et al.*, 2015) and it surprisingly shows very important structural and functional differences that may represent real phylogenetic diversity or they can be the result of a misunderstanding of the structures described in *P. albinotata* (i.e. broken sensilla; lack of ultrastructural analysis). The sensilla trichodea T1 and T2 in the labium tip are similar to those found in closely related species (Dai *et al.*, 2014; Leopold *et al.*, 2003; Wang *et al.*, 2015). These sensilla belongs to the aporous type, these hairs transmit the signal of their deflection to the dendrite tip connected to the articulated base, therefore acting as mechanosensory receptors. This functional hypothesis is strongly supported by the absence of pores and the presence of a single sensory neuron ending at the sensilla base in a typical tubular body (Keil, 1997). Sensilla chetica sensilla (C1 and C2) belong to the group of terminal pore sensilla already described in several arthropodes (Altner and Prillinger, 1980). The single apical pore, the unbranched outer dendritic segments inside the sensilla shaft and the presence of a sensory neuron ending in a tubular body, represent typical features of bi-modal contact-chemosensory sensilla (Zacharuk, 1980). The Sensillum Basiconicum (B) belongs to the wall pore sensilla type (Altner *et al.*, 1977; Zacharuk, 1980); the pores allow odorant molecules to diffuse inside the lumen where the outer dendritic segments are organized in several dendritic branches. These sensilla were abundantly described in many

studies as olfactory sensilla (Altner *et al.*, 1977; Altner and Prillinger, 1980). The presence of a sensory neuron with tubular body at the base of this sensillum suggests that it could have a combined mechano-olfactory function. Generally speaking, the apical part of the labium is equipped with different sensilla tuned to the perception of mechanical stimuli, as well as chemical (both gustatory and olfactory) cues. Olfactory sensilla were already described in the labium of different homopterans but often in a sub-apical position (Brozek and Bourgoin, 2013; Dai *et al.*, 2014). Wang *et al.* 2015 report no wall pores sensillum in the labium tip of the closely related species *P. albinotata*; despite that, in the same position we found the olfactive sensilla (external group of the sensory field), *P. albinotata* showed several broken structures (Wang *et al.*, 2015). The presence/absence of a putative olfactive structure in the tip could represent a very important functional and phylogenetic trait, therefore an in-depth analysis of *P. albinotata* is suggested to have a better comparative study. Precibarial sensilla (PS and BS) of *P. spumarius*, shows almost identical structures and bauplan of those of different cicadellidae species (Backus, 1985; Backus and McLean, 1983), both xylem- and phloem-sap feeders. Their role in gustatory stimuli perception was already proven by histological analysis (Backus and McLean, 1982) and by means of surgical dissection of the hepipharynx nerve (Backus and McLean, 1985). Since they have a critical role in stimuli perception associated with probing and host plant acceptance, the wide variation in feeding habits and preference expressed by those species, is probably due to the different sensitivities of neurons connected to sensillar apparatus than ultrastructural differences (Backus and McLean, 1983). In insect vectors such as *P. spumarius*, the bacterium *Xylella fastidiosa* attaches in the precibarium, potentially covering, due to biofilm formation, sensilla mediating crucial behavioural responses (Hill and Purcell, 1995). The cyclical pattern of *X. fastidiosa* observed in the D-sensilla field (Backus and Morgan, 2011) could be therefore explained in the logic of cleaning of the sensory area. Whether this cleaning is actively mediated by the insect or is the result of a change in the fluid dynamics is still unknown, but the role of these structures is crucial in understanding the mechanism of dislodgement of the pathogen from the area itself. This study provides important information to better understand

P. spumarius hostplant selection and feeding behavior. Since the efficiency of acquisition and inoculation is dependent upon selection of feeding tissue sites, this data could be of direct interest to study the transmission of *X. fastidiosa* by this vector.

Figures

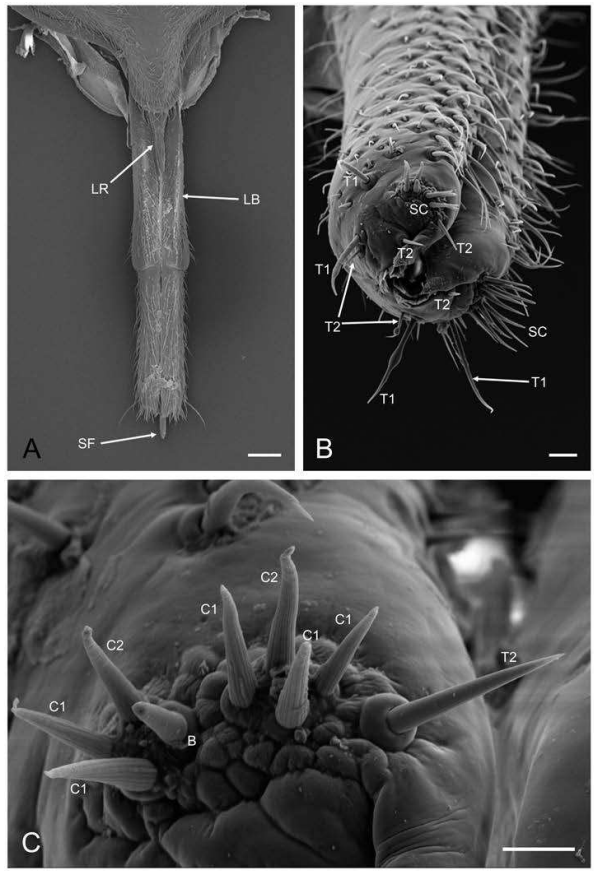

Figure 5: SEM images of *P. spumarius* female rostrum. A) Ventral view of the rostrum made up of a small triangular labrum (LR), a long labium (LB) and a stylet fascicle (SF) running inside a deep groove in the LB. B) Fronto-lateral view of the LB showing the sensilla trichodea type 1 and 2 (T1-2) and the 2 sensory complexes (SC). C) Frontal view of the right sensory complex showing the organization of the sensilla chaetica type 1-2 (C1-2), basiconica (B) and T2. Scale bar: A: 100 µm; B: 20 µm; C: 10 µm.

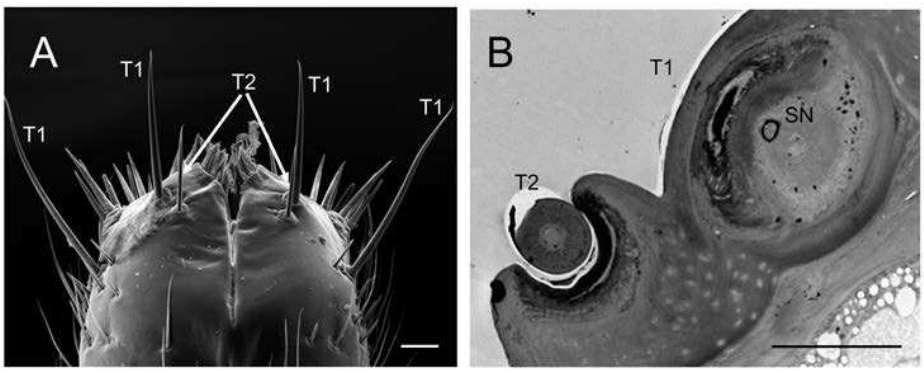

Figure 6: SEM and TEM images of *P. spumarius* female sensilla trichoidea. A) SEM image of the dorsal part of the tip of the labium showing 4 sensilla trichodea type 1 (T1) and 2 sensilla trichodea type 2 (T2). B) Cross section of the insertion level of the T1 and T2 showing the thick cuticular wall and the sensory neuron (SN) ending in a tubular body of the T1. Scale bar: A: 20µm; B: 5 µm.

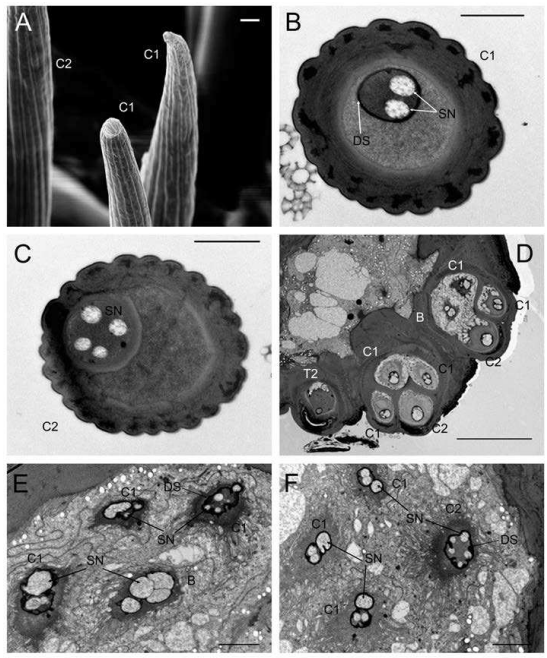

Figure 7: SEM and TEM images of *P. spumarius* female sensilla on sensory complex. A) SEM image of the sensilla chaetica type 1 and 2 (C1-C2) showing grooved surfaces and blunted tips. B) Cross section of the C1 took about in the middle of the peg, showing a thick cuticular wall grooved in the external part, 2 sensory neurons (SN) entering the lumen and surrounded by an electrondense dendrite sheath (DS). C) Cross section of the C2 took about in the middle of the peg, showing a thick cuticular surface grooved in the external part and 4 SN entering the lumen. D) Cross section of the insertion level of the 9 sensilla present on the sensory field. The micrograph shows the arrangement of the 5 C1, the 2 C2, the sensillum basiconicum (B) and sensillum tricodeum type 2 (T2). E) Cross section below the insertion level of the external group of the complex, showing the bundles of sensory neurons belongin to C1, C2 and B sensilla, each bundle surrounded by a thick dendrite sheath. F) Cross section at the inner dendritic segment level of the central group of sensilla in the complex: three C1 and a C2 sensilla. Also in this case, the sensory neurons of each sensillum are surrounded by a thick dendrite sheath. Scale bar: A-E-F: 2 µm; B-C: 1 µm; D: 10 µm.

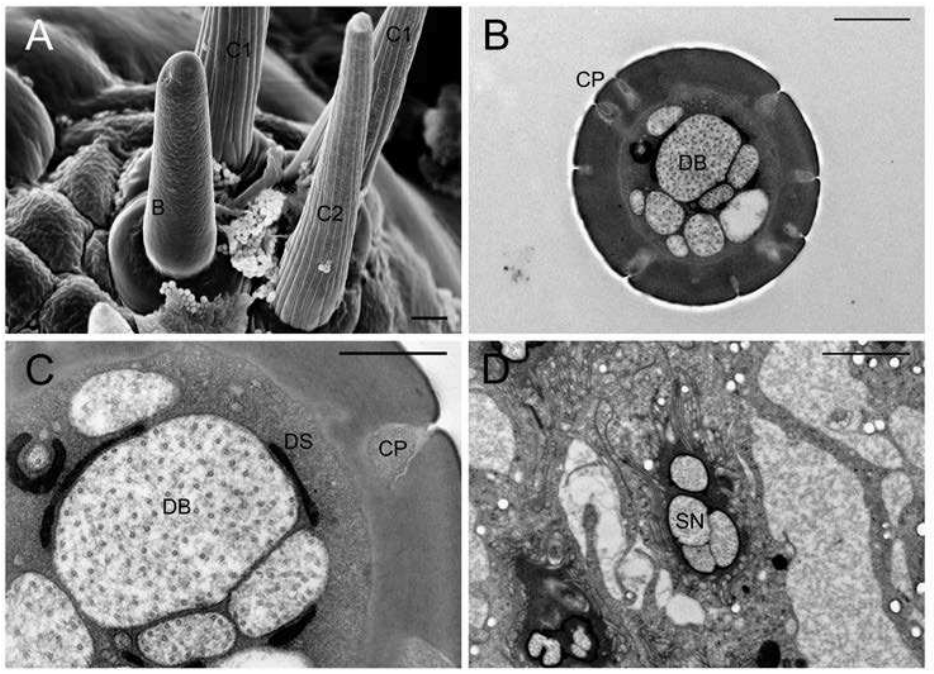

Figure 8: SEM and TEM images of *P. spumarius* female basiconic sensillum. A) SEM image of the sensilla group in the sensory complex, carrying the sensillum basiconicum (B) and sensilla chaetica type 1 and 2 (C1-C2). B) Cross section of the B took about in the middle of the peg, showing the cuticular surface with several cuticolar pores (CP) and several dendrite branches (DB) inside the lumen. C) Cross section of the B showing details of the cuticolar pores (CP) and the dendritic branches with the electrondense dendrite sheath (DS) partially enveloping the neurons. D) Cross section of the sensillum at the inner dendritic segment level showing four sensory neurons (SN) innervating the peg surrounded by the dendrite sheath. Scale bar: A-D: 2 µm; B: 1 µm; C: 0.5 µm.

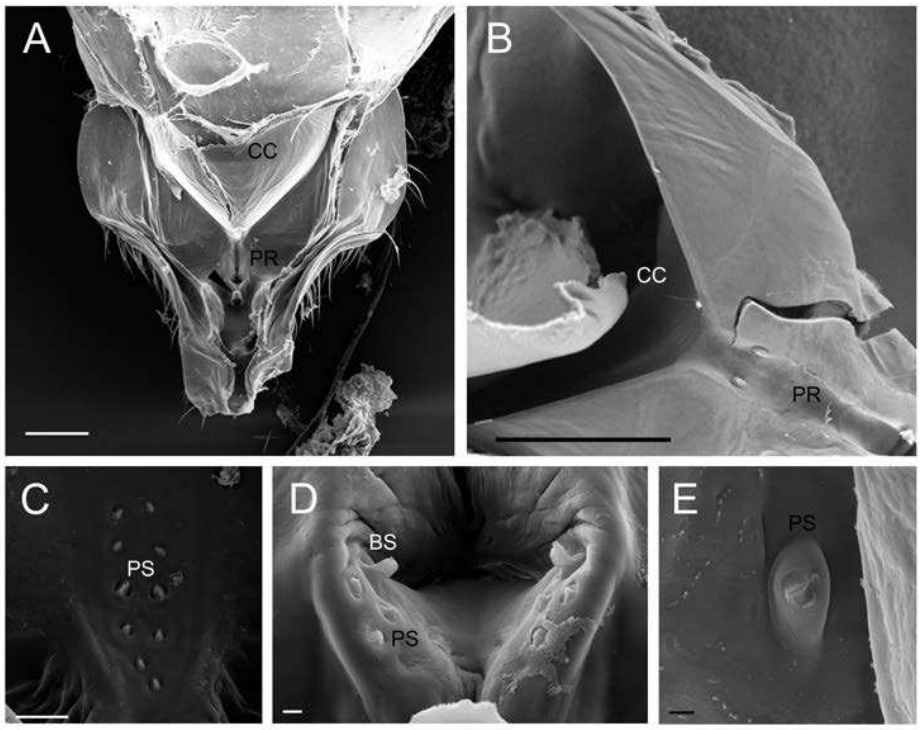

Figure 9: SEM images of precibarium of *P. spumarius* female foreguts. A) SEM image of the epypharinx showing the cibarial chamber (CC) and the precibarium (PR). B) SEM image of the hypopharynx showing the PR and the CCÂň. C) SEM image of the ten papillae structures (PS) located in the distal part of the epypharynx. D) SEM image of the six PS and the 2 bulbous sensilla (BS) located in the proximal part of the epypharynx. E) SEM image of the two PS in the hypopharynx. Scale bar: A-B: 100 µm; C: 10 µm; D-E: 2 µm.

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Chapter 3

Morphological characterization of the retention sites of *Xylella fastidiosa*. Two vectors compared: *Philaenus spumarius* and *Graphocephala atropunctata*.

PRELIMINARY STUDY

Introduction

Xylella fastidiosa is a xylem-limited gram-negative bacterium and is efficiently transmitted by many xylem sap-sucking insects (Baker *et al.*, 2015); this bacterium has a unique feature among all the pathogens spread by arthropods: it multiplies in insect foreguts without being circulative in the hemolymph (Hill and Purcell, 1995). The retention sites inside the vectors are localized in the cibarium (Hemipteran sucking pump) and the precibarium (channel connecting the stylet fascicle to the cibarial pump)(Brlansky *et al.*, 1983; Purcell and Finlay, 1979), but how exactly the insect expels bacteria from these sites is not completely understood. A recent study demonstrated the presence of a cyclical abundance pattern of colonization in the distal part of the precibarium in the sharpshooter vector Graphocehala atropunctata, (Backus and Morgan, 2011). Despite it is uncertain whether the bacteria are mostly ingested or egested, they suggest that the cibarial chamber is the reservoir of bacteria, while the precibarium represents the point from which bacteria are inoculated into the plant. The hydrodynamic model of the insect foreguts also suggests that the cibarium is the area in which cells initially attach, due to the slow speed of the fluid; then, the turbulent movement in the precibarium dislodges the bacteria when multiplication pushes the cells in this area (Rapicavoli *et al.*, 2015). This model apparently does not fit with the many investigations on the localization of *Xylella fastidiosa* in the foreguts of insect vectors. In these studies it seems, in fact, that those described as the attachment sites are the less colonized areas, while the most turbulent areas, such as the precibarium, appear to be the most colonized (Almeida and Purcell, 2006; Alves *et al.*, 2008; Brlansky *et al.*, 1983). The fluid speed model can be very important in understanding the acquisition potential and the transmission mechanisms of the pathogen but to date, a model based on real morphometric data is missing. Moreover, recent investigation on infectivity of the main Italian vector *Philaenus spumarius* shows the presence of a saturation point in the number of cells hosted by the insect. Indeed, the estimated number is from 1 to 2 orders of magnitude smaller then that found in sharpshooters vectors (Cornara *et al.*, 2016; Retchless 2014). This

limited number of cells is also considered to be a consequence of different fluid dynamics in the two species (Cornara *et al.*, 2016), but a morphological comparative study of insect foreguts has not been carried out yet. The aim of this paper is to compare, through micro computed tomography (µCT) scan, the morphometry of the foregut profile of *Philaenus spumarius* with the one of the sharpshooter vector *Graphocephala atropunctata*, in order to estimate important features such as available colonizing areas and difference in dimensions. Moreover this data will represent a starting point to build accurate model to understand the role played by hydrodynamic interactions in the *Xylella fastidiosa* acquisition and transmission.

Materials and Methods

Insects.

Philaenus spumarius and *G. atropunctata* used in µCT and Scanning Electron Microscopy (SEM) analysis were collected in January, in the laboratory of University of California - Berkeley, from healthy grapevines seedlings.

µCT Sample Preparation.

Freshly collected samples were anesthetized by exposure to cold temperatures (−18 °C) for 60 s, then immediately immersed into a solution of glutaraldehyde and paraformaldehyde 2.5% in 0.1M cacodylate buffer + 5% sucrose, pH 7.2-7.3 and left at 4 °C overnight. The specimens were then post-fixed in 1% OsO_4 (osmium tetroxide) for 1.5 h at room temperature and rinsed in 0.1M cacodylate buffer. Dehydration in a graded ethanol series from 35% to 99%, was followed by critical point drying.

Image Acquisition, Reconstruction and Analysis.

Fixed specimen were analyzed through a SkyScan1272 at the MicroPhotonics facility (Micro Photonics Inc. Allentown, PA). The beam energy was 50 KV in a current source of 200 µA. Images pixel size resolution was 1.999974

µm and pictures were captured through rotation step of 0.1° using 360° rotation. The 3D reconstruction was done using NRecon Version 1.6.10.4 and images were then analyzed through visualization softwares. Linear measurement of different tomograms were calculated in DataViewer V1.5.2.4 using no resizing factor and volume reconstruction and quantification was carried out in ImageJ binarizing the tomograms by setting grey-level threshold (Max Entropy method), above which voxels (volume-pixels) are considered part of the insect and below which voxels are considered part of the background. 3D reconstruction was analyzed by 3D viewer plugin in ImageJ, CTVox and Amira® 5.2.2 software. In the analyis, the valve area was excluded because it was considered to be a non-constant dimension area. Pictures of precibaria colonized by *X. fastidiosa* were analyzed to integrate data in calculation analysis (Almeida, unpublished data).

Scanning Eelectron Microscopy (SEM).

Thirty individuals per species from each sex were used for the observations. Insects were anaesthetized by exposure to cold temperatures (−18 °C) for 60 s, then they were dipped in 60% alcohol. Individuals were dissected removing the head capsule from the rest of the body, Specimens were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min each. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma®) and the specimens were allowed to dry under a hood, at room conditions. On each aluminum stub, 5 samples were mounted. The observations were carried out using a FE-SEM Zeiss® SUPRA 40 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and a Hitachi® TM-1000 SEM.

Statistical Analysis.

Relative Standard Deviation (RSD) was calculated on 30 individual (M:F; 1:1) of *P. spumarius* and 30 individual of *G. atropunctata*. RSD is expressed in percentage and was calculated with the linear measurement of SEM pictures.

Results

Precibarium Profile.

The precibarium (Pr) is a narrow canal starting from the hypopharyngeal extension, that inserts into the food canal formed by the stylets, and it ends in the cibarial chamber (Ci). The profile is generally narrow in the distal part while it enlarges quickly after the valve, until it connects with the Ci (Fig.10-11). In *P. spumarius* the Pr linear length is ∼215 µm (Fig. 10A-C) with a relative standard deviation of 6.9%. The volume is ∼52584 μm^3, the average diameter of the distal part (from the stylet insertion to the valve) is ∼19 µm while in the proximal part the average diameter Is ∼24.46 µm. Considering an average density of 6.7 cells per μm^2 in a fully colonized Pr, *P. spumarius* shows a potential capacity of harboring ∼66654.11 cells. The reduction of Pr mean diameter calculated after *X. fastidiosa* colonization is ∼23.75% (final ø= 14.49 µm) in the distal area and 18.67% (final ø= 19.89 µm) in the proximal one. The estimation of the Ci volume, based on linear measurement is ∼0.1538mm^3. In *G. atropunctata* the Pr linear length is ∼184 µm (Fig. 10B) with a relative standard deviation of 5.1%. The volume of the part is ∼20224 μm^3, the average diameter of the distal part is ∼11.25 µm and 16.79 µm in the proximal part. G. atropuncatata has a *X. fastidiosa* harbor capacity of ∼36156.43 cells. The reduction of Pr mean diameter calculated after bacterial colonization is ∼39.39% (final ø= 6.82 µm) in the distal area and 26.80% (final ø= 12.29) in the proximal one. The estimation of the Ci volume is ∼0.087mm^3.

Discussion

Xylella fastidiosa attaches to, and persistently colonizes the cuticular lining of the foregut of insect vectors (Purcell *et al.*, 1979). Our series of morphometric comparisons between *P. spumarius* and *G. atropunctata* using µCT scan, indicate that *P. spumarius* could potentially harbor twice as many cells as *G. atropunctata*. These data suggest that the responsible for the bacterial population size discrepancy observed in these two vectors by

molecular analysis (Cornara *et al.*, 2016) is probably the fluid dynamics, and not cuticular surface available for *X. fastidiosa* colonization. Fluid speed in the two vectors should have be considered different due to the different morphometry we observed in cibarium and precibarium size, volume and surface sections. The speed of the intake sap it is proposed to be similar in the two species (Cornara *et al.*, 2016; Purcell *et al.*, 1979), nevertheless our data show no evidences to suppose similarities in the hydrodynamic model. On the contrary, this difference could be the base of the difference colonization of the pathogen in the two vectors. The full colonization of the precibarium, in both species, results in a considerable reduction of diameters; this reduction affects more *G. atropunctata* than *P. spumarius*, and more the distal area (between precibarial valve and stylets) than the proximal one (between cibarium and the valve). The reduction of the section diameter, by the continuity equation for the steady flow, deeply affects the speed of the fluid. Considering that, our evidences seem to be correlated with the cyclical pattern of colonization observed in the distal area of *G. atropunctata* (Backus and Morgan, 2011). The increase of the fluid speed, in fact, could produce and higher friction action against the biofilm and re-suspend part of the pathogen population. Based on previous model, the cibarium is proposed as the reservoir of bacteria due to the less turbolent movement of the fluid inside it (Rapicavoli *et al.*, 2015). Our data highlight the importance of building more accurate model, based on real morphometrical clues to study fundamental dynamics affecting the transmission of the pathogen itself. Indeed, it is not excluded that a permanent light bacterial biofilm in the precibarium may resist the uptake flow and act as a reservoir where cells multiply and are subsequently dislodged into the plant by egestion.

Figures

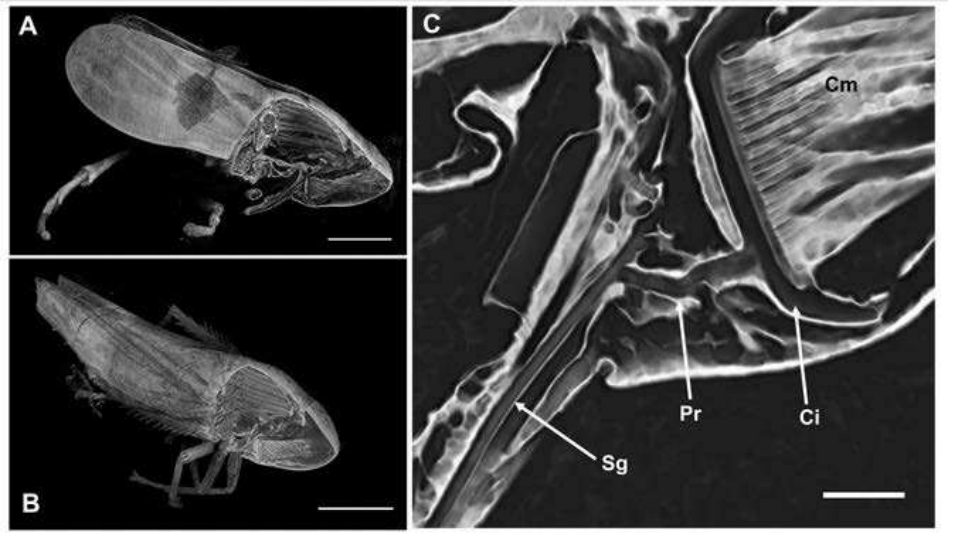

Figure 10: A) Micro computed tomography (µCT) 3D reconstruction of *Philaenus spumarius* total body with quadrat-shaped front-lateral section of the head. B) µCT 3D reconstruction of *Graphocephala atropunctata* total body with quadrat-shaped front-lateral section of the head. C) Sagittal view tomogram of *P. spumarius* showing part of head and torax. Sg = stylet groove; Pr = precibarium; Ci = cibarium; Cm = cibarial muscles. Scale bar, A,B:1mm; C: 125 µm.

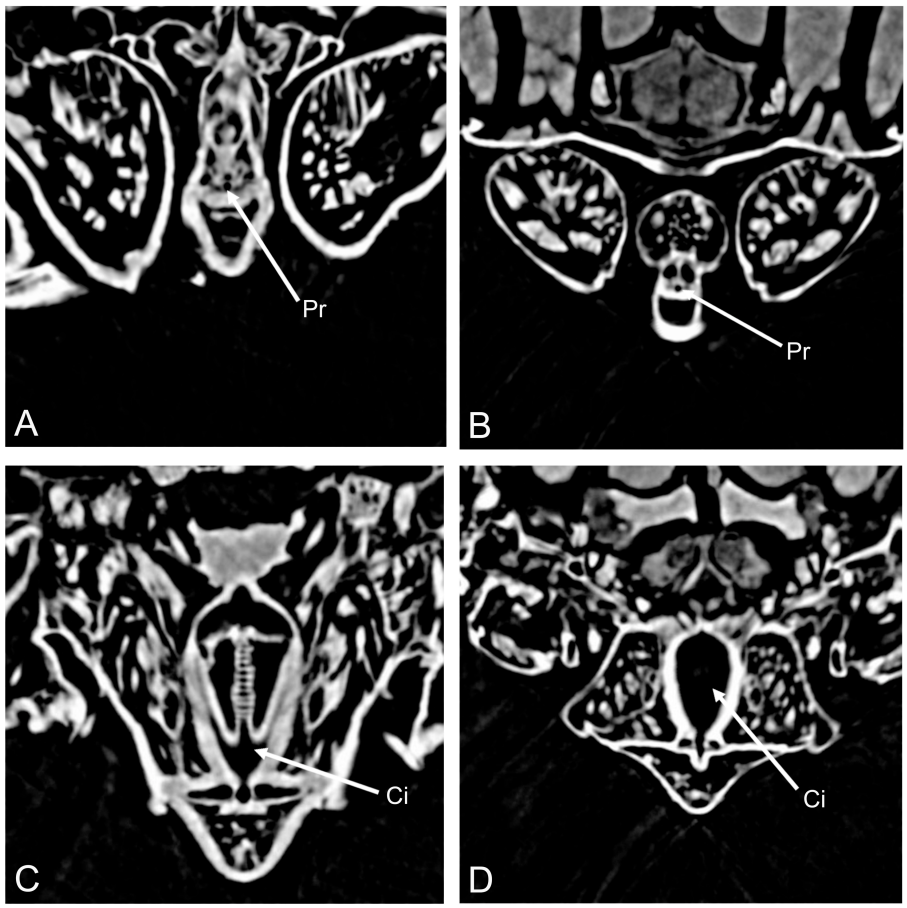

Figure 11: Transaxial view of *Philaenus spumarius* and *Graphocephala atropunctata* tomograms. A) *Philaenus spumarius* precibarium (Pr) tomogram took at the level in which hypopharyngeal extension inserts into the food canal. B) *Graphocephala atropunctata* precibarium (Pr). C) Tomogram of *P. spumarius* cibarium took at the level in which precibarium connect with the cibarium (Ci). D) Tomogram of *G. atropunctata* cibarium took at the level in which precibarium connect with the cibarium (Ci).

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Chapter 4

Functional anatomy of *Philaenus spumarius* precibarial valve.

PRELIMINARY STUDY

Introduction

In phytophagous piercing-sucking species, their particular mouthpart adaptation allows them to feed on host plants in a partially non-destructive way, by inserting stylets in specific tissues (i.e. phloem, xylem). They have a muscle-dependent pump known as cibarium, inserted in the head capsule. With inflating and compressing movements, the cibarium is responsible of the fluid uptake from the plant and the dislodgement of the sap in the esophagous. All this system works thanks to 2 valves alternatively operating. The first, known as the cardiac valve, is a pressure-dependent structure located in the esophagous, that is closed when the fluid is uptake from the plant to the cibarium. The second is a valve located in the precibarium (a canal connecting the stylet fascicle to the ciabrium) that is closed when cibarium is compressed, pushing the fluid in the esophagous. The precibarial valve in Cicadellidae is a well known structure located in the middle of the precibarium, in the epypharynx side of it. It was first described on leafhopper species *Macrosteles fascifrons* (Backus and McLean, 1982), and the operative mechanism, was described as a muscle dependent flap-like hinged structure (Ullman and McLean, 1986). Contraction of valve muscles (independent from cibarial muscles) makes the valve closes the precibarium lumen while relaxation of muscles, makes it open. Moreover, the authors suggested that the valve can also act as a pressure-sensitive check valve preventing the flow of fluids from the cibarium back into the stylets. This sort of valve functioning seems to be unique among homopterans. Morphological studies carried out on aphids (Mclean and Kinsey, 1984), psyllids (Ullman and McLean, 1986) and aleyrodidae (Hunter *et al.*, 1996) reveal the absence of such structure and valve muscles work accordingly with cibarial ones even if in an independent way. Moreover, other phytophagous sap-sucking taxa such as Thrips, show similar structure (Hunter and Ullman, 1994). In all those species the contraction of muscles makes the valve open and permit the uptake of fluids. This structure assumed a different relevance and attracted the interest of scientists, after the discovery of the precibarium as the retention site of *Xylella fastidiosa* (Hill and Purcell, 1995): a xylem limited bacterium transmitted by many xylem-sap sucking insects. The valve

was hypothesized to be responsible of the dislodgement of bacteria from the precibarium due to mechanical friction (Almeida and Purcell, 2006). To date, the many investigations through electronic microscope approaches reveal some perplexity on the functioning mechanism of this valve and many features where not sufficiently investigated. The aim of this paper is to investigate the functional anatomy of the precibarial valve of *P. spumarius* through optical microscopy to better understand its functioning.

Materials and Methods

Insects.

Philaenus spumarius used in µCT and Scanning Electron Microscopy (SEM) analysis were collected in January in the laboratory of University of California - Berkeley, from healthy grapevines seedlings breeding.

Scanning Electron Microscopy (SEM).

Thirty individuals per species from each sex were used for the observations. Insects were anaesthetized by exposure to cold temperatures (−18 °C) for 60 s, then they were dipped in 60% alcohol. Individuals were dissected removing the head capsule from the rest of the body, Specimens were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min each. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma®) and the specimens were allowed to dry under a hood, at room conditions. On each aluminum stub, 5 samples were mounted. The observations were carried out using a FE-SEM Zeiss® SUPRA 40 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and a Hitachi TM-1000 SEM.

Light Microscopy.

Twenty individuals of each sex were anesthetized by exposure to cold temperatures (−18 °C) for 60 s, then immediately immersed into a solution of glutaraldehyde and paraformaldehyde 2.5% in 0.1 M cacodylate buffer +5%

sucrose, pH 7.2-7.3. Each head capsule was detached from the rest of the body and sectioned along the fronto-clypeus sutures. Each specimen was then deep in fixative and left at 4 °C for 2 h. The specimens were kept at 4 °C overnight in 0.1 M cacodylate buffer +5% sucrose, pH7.2-7.3, then the specimens were post-fixed in 1% OsO_4 (osmium tetroxide) for 1 h at 4 °C and rinsed in the same buffer. Dehydration in a graded ethanol series from 60% to 99%, was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Semi-thin sections were taken with a diamond knife on a LEICA ULTRACUT R ultramicrotome (Leica®), mounted on slides and stained with Toluidine blue for 1 min at room temperature. Slides were observed using a model Nikon® Eclipse E600 microscope.

Results

Precibarial Valve.

The precibarial valve is a complex structure located about in the middle of epypharynx and composed of three main parts: a Knocker-Flap (KF), a Bell-like invagination (BE) and a Valve Membrane (VM). These parts act together to selectively open or close the precibarium to the fluid movement (Fig. 12). The KF is a small tab (about 20 µm of linear length) rounded in the apical part and directly connected with the BE aperture (Fig. 12A). When the valve is closed, the KF is pushed against the seat of the valve in the hypopharynx: the precibarial Valve Receptacle (VR), blocking the area. Series of longitudinal and cross sections, reveal that the BE invagination is a hollow space, hidden below the KF (Fig. 13B-C). The outermost part of the BE is a rigid circular Ring of chitin (RI; ø: 15-17 µm), while the internal part is a tube-like structure length ∼60 µm and ending in a dead-end cuticular wall. A Glandular tissue (GL) is wrapped around the thin cuticle of BE (Fig. 13C-D). The VM is a thin membrane (<2 µm), it appears like an infolding of the chitin along two axis, forming two sutures in the surface (Fig. 13B-D). The first is a Coronal Suture (CS) placed in the proximal part of the valve, connecting the thin cuticle of the VM with the thick surface of the rest of the epypharynx. The second is a Sagittal

Suture (SS) running longitudinal to the epypharynx. In the internal part, a putative Tendon (TE) connect the SS to the valve Muscles (MU) attached to the fronto-clypeus cuticle (Fig. 13B-D).

Discussion

The precibarial valve role in the egestion is nowadays under debate (Almeida and Purcell, 2006), and there is still a lack of information on the complete functioning mechanism. Our observations, opposite of previously descriptions (Backus and McLean, 1982; Ullman and McLean, 1986), suggest that when muscles are relaxed, the valve apparently closes through cuticular tension, thus the valve is normally closed. The valve is opened by a set of dilator muscles (independent from cibarial muscles) attached to the TE connecting to the internal surface of the SS. The thin cuticle of the VM and the invagination of the SS, indeed, resemble the same working mechanism proposed for aphids (Mclean and Kinsey, 1984), psyllids (Ullman and McLean, 1986) and whiteflies (Hunter *et al.*, 1996). When the valve is closed through cuticular tension against the VR, the distal part of the precibarium connects directly with the BE, forming a sort of sealing cap. The big glandular tissue surrounding the cap seems to secrete directly on the BE (Ranieri, unpublished data), but ultrastructural observations are necessary for a complete characterization of the structure. The mechanism proposed is unique among other piercing-sucking species but it seems to be present in both Cicadellidae and Aphrophoridae (Backus and McLean, 1983, 1982), both phloem- and xylem-sap sucking species, thus representing an important phylogenic and physiological traits. The morphological analysis discussed in this study are fundamental in the understanding of the biology of sap-sucking species and of the interaction between *X. fastidiosa* and the vectors' foreguts.

Figures

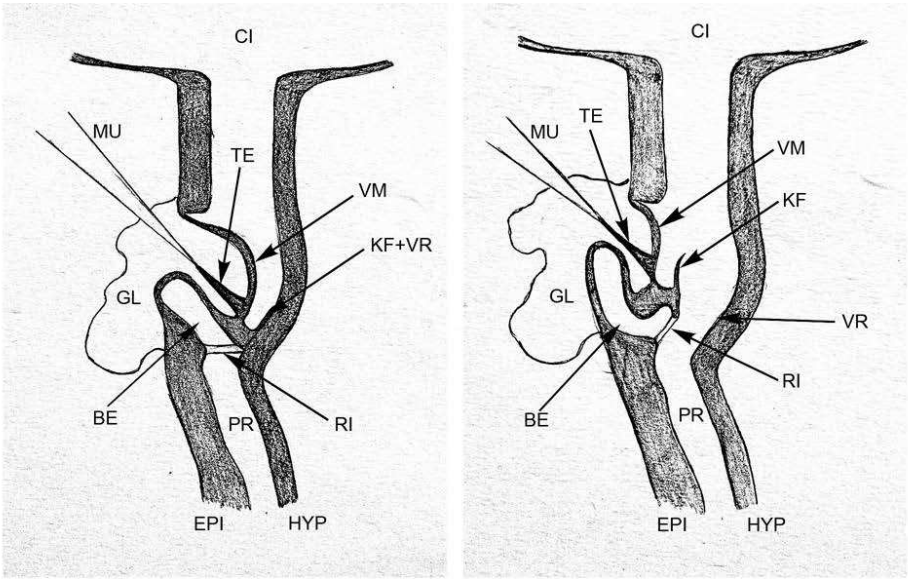

Figure 12: Stylized sagittal view of an adult of *Philaenus spumarius* precibarium, showing the operative mechanism and the different structures A) Precibarium when valve is closed; B) Precibarium when the valve is open. CI: cibarium; PR: precibarium; EPI: epipharynx; HYP: hypopharynx; MU: valve muscles; TE: tendon; GL: glande; VM: valve membrane; KF: knocker flap; VR: valve receptacle; BE: bell-like structure; RI: cuticular ring.

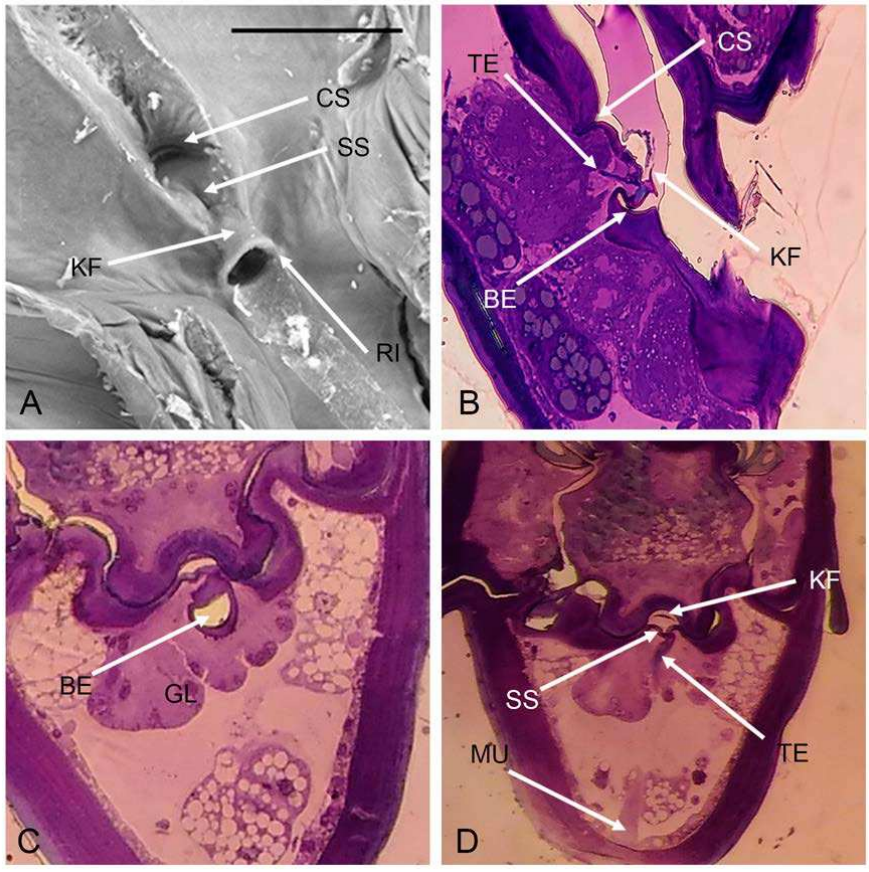

Figure 13: SEM and optical microscope pictures of *Philaenus spumarius* precibarium. A) SEM picture of *P. spumarius* valve showing both suture: Corotonal (CS) and Sagittal (SS) and the cuticular ring (RI) at the base of the knocker flap (KF). B) Optical microscope picture of a longitudinal section of the precibarium, showing the CS, the tendon (TE) connected to the valve membrane and the bell-like structure (BE) starting from the ring below the KF. C) Optical microscope cross section of the precibarium showing the gland (GL) around the BE. D) Optical microscope cross section of the precibarium, showing part of the KF, the AP attached to the SS and part of the valve muscle (VM) connected with the cuticle. Scale bar: A: 50 µm.

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Chapter 5

Spatio-temporal distribution patterns of xylem-sap feeding insects in a central Italy olive agroecosystem.

DRAFT

Introduction

Xylella fastidiosa Wells is a vector-transmitted bacterial pathogen inhabiting the xylem of many plant species (Wells *et al.*, 1987; Baker *et al.*, 2015), mostly without causing disease. In susceptible hosts, bacterial population restricts water movement by occluding xylem tissues, and highly colonized and blocked vessels are associated with disease symptom development (Newman *et al.*, 2003). Recently, the bacterium has been found in the Salento peninsula (Apulia, Italy), associated with the Olive Quick Decline Syndrome (OQDS), a rapidly spreading decline of olive trees (Saponari *et al.*, 2013; Martelli *et al.*, 2015). In Italy, this recent pathogen outbreak is considered a relevant phytosanitary threat due to the high probability of spread from established infested areas. Indeed, this risk is nowadays assessed as very likely, mainly because of the existence of a large number of confirmed or potential host plants and the abundance and widespread distribution of known or potential vectors (Baker *et al.*, 2015). *Xylella fastidiosa* interactions with insects are poorly specific, therefore, any xylem sap-feeder could be a potential vector until further contrasting evidences (Frazier, 1944; Purcell, 1989; Almeida *et al.*, 2005). So far, high specificity has been indeed reported only once between a *X. fastidiosa* strain and insect vector (Lopes *et al.*, 2009), while many other interactions were demonstrated to be very unspecific (Damsteegt *et al.*, 2006). All the xylem-fluid feeding insects belong to the Auchenorrhyncha group; among them, at present, *X. fastidiosa* vectors are reported only in three superfamilies: Cicadoidea (cicadas) (Baker *et al.*, 2015), Membracoidea (leafhoppers) and Cercopoidea (spittlebugs and froghoppers). The role of cicadas as vectors is largely unknown, as only few reports are available (Paiao *et al.* 2002; Krell *et al.*, 2007) and the uncertainty of their role is still high (Baker *et al.*, 2015). Membracoidea that feed on the xylem belong only to the subfamily Cicadellinae (sharpshooter) which is the *taxon* with the largest number of vector species identified in the Americas; in the same area, Cercopoidea species are considered to have a marginal role. Studies conducted in the Americas, where the pathogen is endemic, have provided several clues about which vector species are the most important to the disease spread, and which plants are sources of inocu-

lum of the pathogen (Redak *et al.*, 2004; Baker *et al.*, 2015). The European context is different: first, there are only few species of sharpshooters while spittlebugs are relatively more abundant (Aphrophoridae and Cercopidae) (Baker *et al.*, 2015); moreover, a good knowledge on the biology and ecology of the xylem feeders is still unavailable. In fact, many of them were not considered economically relevant until the *X. fastidiosa* outbreak in Europe. To date, the meadow spittlebug *Philaenus spumarius* is considered the main vector of the Italian strain of *X. fastidiosa* for several reasons: it is abundant in Italy, commonly found positive to the pathogen and it was the only species proven to be able to acquire and transmit the pathogen from/to different host plants and olive trees (Cornara *et al.*, 2016; Saponari *et al.*, 2014). Nevertheless, considering the complex scenario depicted by *X. fastidiosa* epidemiology, to have an accurate spread-risk assessment, would be important to evaluate both potential and confirmed vectors' role. The importance of a specific insect for spreading the disease, in fact, depends not only on their transmission ability but also on ecological factors such as habitat, host selection, vector density, mobility and spatial and temporal distribution (Purcell 1985; Almeida *et al.*, 2005). Moreover, these factors are related with insect's physiology and stimuli perception that depend on the phenological stage and sex. Cercopidaea and Cicadidae nymphs live below the ground, on the roots of different plants, thus having a very limited dispersion ability; similarly, Aphrophoridae nymphs tend to live sedentarily inside froth masses in host plants (Weaver and King, 1954; Yurtsever, 2000). On the contrary, the potential of adults' movement and migration in ecosystem, is generally higher. In addition, in many Auchenorryncha species, males are known to have high dispersal ability whereas females tend to remain sedentary on their host plant (Bosco *et al.*, 1997; Denno and Roderick, 1990). From a managing perspective, the knowledge of vectors' movement strategies in the agroecosystem plays a crucial role (Stinner *et al.*, 1983) and the lack of information about it, was the starting point for this study. In the current work, the potential vectors of *X. fastidiosa* were studied in a central Italy olive orchard. To this aim yellow sticky traps were used, since their efficiency in sampling adult leafhoppers was widely proven in previous studies (Bosco *et al.*, 1997; Lessio *et al.*, 2007; Oren-

stein *et al.*, 2003). Traps were positioned at two different heights because it is known that the height can potentially affect the number and gender of leafhoppers captured (Van Steenwyk *et al.*, 1990; Minuz *et al.*, 2013). The xylem-sap feeding fauna was subsequently analyzed by studying: 1) their abundance and temporal population dynamics throughout the year; 2) their vertical distribution studying the effects of trap height on their capture; 3) their dispersal pattern inside the agroecosystem; 4) their relationships with the wild-plant abundances, land usage and pedoclimatic condition. This data can provide a better understanding of the dispersal dynamics of potential and known vectors; this is critical to understand their role in the epidemiological cycles of the *X. fastidiosa* diseases and to facilitate their management.

Materials and Methods

Study Site.

The study was conducted in an area located in Central Italy (district of Gallignano, Marche region −43.566° lat 13.431° long, 152 m of average elevation), inside the Marche Polytechnic University experimental field station, which is subject to the Italian organic farming regulations. The area included an olive orchard and its surrounding areas (Fig. 14) about 18000 m^2. The olive trees were distributed in 10 rows oriented from NW to SE, parallel to the main slope on the field (NE to SW). The rows were irregular in number of plants due to the cuttings of trees in the past years. The orchard was bordered along three sides by wood hedges (NW, NE, SE); between the SE border of the orchard and the wood hedge there was a young truffle tree nursery (average height of the trees ∼1.5m). The SW border was represented by a meadow and a tree hedge. The entire study area was covered by an abundant wild vegetation. The spontaneous vegetation in the olive orchard and in the truffle tree nursery, was mowed 3 times, in March, June and October. Pesticide treatments with 'Spintor Fly'® were applied 6 times, every 2 weeks, starting from August.

Insect Sampling.

Insects were sampled positioning yellow sticky traps (25x10cm; Glutor, Biogard division, Grassobbio, Italy) in a 20 meters grid centered on the olive orchard, and covering the truffle tree nursery field and all the hedges. The sampling points were georeferenced by using a handheld global positioning system (GPS 60 Garmin, Garmin International Inc., Olathe, KS). Each trap point includes two traps positioned at different heights: at 0.3m (low) and 1.7m (high) above the soil (total 108 traps). The traps were replaced every 2 weeks, from the first week of July 2015 to the last of June 2016. The traps were wrapped inside a plastic film, labeled, and stored at 20 °C. The specimens were removed from the traps using drops of limonene (Carlo Erba, Milan) and stored in 99% ethanol in Eppendorf vials (Carlo Erba, Milan) until their identification according to the taxonomic keys in the literature (Ribaut, 1952; Ossiannilsson, 1981; Holzinger *et al.*, 2003).

Vegetation Surveys.

Two surveys were carried out during 2016, one in April and one in July, over an area of $\sim$30 m^2 around each georeferenced point. The Braun-Blanquet method was carried out for the phytosociological analysis (Braun-Blanquet, 1932). The method consists in a rapid visual assessment technique that allows a range of values of cover abundance to be assigned for each plant species in the field (Braun-Blanquet, 1932; range, + to 5). Braun-Blanquet values were then converted through Van der Maarel method (van der Maarel, 2007) to obtain a fair approximation of a metric scale (range, 1-9), which is needed for the correlation analysis. Samples of each plant species observed, were collected for taxonomic identification following the keys in the literature (Pignatti 1982). Among the plants observed, sporadic occurences were excluded from the analysis.

Data Analysis.

The total numbers of insects captured in the high and low traps, and the total numbers of males and females captured in the high and low traps,

were prior tested for normality with the Shapiro-Wilk W Test (p=0.05), and then compared by using Wilcoxon Signed-rank tests (Wilcoxon, 1945; Woolson, 2008), with R software (R Core Development Team 2008) using traps as replicates. To detect the two dimensional spatial patterns of insect cumulative captures within the year, Spatial Analysis by Distance IndicEs (SADIE) was performed grouping high and low trap captures per grid point. The method works through equating the degree of spatial pattern in an observed arrangement of counts to the minimum effort that the individuals in the population would need to expend to move to a completely regular arrangement in which abundance was equal in each sample unit (Perry *et al.*, 1999). For each sampling point the dimensionless indexes of clustering is calculated, in means of local contribution to patch (v_i) and to gap (v_j). Large values of v_i indicate patchiness while large negative values of nj, indicate membership of a gap. An overall test of clustering is then provided by the comparison of the mean value of v_i and v_j with their corresponding values generated under the null hypothesis of a random distribution (α=0.05) (Mori *et al.*, 2015b; Perry *et al.*, 1999). The same spatial analysis was carried out for plant species using the combined Van der Maarel value. Associations between insects and plant species were obtained through SADIE package for spatial association analysis, comparing data outputs obtained in spatial pattern analysis. All analyses used the non-parametric SADIE method, with the maximum number of randomizations and met the recommendation of Holland *et al.* (1999) of containing at least 36 units . The algorithm used determines the local spatial association and derive an overall index of spatial association (X) and its probability level (P_x). This two-tailed test determines whether the clusters of the two species are associated ($P_x<0.025$), unassociated ($0.025<P_x<0.975$) or dissociated ($P<0.975$)(Mori *et al.*, 2015b; Perry *et al.*, 1999; Perry and Dixon, 2002). Two-dimensional cluster/gap spatial distribution map were built for each species using linear kriging with 0 nugget variance (Decante and Van Helden, 2008; Goovaerts, 1999) in SURFER 13® (Golden Software Inc.). The species *Evacanthus acuminatus* was excluded from all the statistical analysis due to the low number of records.

Results

Insect Identification and Abundance.

A total number of 1345 insects were collected among 7 families: 3 Aphrophoridae, 2 Cicadellidae, 2 Tibicinidae and 1 Cicadidae. It was not possible to identify the sex of many insects, due to the bad condition of the samples, especially in Cicadidae and Tibicinidae. The most abundant species sampled was *P. spumarius* (744 total; 475 Male /268 Female /1 Unknown), followed by *Cicadella viridis* (207; 153/33/21), *Cicada orni* (183; 6/30/147), *Tettigetta argentata* (103; 44/39/20), *Cicadetta montana* (41; 23/13/5), *Neophilaenus campestris* (32; 21/10/1), *Aphrophora alni* (30; 6/23/1) and *Evacanthus acuminatus* (5; 2/3). Trap analysis revealed that Cicadidae and Tybicinidae individuals, were highly damaged, presumably due to predation events, thus making difficult sexing the samples. In *C. orni* this unlucky event reaches an impressive peak of 147 individuals on 183 total (∼80%). Moreover, by a visual analysis of the traps we found many insects caught soon, after moulting. The sex ratio was male-biased for *P. spumarius* (W = 6.62 P<0.001) and *C. viridis* (W=5.77, P<0.001) while significantly more female were captured for *A. alni* (W=-3.27, P=0.002) and *C. orni* (W=-2.82, P<0.001). The individuals were significant more abundant in low traps then in high traps for *N. campestris* (W=-3.52, P<0.001), *C. viridis* (W= -6.15, P<0.001), *C. orni* (W= -2.26, P=0.024), *C. montana* (W=-4.28, P<0.001) and *T. argentata* (W=W-3.71, P<0.001). Further comparisons showed higher numbers in high traps within the males for *P. spumarius* (W=3.67, P<0.001) and the females of *T. argentata* (W=-2.35, P=0.02) and *C. orni* (W=-2.55, P=0.016). Significant higher numbers in low traps was found for the male of *P. spumarius* (W=3.36, P=<0.001) and *C. viridis* (W=4.27, P<0.001) and the females of *A. alni* (W=-2.50, P=0.02) and *C. orni* (W=-2.73, P=0.009) (Fig. 15). The temporal patterns and abundance for all the 7 species are shown in Fig. 16. *P. spumarius* was captured from May until February with two flight peaks in May and in October. *N. campestris* shows the same peaks and similar trend but no individual were sampled between the peaks. *Aphrophora alni* and *C. viridis* appear in June

until the first half of December while *E. acuminatus* was limited in June. *C. orni* was collected from July until September, *C. montana* from June to July and *T. argentata* from June to August.

Plants Identification and Abundance.

A total number of 135 different plant species were collected among 37 families. Seventeen plant species among the total sampled are known in literature to be host of *X. fastidiosa*, while other 46 species belong to genus in the host range of the bacterium (Baker *et al.*, 2015).

Spatial Distribution and Relationships with the Plant Communities.

Spatial Analysis by Distance IndicEs detected significant clustering into patch/gap in 4 insect species (*P. spumarius*, *C. montana*, *T. argentata* and *C. orni*) and in 12 plants on 30 analyzed (Tab. 1). *Philaenus spumarius* clustered mainly in the south-west part of the study site (Fig. 17A) where the edge is narrow and there is a row of spontaneous wild cover vegetation. Spatial association analysis revealed its association with *Clematis vitalba*, *Medicago* sp., and *Melissa officinalis* (romana), and dissociation from *Brachypodium rupestre*, *Scabiosa maritima*, *Vicia lutea* and *Veronica* sp. . *N. campestris* was found uniform distributed (Fig. 17B) and associated with *Lotus corniculatus* and *Scabiosa maritima* while it was dissociated from *Clematis vitalba*, *Rubus ulmifolius* and *Melissa officinalis*. *Aphrophora alni* was associated with *Hedisarium coronarium* and *Medicago sativa* while no plant dissociations were observed. *C. viridis* was associated with *Picris echioides* and *Sonchus asper* and it shows the highest number of dissociated plants, 6: *Bromus hordeaceus*, *Dactylis glomerata*, *Lotus corniculatus*, *Medicago* sp., *Vicia sativa* and *Geranium dissectum*. Both *A. alni* and *C. viridis* showed no pattern distribution, (Fig 17C-D).Tibicinidae showed spatial cluster distribution, *C. montana* seems to be distributed mainly in the northern corner of the field (Fig 18A), associated with 8 different plant species and dissociated with *Melissa romana* and *Clematis vitalba*. *Tet-*

tigetta argentata was found distributed in the eastern corner of the field (Fig 18B), associated with *Crepis vesicaria*, *Hedisarium coronarium*, *Centaurium erythrea*, *Brachipodium rupestre* and dissociated with *Medicago* sp. and *Melissa romana*. Finally *C. orni* was found distributed mainly within the olive canopy (Fig 18C), associated with 8 plant species and dissociated with *Rubus ulmifolius* and *Melissa romana*.

Discussion

In the current study, we described the abundance, population dynamics, two-dimensional dispersal patterns and plant relationships of 8 xylem-sap feeding insect species, according to the olive orchard agroecosystems characteristics, managements and time of the study. The spatial analysis approach was largely used in studying many other vector species to identify host weed association (Decante and Van Helden, 2008; Minuz *et al.*, 2013; Mori *et al.*, 2015). In our study, Cicadidae and Tibicinidae species distribution was partially biased by the sampling method used, therefore, the analysis of the vertical distribution of these insects could lead to a misunderstanding of their real distribution and their significant high number in low traps could be just a matter of fate. Despite that, the association analysis revealed that these species have the highest number of connected plants. The estimation of Cicadidae and Tibicinidae abundance has always been a difficult task since the larvae live deep in hard soil while the adults are cryptic, and often high in tree canopies. Several methods were used to estimate the field density, among them the most relevant are: counts of emergence holes, counts of exuviae, emergence-traps and the estimation of cicada numbers based on sound level they produce (Andersen, 1994; Dean and Milton, 1991; Milton and Dean, 1992; Patterson *et al.*, 1997; White and Sedcole, 1993; Wolda, 1989). Nevertheless, in some cases, these techniques have been recognized as poor or biased estimators of cicada densities (Andersen, 1994; Lane, 1993). Cicadas were proven to be able to transmit *X. fasidiosa* to healthy grapevines (Paiao *et al.* 2002; Krell *et al.*, 2007), while no records were assessed for Tibicinidae. More researches are necessary in

evaluating their transmission efficiency and their abundance to better understand their potential role in the spread of *X. fastidiosa*. The trap system we used here was efficient in sampling *P. spumarius* population. Indeed, it was the most abundant among all xylem-sap feeders and it was present for a broad span of time. The two peaks in population fluctuations were observed so far (Wiegert, 1964) and we found similar phenomenon also in the closely related species *N. campestris*. Some authors suggest the main reason of this particular phenology is the host plant shifting of the species during dry periods (Cornara, 2014). However, the authors themselves suggest the importance of the synergic use of different sampling methods for a more accurate estimation of the insect movement. *P. spumarius*, as well as the other xylem-sap feeders, is highly polyphagous and hundreds of plant species are known as hosts (Halkka *et al.*, 1967; Nickel, 2003; Weaver and King, 1954). The main insight on xylem sap-feeders host preference is oriented on those hosts that provide an high amino-acid concentration in the xylem sap, such as nitrogen fixing herbaceous legumes (Fabaceae) and some other plant species (Thompson, 1994). Among our three plant species linked with *P. spumarius*, only one of them belongs to the Fabaceae (*Medicago* sp), but the strongest association observed was with *Clematis vitalba*, a shrub of the Ranunculaceae family. Moreover it shows an unexpected dissociation from the nitrogen fixing plant *Vicia lutea*. Comparing the distribution map of *P. spumarius* with the morphology of the study site, it appears that fallow field and border area play a crucial role in insect distribution. In the west corner of the field (Fig 14-17A), the wood hedge is very narrow compared with the other three borders, and it is connected with a meadow corridor. The present paper suggests, in this condition, that the border texture could be fundamental in regulating the abundance of the insect coming from surrounding habitats. Xylem-sap sucker within Cicadellidae are epidemiologically relevant vectors in many countries (Redak *et al.* 2004), however, in Europe this group is poorly represented. At present only 7 species are described in Italy: *Cicadella viridis* L., *Graphocephala fennahi* Young, *Evacanthus acuminatus* Fabricius, *Evacanthus interruptus* L., *Evacanthus rostagnoi* Picco, *Errhomenus brachypterus* Fieber and *Anoterostemma ivanoffi* Lethierry. While *Evacanthus acuminatus* was very

uncommon (5 individuals) in our experiment, *C. viridis* was the second most abundant xylem-sap feeding insect sampled in the field. Nowadays, the probability of *C. viridis* to be a *X. fastidiosa* vector is assessed as 'moderate to high' due to two criteria: it is a very common insect species and it has a wide host range (Baker *et al.*, 2015). In our findings, this sharpshooter is strongly associated with ground cover vegetation, indeed, it was rarely found in high traps. *X. fastidiosa* has a broad range of hosts among herbaceous species; in this context *C. viridis* role could be relevant, moreover it was also found connected with *Sonchus asper*, a very common herbaceous species that has already been found positive to *X. fastidiosa* (Freitag, 1951). A similar distribution was observed in *N. campestris*, even if with a considerably lower number of individuals. Its role as vector is nowadays considered to be unlikely critical because always very few individuals have been found in the italian orchards and its transmission efficiency seems to be neglectable in laboratory conditions (Cornara *et al.*, 2016). However, it is noteworthy how very close-related species such as *P. spumarius* and *N. campestris* show different potential preferred host plants: *Clematis vitalba* and *Melissa romana* are associated with *P. spumarius* but both dissociated with *N. campestris*. *Aphrophora alni*, basing on our insights, showed no significant preference between high and low traps and it was homogenously distributed. Despite that, its abundance in the ecosystem is even less then *N. campestris*. To date there are still many unsolved questions and further investigations in the study of the *X. fastidiosa* pathosystem are necessary. Future investigations through multi-disciplinary approaches could be aimed to study: i) the role played by Cicadidae and Tibicinidae in *X. fastidiosa* epidemiology; ii) the behavior of *P. spumarius* with a particular focus to its phenology, its population fluctuations, its host preference and volatile compound perception.

Tables

Table 1: Average indexes of clustering into patch (mean v_i) and into gap (mean v_j) with associated probability (P) from randomization test.

		mean v_i	P(mean v_i)	mean v_j	P(mean v_j)
Insects					
Aphrophoridae	*Aphrophora alni*	1.139	0.1828	-1.121	0.2122
	Neophilaenus campestris	1.243	0.0868	-1.256	0.0811
	Philaenus spumarius	**2.55**	**<0.0001**	**-2.868**	**<0.0001**
Cicadidae	*Cicada orni*	**1.353**	**0.0431**	**-1.419**	**0.0325**
Cicadellidae	*Cicadella viridis*	1.071	0.2571	-0.959	0.5076
Tibicinidae	*Cicadetta montana*	**2.631**	**<0.0001**	**-2.517**	**<0.0001**
	Tettigetta argentata	**1.232**	**0.0012**	**-1.806**	**0.0013**
Plants					
Asteraceae	*Bellis perennis*	1.504	0.0188	-1.481	0.0236
	Crepis vesicaria	1.282	0.0665	-1.24	0.0888
	Pallenis spinosa	1.174	0.1249	-1.21	0.1061
	Picris echioides	**1.716**	**0.003**	**-1.792**	**0.0013**
	*Sonchus asper**	1.055	0.295	-1.128	0.1892
Convolvulaceae	*Convolvulus arvensis**	1.119	0.1961	-1.096	0.2296

Dipsacaceae	*Scabiosa maritima*	**1.896**	**0.001**	**-1.858**	**0.0012**
Fabaceae	*Hedysarum coronarium*	**1.741**	**0.0025**	**-1.852**	**0.001**
	Lathyrus pratensis	0.931	0.5958	-0.953	0.5246
	Lotus corniculatus	**1.361**	**0.0323**	**-1.341**	**0.0421**
	*Medicago sativa**	1.105	0.2053	-1.097	0.2172
	*Medicago sp.**	1.041	0.3263	-1.064	0.2839
	Vicia lutea	1.087	0.2391	-1.11	0.2053
	Vicia sativa	1.182	0.1337	-1.22	0.1148
	*Melilotus**	1.22	0.0962	-1.22	0.0979
Gentianaceae	*Blackstonia perfoliata*	**1.445**	**0.024**	**-1.419**	**0.031**
	Centaurium erythraea	**1.433**	**0.0221**	**-1.413**	**0.03**
Geraniaceae	*Geranium dissectum**	1.027	0.3402	-1.033	0.3295
Graminaceae	*Lolium multiflorum*	**1.852**	**0.0005**	**-1.948**	**<0.0001**
Lamiaceae	*Calamintha nepeta*	1.214	0.0987	-1.26	0.0766
	*Melissa romana**	**1.683**	**0.004**	**-2.021**	**<0.0001**
Poaceae	*Avena* sp.	**1.352**	**0.0434**	**-1.42**	**0.029**
	Brachypodium rupestre	**1.808**	**0.001**	**-1.748**	**0.0022**
	*Bromus diandrus**	0.887	0.734	-0.895	0.7044
	Bromus hordeaceus	1.063	0.2707	-1.093	0.2309
	Dactylis glomerata	**1.457**	**0.0179**	**-1.482**	**0.0184**

Plantaginaceae	*Veronica* sp*	1.092	0.2291	-1.086	0.235
Polygonaceae	*Rumex* sp.	1.253	0.0818	-1.284	0.0655
Ranunculaceae	*Clematis vitalba*	**1.63**	**0.0042**	**-1.64**	**0.0042**
Rosaceae	*Rubus ulmifolius*	1.253	0.0818	-1.284	0.0655

Table 2: Probability associated to SADIE spatial association index.

		PS	NC	CV	AA	CO	CM	TA
Ast	*Bellis perennis*	0.1735	0.5981	0.9634	0.8521	0.0653	0.7844	0.2061
	Crepis vesicaria	0.9682	0.0772	0.3117	0.2483	0.2333	0.5241	0.017
	Pallenis spinosa	0.8913	0.0327	0.9094	0.3453	**0.0036**	0.0316	0.3303
	Picris echioides	0.9688	0.0721	**0.0093**	0.4631	0.1186	**0.0143**	0.5255
	*Sonchus asper**	0.8938	0.0581	**0.0246**	0.3032	0.3788	0.5059	0.507
Con	*Convolvulus arvensis**	0.1284	0.3986	0.3018	0.8426	0.5952	0.6516	0.866
Dip	*Scabiosa maritima*	0.9949	**0.0003**	0.3644	0.0496	0.2206	0.0003	0.0277
Fab	*Hedysarum coronarium*	0.8201	0.1555	0.8928	**0.0027**	**0.022**	**0.0062**	**0.0131**
	Lathyrus pratensis	0.154	0.1196	0.9363	0.625	0.3244	0.5826	0.8506
	Lotus corniculatus	0.8973	**0.0028**	0.9787	0.2131	**0.0075**	**0.0035**	0.2968
	*Medicago sativa**	0.3303	0.8332	0.9052	**0.0041**	0.3657	0.8837	0.1218
	Medicago sp*	**0.0142**	0.9574	0.9789	0.1638	0.1301	0.8653	0.9941
	Vicia lutea	0.9778	0.0317	0.8292	0.1288	0.0328	**0.011**	0.1388
	Vicia sativa	0.0311	0.3114	0.9994	0.4683	0.0413	0.4756	0.8054
	Melilotus sp.*	0.2684	0.349	0.53	0.7842	0.2206	0.4313	0.9169
Gen	*Blackstonia perfoliata*	0.9707	0.1917	0.8356	0.04	**0.0029**	0.0285	0.2406

	Centaurium erythraea	0.9603	0.1429	0.7261	0.0285	**0.0084**	**0.0031**	**0.0172**
Ger	*Geranium dissectum**	0.3765	0.8388	0.9897	0.096	0.6058	0.8525	0.6287
Lam	*Calamintha nepeta*	0.3793	0.1416	0.972	0.7349	0.014	0.2905	0.1913
	*Melissa romana**	**0.0001**	0.9993	0.745	0.8976	0.9988	0.9999	0.9964
Poa	*Avena* sp.	0.8068	0.0526	0.6584	0.9676	**0.0087**	**0.0205**	0.8866
	Brachypodium rupestre	0.9932	0.0768	0.947	0.1406	**0.0072**	**0.004**	**0.0048**
	*Bromus diandrus**	0.4078	0.8996	0.4216	0.6886	0.8577	0.7165	0.8432
	Bromus hordeaceus	0.471	0.5438	0.998	0.1398	0.5815	0.7235	0.8407
	Dactylis glomerata	0.1476	0.9392	0.9879	0.4037	0.4064	0.6472	0.3388
	Lolium multiflorum	0.439	0.4698	0.1493	0.7165	0.3315	0.37	0.9717
Pol	*Rumex* sp.	0.5231	0.607	0.0642	0.9617	0.2206	0.217	0.8925
Pla	*Veronica* sp.*	0.9975	0.2182	0.7519	0.0827	0.2305	0.0547	0.1697
Ran	*Clematis vitalba*	**0.008**	0.9895	0.5712	0.9554	0.9715	0.9998	0.9306
Ros	*Rubus ulmifolius*	0.3497	0.9991	0.4713	0.0298	0.9998	0.8588	0.267

1

[1] Asterisks indicate plant already tested positive for *Xylella fastidiosa*. Numbers in bold indicate associations (P<0.025), while numbers in red indicate dissociations (P>0.975).
PS = *P. spumarius*; NC = *N. campestris*; CV = *C. viridis*; AA = *A. alni*; CO = *C. orni*; CM = *C. montana*; TA = *T. argentata*.
Ast = Asteraceae; Con = Convolvulaceae; Dip = Dipsacaceae; Fab = Fabaceae; Gen = Gentianaceae; Ger = Geraniaceae; Lam = Lamiaceae; Poa = Poaceae; Pol = Polygonaceae; Pla = Plantaginaceae; Ran = Ranunculaceae; Ros = Rosaceae.

Figures

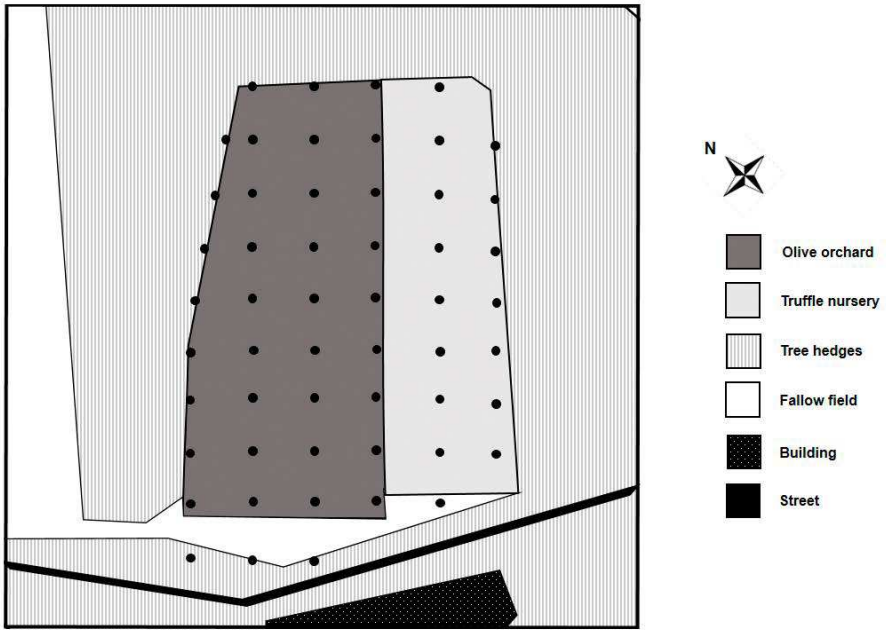

Figure 14: Layout of the study site, indicating the agroecosystem features and sampling points.

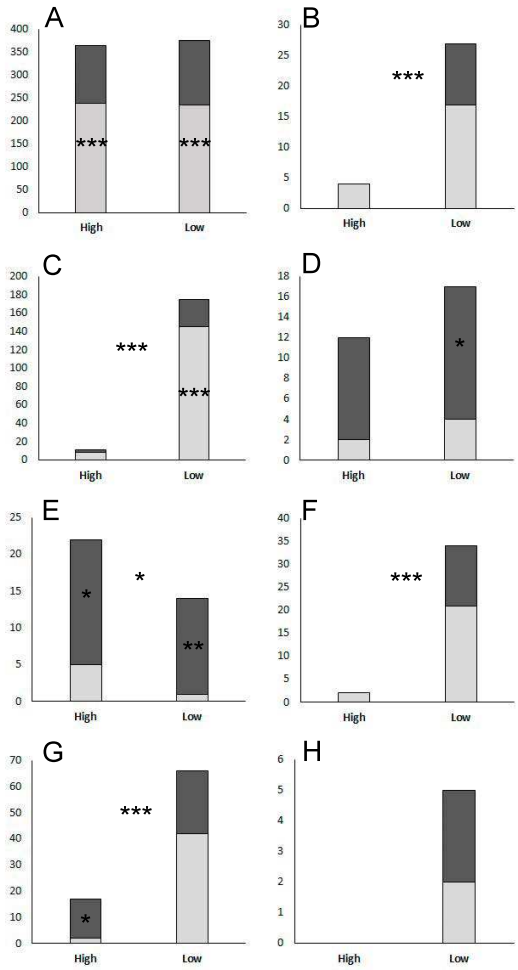

Figure 15: Total numbers of *Philaenus spumarius* (A), *Neophilaenus campestris* (B), *Cicadella viridis* (C), *Aphrophora alni* (D), *Cicada orni* (E), *Cicadetta montana* (F), *Tettigetta argentata* (G) and *Evacanthus acuminatus* (H) in high and low traps, between male (light grey) and female (dark grey) individuals. Asterisks inside the columns, signifcance in comparisons captured within male and female; asterisks between the columns, significance in comparisons between high and low traps. (*P$<$0.05; **P$<$0.01; ***P$<$0.001, Wilcoxon Signed-rank tests)

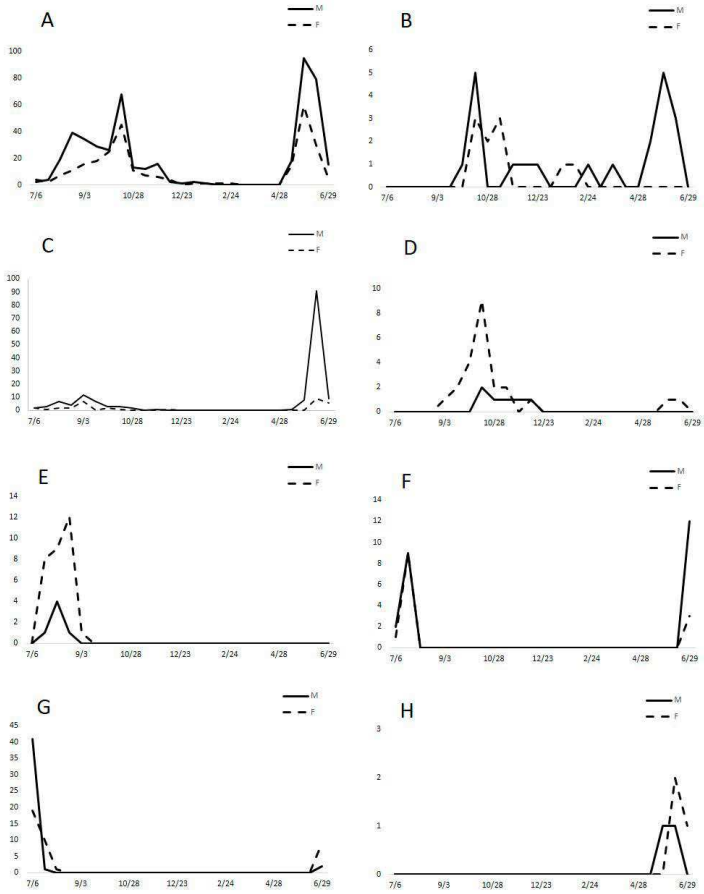

Figure 16: Seasonal flight activities of males and females of *Philaenus spumarius* (A), *Neophilaenus campestris* (B), *Cicadella viridis* (C), *Aphrophora alni* (D), *Cicada orni* (E), *Cicadetta montana* (F), *Tettigetta argentata* (G) and *Evacanthus acuminatus* (H).

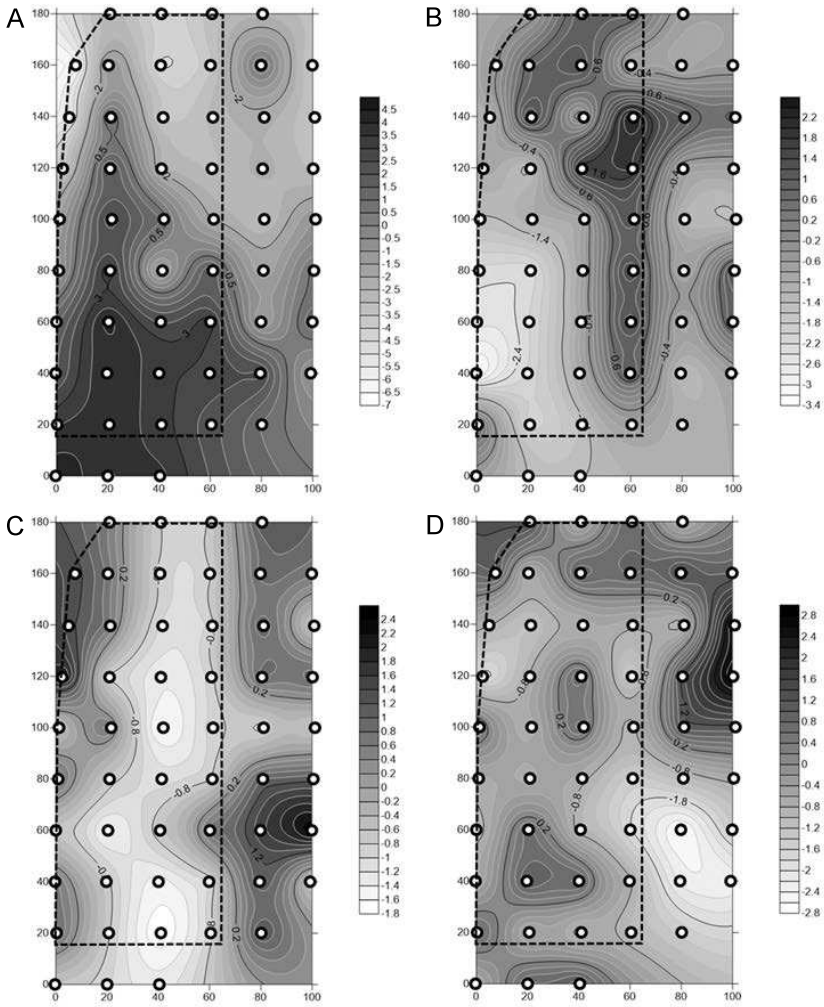

Figure 17: Contour maps of cluster distribution in *Philaenus spumarius* (A); *Neophilaenus campestris* (B); *Cicadella viridis* (C) and *Aphrophora alni* (D); white dots are the sampling points; dotted line represent the olive canopy border; gray scale on the sides represents estimated insect patch/gap index of aggregation.

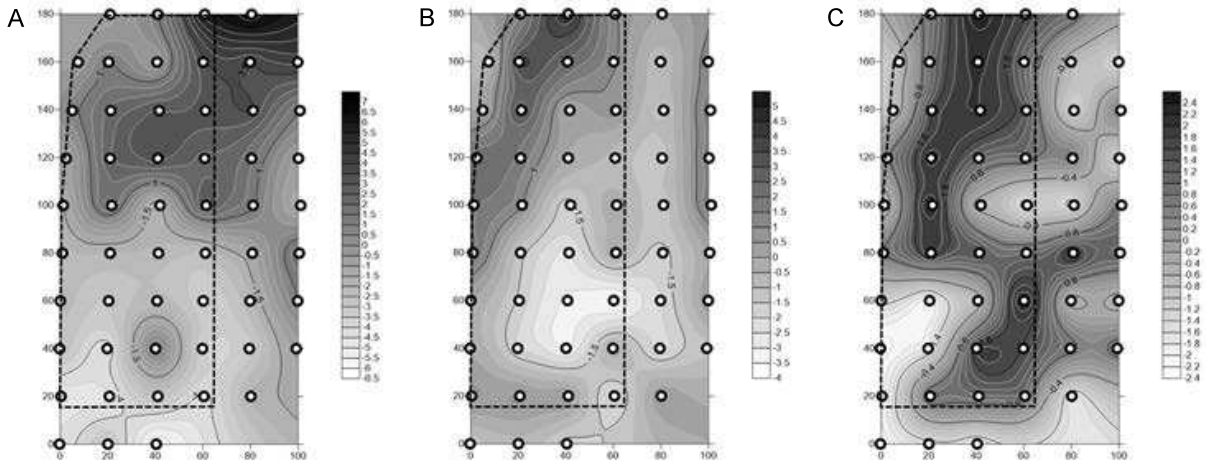

Figure 18: Contour maps of cluster distribution in *Cicadetta montana* (A) *Tettigetta argentata* (B); *Cicada orni* (C); white dots are the sampling points; dotted line represent the olive canopy border; gray scale on the sides represents estimated insect patch/gap index of aggregation.

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Concluding Remarks

Xylella fastidiosa epidemics in Italy and in Europe will prove to be a very challenging problem and a critical menace to many agricultural contexts. European commission is making any necessary efforts to control the situation and to find correct answers to such a difficult issue. Also the answer of the scientific community was strong and worldwide, researches involving *X. fastidiosa* and its vectors has increased dramatically in recent years (Almeida *et al.*, 2005). Despite we learned a lot, recent previsions on the epidemics are discouraging: network analysis revealed that the chances of eradicating *X. fastidiosa* from Italy are extremely slim and is very likely that the spread of the pathogen will continue (Strona *et al.*, 2017). The studies presented in this thesis aimed to shed light on some dark corners about the functional anatomy and the ecology of the main vector of the disease, *Philaenus spumarius*, and to analyze the other risk-factors related to other potential vectors. In chapters 1 and 2, the sensory tructures in the antennae, in the labium tip and in the precibarium of *P. spumarius*, were studied. These results will help to understand the nature of the stimuli perceived by the insect during the search, the evaluation and final selection of the host plant. These stimuli, indeed, are the base to understand the behavior of the insect in the field. Overall *P. spumarius* apparatus showed several similarities with other leafhoppers and planthoppers, xylem- or phloem-sap feeders, polyphgous or oligophagous. Despite that, some interesting peculiarities were found (i.e. olfactive structures on the labium). The role of specific odorant, gustative and visual stimuli remains to be assessed with precise electrophysiological and behavioural tests to complete the investigation. These works were carried out with the tutorship and collaboration of the people of the Entomology labs of the Marche Polytechnic University (Prof. Nunzio Isidoro; Prof.ssa Paola Riolo; Dott.ssa Sara Ruschioni) and University of Perugia (Prof. Roberto Romani; Dott. Cesare Dentini). Moreover, technical help was provided by the people of the Laboratorio Microscopia Elettronica a Scansione (SIMAU; Università Politecnica delle Marche, Italy) and Centro Universitario di Microscopia Elettronica (CUME; Università degli Studi di Perugia, Italy). In chapters 3 and 4, we investigated the precibarium and the precibarial valve, since this area is a critical part in the interaction with *X. fastidiosa* cells. The

preliminary data presented are encouraging results to define important parameters in the transmission of the pathogen itself and in the phylogeny of the insects. Despite these two works have to be implemented with further anatomical and ultrastructural studies, the data obtained are sufficient to reconsider important pardigms about the insect-plant-pathogen interaction. First, the fluid dynamics in the foreguts was considered to be similar in many leafhopper species and previous hydrodynamic model was not built on real morphological data. Second, the data allowed us to obtain a new description of the precibarial valve, which will hopefully replace a 35-years-old-model and clarify the role of the valve itself. These works were carried out with the tutorship and collaboration of the group of the Entomology labs of the Marche Polytechnic University (Prof. Nunzio Isidoro; Prof.ssa Paola Riolo; Dott.ssa Sara Ruschioni) and University of California - Berkeley (Prof. Rodrigo P. P. Almeida; Prof. Emer. Alexander Purcell). Moreover, technical help was provided by the people of the Electron Microscopy Lab (University of California - Berkeley) and Micro Photonics Lab (Pennsylvania - USA). In chapter 5, is presented the results of a deep ecological investigations on *P. spumarius* (youngs and adults), xylem-sap feeding species. These are the first works on Homoptera in an olive orchard agroecosystem, analyzed with seasonal activities, distribution. Moreover, insight on plant host species connected with those insects is given, on the basis of spatial association. This study can be successively implemented and linked with epidemiological models, behavioural tests, and it can represent the base of a pest-risk assessment in the Marche region. These studies were carried out with the tutorship and collaboration of the people of the Entomology and Botany labs of the Marche Polytechnic University (Prof. Nunzio Isidoro; Prof.ssa Paola Riolo; Prof.ssa Simona Casavecchia; Dott.ssa Roxana Minuz; Dott. Nino Loreto, MSc student Dr. Francesco Marchetti). Future dedicated work is necessary to complete the investigation on vectors' role, behavior and management strategies.

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Appendixes

QR-Code link to the Figures presented.

Italian Xylem-Sap Feeding Fauna.

Table 3: Hemipteran Xylem-Sap feeding insects in the Italian mainland

Family	**subfamily**	**tribe**	**Species**
Cicadidae	Cicadinae	Cicadini	*Cicada orni* Linnaeus
			Cicadatra atra (Olivier 1790)
	Platypleurinae	Cryptotympanini	*Lyristes plebejus* (Scopoli 1763)
Tibicinidae	Tibicininae	Cicadettini	*Cicadetta mediterranea* (Fieber 1876)
			Cicadetta montana (Scopoli 1772)
			Cicadivetta tibialis (Panzer 1798)
			Tettigetta argentata (Olivier 1790)
			Tettigetta brullei (Fieber 1876)
			Tettigetta dimissa (Hagen 1856)
			Tettigetta pygmaea (Olivier 1790)
		Tibicinini	*Tibicina haematodes* (Scopoli 1763)
			Tibicina *nigronervosa* (Fieber 1876)
			Tibicina *picta* (Fabricius 1794)
			Tibicina *tomentosa* (Olivier 1790)
Cercopidae			*Cercopis arcuata* (Fieber 1844)
			Cercopis intermedia (Kirschbaum 1868)

			Cercopis sanguinolenta (Scopoli 1763)
			Cercopis vulnerata (Rossi 1807)
			Haematoloma dorsata (Ahrens 1812)
Aphrophoridae			*Aphrophora alni* (Fallen 1805)
			Aphrophora corticea (Germar 1821)
			Aphrophora major (Uhler 1896)
			Aphrophora pectoralis (Matsumura 1903)
			Aphrophora salicina (Goeze 1778)
			Lepyronia coleoptrata (Linnaeus 1758)
			Neophilaenus albipennis (Fabricius 1798)
			Neophilaenus campestris (Fallen 1805)
			Neophilaenus exclamationis (Thunberg 1784)
			Neophilaenus infumatus (Haupt 1917)
			Neophilaenus limpidus (Wagner 1935)
			Neophilaenus lineatus (Linnaeus 1758)
			Neophilaenus minor (Kirschbaum 1868)
			Philaenus italosignus (Drosopoulos & Remane 2000)
			Philaenus spumarius (Linnaeus 1758)
Cicadellidae	Cicadellinae	Cicadellini	*Cicadella viridis* (Linnaeus 1758)
			Graphocephala fennahi (Young 1977)

Evacanthini	*Evacanthus acuminatus* (Fabricius 1794)
	Evacanthus interruptus (Linnaeus 1758)
	Evacanthus rostagnoi (Picco 1921)
Errhomenini	*Errhomenus brachypterus* (Fieber 1866)
Anoterostemmatini	*Anoterostemma ivanoffi* (Lethierry 1876)

Partial collection of Homoptera species sampled during Chapter 5 survey

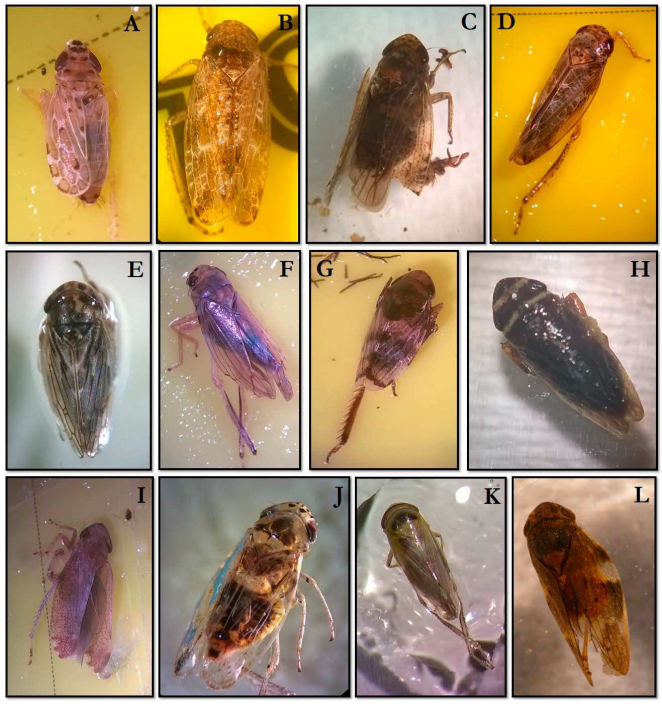

Figure 19: A: *Adarrus multinotatus*; B: *Allygidius atomarius*; C: *Allygidius furcatus*; D: *Allygus modestus*; E: *Anaceratagallia* sp.; F: *Anoplotettix fuscovenosus*; G:*Anoscopus albifrons*; H: *Aphrodes bicinctus*; I: *Fiebereiella florii*; J: *Euscelis lineolatus*; K: *Exitianus capicola*; L: *Aphrophora alni*.

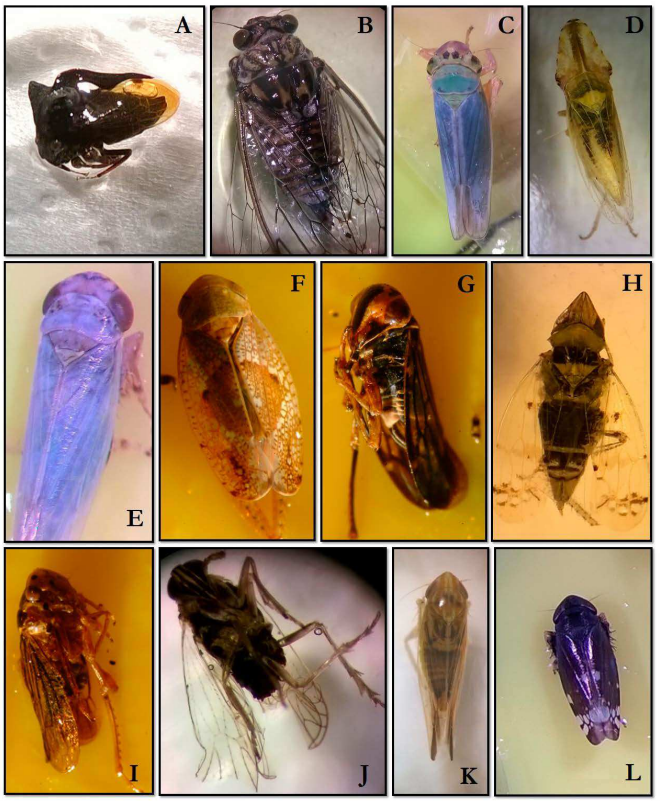

Figure 20: A: *Centrotus cornutus*; B: *Cicada orni*; C: *Cicadella viridis*; D: *Eupelix cuspidata*; E: *Grypotes staurus*; F: *Hishimonus hamatus*; G: *Idiocerus notatus*; H *Japananus hyalinus*; I: *Macropsis fuscula*; J: *Laodelphax striatella*; K: *Mocydia crocea*; L: *Neoaliturus fenestratus*.

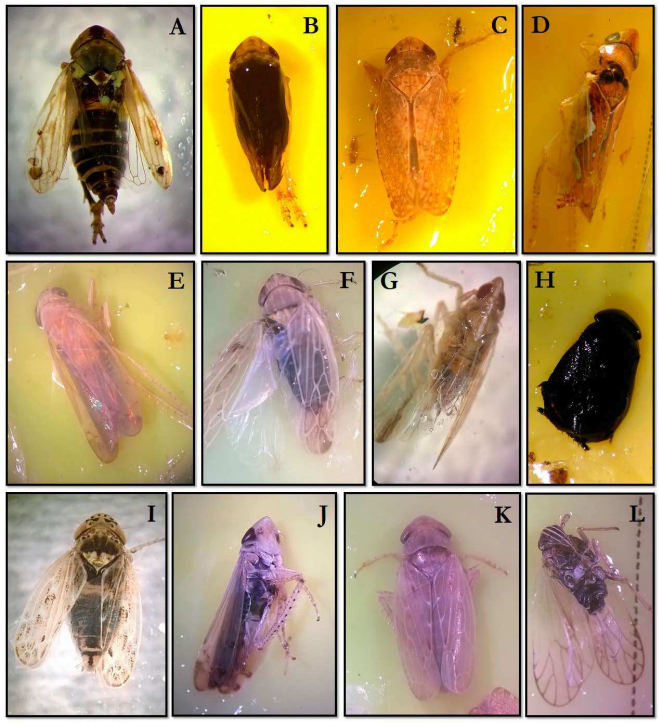

Figure 21: A: *Neophilaenus campestris*; B: *Philaenus spumarius*; C: *Phlepsius intricatus*; D: *Platymetopius major*; E: *Placotettix taeniatifrons*; F: *Psammotettix alienus*; G: *Stenocranus major*; H *Tettigometra atra*; I: *Recilia schmidtgeni*; J: *Synophropsis lauri*; K: *Thamnotettix dilutior*; L: *Toya propinqua*.

Acknowledgements

I wish to thank anyone who helped me, in these three years. Shall your names be in my flowing cups freshly remembered as household words in happy times.