Feeding behavior in relation to spittlebug transmission of *Xylella fastidiosa*

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Received: 29 July 2019 / Revised: 11 January 2020 / Accepted: 13 May 2020 / Published online: 22 May 2020
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Abstract

Here, we provide the first insights into the transmission dynamics of the bacterium *Xylella fastidiosa* by the meadow spittlebug *Philaenus spumarius*, gathered through DC EPG (electrical penetration graph)-assisted transmission tests and comparative observations of the probing and feeding behavior of infective versus non-infective vectors on healthy olive plants. Bacterial cells binding to *P. spumarius*’ foregut occurred at a very low rate and in a time as short as 15 min spent by the insect in xylem ingestion or activities interspersed with xylem ingestion (interruption during xylem ingestion and resting). *P. spumarius* inoculation of bacterial cells into the xylem was exclusively associated with an early (ca. 2 to 7 min after the onset of the first probe) and occasional behavior, provisionally termed waveform Xe, presumably related to egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs compared to non-infective ones exhibited: (i) longer non-probing and shorter xylem ingestion; (ii) longer duration of single non-probing events; (iii) fewer sustained ingestions (ingestion longer than 10 min) and interruptions of xylem activity (N); and (iv) longer time required to perform the first probe. These observations suggest difficulties in feeding of infective *P. spumarius* probably caused by the presence of *X. fastidiosa* within the foregut. Overall, our data indicate that likely short time—few minutes—is required for *X. fastidiosa* transmission by *P. spumarius*; thus, vector control strategies should aim at preventing spittlebug access to the host plant. Furthermore, our findings represent an important contribution for further research on the disruption of spittlebug–bacterium interactions.

Keywords *Philaenus spumarius* · EPG · Olive · Oleander · Transmission dynamic · Fastidious bacterium

Key message

- Here, we provide the first insights into the transmission dynamics of the bacterium *Xylella fastidiosa* by its main European vector, the spittlebug *Philaenus spumarius*. Acquisition occurs at a very low rate during the first minutes the insect is ingesting the xylem sap. Inoculation is likely related to an occasional behavior that occurs early in the probe, possibly egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs exhibited feeding difficulties possibly caused by the presence of the bacterium within the foregut.
- Given the short time required for *X. fastidiosa* transmission, vector control strategies should aim at preventing spittlebug access to the host plant.

Introduction

Since the first report of a grapevine disease (Pierce 1892) later found to be caused by a vector-borne microorganism successively identified as a bacterium (Davis et al. 1978), named *Xylella fastidiosa* (Wells et al. 1987), research has clarified many aspects of bacterium–vector–host plant interactions. Nevertheless, an essential question still remains unanswered: What are the vector behaviors necessary for bacterial transmission to plants (Almeida 2016)?
X. fastidiosa is a xylem-limited bacterium, whose natural spread relies on insects specialized for feeding on xylem sap (Houston et al. 1947; Frazier 1965). Therefore, it is assumed the vector should access xylem vessels to acquire the bacterium as well as to inoculate it (Houston et al. 1947); however, the exact behaviors or sequence of behaviors resulting in transmission are unknown. Vector acquisition efficiency is a direct function of vector access period to the source plant and of the bacterial population inside the infected tissue (Purcell and Finlay 1979; Hill and Purcell 1997). Following acquisition, X. fastidiosa cells bind to the vector foregut, putatively to the portion of the pre-cibarium proximal to the cibarium, behind the pre-cibarial valve (Almeida and Purcell 2006). The bacterium persists in its vectors during the entire insect life span, but is shed with molting (Purcell and Finlay 1979; Purcell et al. 1979; Almeida and Purcell 2006). The loss of vector infectiousness with molting suggests that the foregut is the essential retention site of X. fastidiosa. Given the heterogeneous distribution of X. fastidiosa in the host plant and the turbulent rapid flow of xylem sap into the vector foregut upon uptake, bacterial cells’ binding to the foregut is thought to be a rare event, with the majority of the bacterial cells swallowed rather than retained (Retchless et al. 2014). X. fastidiosa inoculation positively correlates with the access period to the recipient plant (Hill and Purcell 1995; Almeida and Purcell 2003), the number of infective vectors on the host plant (Daugherty and Almeida 2009), and the number of probes performed by the single infective vector (Jackson et al. 2008). Bacterial inoculation can occur as soon as one hour after acquisition; thus, bacterial multiplication and biofilm formation are not required (Purcell and Finlay 1979). Backus et al. (2009) proposed that vectors introduce X. fastidiosa into plants through a mechanism defined as “salivation–ingestion–egestion”: Once stylets reach a xylem vessel, the insect might ingest a mixture of saliva (previously secreted during the formation of the salivary sheath) and xylem sap that is swished through the pre-cibarium and sensed by the pre-cibarial sensilla. This process could lead to an enzymatic (saliva) and mechanical (fluid turbulence) detachment of X. fastidiosa cells within the foregut. These loosened cells could be inoculated into the xylem vessel through egestion, the putative active expulsion of fluid from the food canal (Ramirez et al. 2008; Backus et al. 2012; Backus 2016). Although indirect evidences support this theory, a final correlation between the occurrence of such sequence of behaviors and X. fastidiosa inoculation to a recipient plant is missing (Almeida 2016). Identifying the inoculation mechanism of a plant pathogen by its vector involves the real-time observation of the probing behavior of an infective insect given access to a healthy plant; the probe should be terminated when the putative inoculation behavior is performed (Backus 2016). The EPG (electrical penetration graph) is a technique that permits the real-time monitoring of hemipterans’ probing and feeding behavior (McLean and Kinsey 1964; Tjallingii 1978; Backus and Bennett 2009); the use of the EPG has been crucial in determining the behaviors associated with acquisition and inoculation of several vector-borne plant pathogens (Prado and Tjallingii 1994; Martin et al. 1997; Moreno et al. 2012; Antolinez et al. 2017; Jiménez et al. 2018). However, similar studies on X. fastidiosa have failed because of the very low inoculation efficiency per individual vector and per probe (Backus 2016). Regarding the X. fastidiosa–vector relationship, the idea of the insect as a mere carrier of the bacterium has been recently challenged by the finding of bacterial exploitation of vector cuticle as carbon source, with possible detrimental effects for the insect (Killiny et al. 2010; Labrousaa et al. 2017). However, to the best of our knowledge, no qualitative or quantitative data on bacterial-mediated effects on the behavior of infective vectors have been produced so far. Such effects of the bacterium on its vectors may have direct consequences on the epidemiology of X. fastidiosa-related diseases. Most of the background on X. fastidiosa transmission dynamics and bacterium–vector interactions exposed above come from studies on the X. fastidiosa–grapevine–sharpshooter pathosystem in California (USA) (Rapicavoli et al. 2018). However, vectors other than sharpshooters, i.e., spittlebugs, seem to play the key role in X. fastidiosa spread in Europe (Cornara et al. 2018a, 2019). Indeed, the meadow spittlebug Philaenus spumarius L. (1758) (Hemiptera: Aphrophoridae) has been proved to be the main vector of X. fastidiosa to olive in South Italy and is likely involved in bacterial spread in all the European outbreaks reported so far (Saponari et al. 2014; Cornara et al. 2017a, b; Cruaud et al. 2018; Morente et al. 2018; Cornara et al. 2019). P. spumarius has some different features with respect to its relationship with X. fastidiosa compared to sharpshooters. These differences may relate to spittlebug feeding behavior and the dynamics of fluids within the foregut (Cornara et al. 2016, 2018b; Sicard et al. 2018; Ranieri et al. 2019). Such differences might have major implications on the spittlebug-mediated transmission of the bacterium that could so far differ in some extent to what has been described for sharpshooters. Therefore, X. fastidiosa transmission dynamics by P. spumarius and spittlebug–bacterium interactions must be investigated in detail. We began to explore the transmission dynamic of X. fastidiosa by P. spumarius by using EPG in experiments to study the relationship of vector feeding behavior to transmission. We addressed three main questions: (i) What is the behavior/sequence of behaviors leading to bacterium acquisition by the meadow spittlebug?; (ii) What is the P. spumarius behavior/sequence of behaviors leading to bacterium inoculation to the host plant?; and (iii) Are there any differences in probing and feeding behavior between infective and non-infective P. spumarius? The data presented here constitute an essential step for research on spittlebugs transmission of X. fastidiosa.
Materials and methods

Collection and rearing of Philaenus spumarius

Philaeus spumarius individuals used to study the acquisition behavior and for comparison of the feeding behavior of infective versus non-infective spittlebugs were collected at the nymphal stage with a fine-tip brush on ground vegetation in a X. fastidiosa-free olive orchard in Apulia region (Southern Italy) on March 2018. The nymphs were reared until adulthood on a mix of different suitable plant species (Conyza sp., alfalfa (Medicago sativa), oat (Avena sp.), and vetch (Vicia sativa)) inside a cage (2 × 1 × 1 m) placed beneath an olive tree inside an experimental field in the premises of the University of Bari (Apulia region, Southern Italy). Three to four weeks after emergence, adult spittlebugs (males and females) were collected using a mouth aspirator, transferred to a plastic aerated empty cylinder, and moved to an indoor facility located in the X. fastidiosa-infected area (Racale (LE), South Italy) where the transmission experiments took place. Before the experiments, the spittlebugs were pre-screened for X. fastidiosa by caging them on periwinkle (Catharanthus roseus) plants, in groups of five per plant, for an IAP of four to seven days, inside an insect-proof air-conditioned chamber (25 ± 2 °C, 40% HR). The prescreened periwinkles, tested for X. fastidiosa ca. 50 days after the IAP by qPCR (following the protocol by Locono-sole et al. (2014)), were negative for the bacterium.

To assess the inoculation behavior, P. spumarius adults (males and females) were collected in the X. fastidiosa-infected area (Salve (LE), Apulia region). Briefly, in August 2017 insects were collected by sweep net and mouth aspirator in an olive grove with a high disease prevalence (ca. 80% of the olives exhibiting clear symptoms of Olive Quick Decline Syndrome caused by X. fastidiosa), mainly on the bordering trees and shrubs (oak (Quercus ilex), lentisk (Pistacia lentiscus), persimmon tree (Diospyros kaki), orange (Citrus sinensis), pomegranate (Punica granatum), and cypress (Cupressus sempervirens)). The spittlebugs were then caged in groups of six per plant on 15-day-old vetch plants inside an insect-proof air-conditioned chamber (25 ± 2 °C, 40% HR) until the transmission experiments were performed. In 2018, insects were collected in the same olive grove, but during the months of June and July, and on olive plants (Olea europaea) in addition to the plants described above for the 2017 collection.

Experimental plants

Seedlings of Conyza sp., alfalfa, oat, and vetch were used to rear the juveniles, while plants of vetch were used to maintain the adult spittlebugs until the EPG experiments. Source plants for the acquisition of the bacterium (X. fastidiosa subsp. pauca, ST53) consisted of olive seedlings infected in 2014 (transmission experiments details described by Cornara et al. 2017b). The recipient plants used for the inoculation varied according to the EPG experiment and consisted of (i) two-year-old olive seedlings (20–30 cm height); (ii) 4-month-old self-rooted oleander plants; and (iii) 3-month-old periwinkle plants. The plants used for the EPG-assisted transmission experiments were grown in soil, sand, and vermiculite (6:3:2).

Philaenus spumarius probing and feeding behavior: EPG waveforms

The probing and feeding behavior of P. spumarius has been characterized through a combination of EPG, video-assisted observations and micro-computed tomography (Cornara et al. 2018b). Five distinct main EPG waveforms were described in that study, with each waveform corresponding to a different behavior during the probe: C (pathway waveform, corresponding to stylet penetration activities during the pathway phase, salivation and build-up of the salivary sheath, and tissue exploration while stylets move toward the xylem vessels); Xc (xylem contact/pre-ingestion, representing the first contact with a xylem vessel and possible pre-ingestion or trial ingestion); Xi (active xylem sap ingestion); R (a resting phase alternated with xylem ingestion); N (brief interruption during the xylem phase, either Xc or Xi, of unknown biological meaning). Waveform N is not considered a “proper” interruption of xylem activities, i.e., in case of occurrence of N, the activities preceding and following N are always (or almost always) displayed during all P. spumarius probes, as observed on olive, grapevine, vetch, and other plants (Cornara et al. 2018b; Markheiser et al. 2019). Other occasional patterns not previously described represent exceptions to these stereotypically repeated events. Therefore, in this work, for the nomenclature of the waveforms (thus the behavioral patterns), we adopted the one used by Cornara et al. (2018b) specifically for P. spumarius. Main waveforms produced by P. spumarius are reported in Fig. 1.

Acquisition behavior

After pre-screening, non-infective adult spittlebugs were transferred to two-week-old vetch plants (non-host of the ST53 strain used in this experiment), in groups of ten per plant, inside an insect-proof air-conditioned chamber (same conditions described for pre-screening) until the EPG-assisted AAP (Acquisition Access Period) (one to 14 days). The probing and feeding behavior of...
pre-screened insects on *X. fastidiosa* olive source plants was monitored through EPG, in order to identify the behavior(s) associated with bacterial acquisition. There were four treatments, with at least 30 replicates per treatment: interruption of the probe during pathway (C); interruption of the probe during xylem contact (Xc); one-hour AAP on an olive infected plant; and three hours of AAP on an olive infected plant (Table 1). *X. fastidiosa* source plants for the EPG-assisted AAP were three infected olives showing approximately the same vegetative conditions. The plants were trimmed at 30 cm of height one week prior to the beginning of the experiment and pruned leaving non-symptomatic green lateral shoots more suitable for meadow spittlebug settling and probing (Cornara et al. 2018a). For each of the infected source plants, we selected a middle non-symptomatic shoot; one week before the EPG, half of the leaves of the selected shoots were tested by qPCR (following the protocol by Loconsole et al. (2014)) and found positive to the bacterium. Additionally, at the end of the acquisition experiment, the shoots offered to the spittlebugs were re-tested by qPCR (pooling together the leaves and the stems); all the tissues selected for the acquisition tests showed similar bacterial population (ranging from 8.34E+04 to 3.38E+05 CFU/ml). We EPG-recorded three spittlebugs per time, each on one EPG channel and on one source plant, and all the three subjected to the same treatment (interruption of the probe during the waveform C, or Xc, or after 1 h or 3 h). Following the EPG-assisted AAP, each spittlebug was gently removed from the infected source plant with a paint brush, and caged on a non-infected periwinkle plant for an IAP of 96 h, inside an insect-proof air-conditioned chamber (25 ± 2 °C, 40% HR). At the end of the IAP, the insects were collected and stored in ETOH 70% at -20 °C; the receptor plants were maintained in an insect-proof air-conditioned chamber at 27 ± 2 °C 40% HR and watered twice a week. Insect infectivity and periwinkle infection status (the latter assessed ca. 40 to 60 days after the IAP) were tested by qPCR, following the protocols by Harper et al. (2010) and Loconsole et al. (2014). We considered acquisition as having occurred if at least one of the two samples per replicate (the insect and the receptor plant) tested positive by qPCR.

Fig. 1 EPG waveforms (behavioral patterns) displayed by *Philaenus spumarius*. **a** Waveform C (pathway); **b** waveform Xc (xylem contact/trial ingestion); **c** waveform Xi (xylem ingestion); **d** waveform N (interruption during the xylem activity); **e** waveform R (resting phase); **f** waveform Xe (spikelet burst). Time (s) is reported on the x axis; voltage (V) is reported on the y axis. Images a to e are derived from EPG recordings made with *P. spumarius* on olive plants; image f is derived from a recording made with *P. spumarius* on oleander.
Inoculation behavior

The spittlebugs were given a seven to ten-day AAP on five infected olive seedlings inside a Bugdorm-2 Insect tent (https://shop.bugdorm.com). Following the AAP on the infected olive plants, the spittlebugs were moved to healthy vetch plants until the EPG-assisted inoculation tests began (the spittlebugs remained caged on the healthy vetch plants approximately from two to 20 days after the AAP on infected olive plants). After tethering and connection to the EPG probe, each P. spumarius was placed on a 5 cm portion of a healthy olive seedling stem, having access to at least one leaf. The probing and feeding behavior of P. spumarius on olive receptor plants was monitored through EPG, in order to identify the behavior(s) (EPG’s waveform(s)) associated with bacterial inoculation. Each spittlebug was left probing until the occurrence of the waveform of interest; once the waveform occurred, the insect was removed from the receptor plant with a paint brush, and stored in ETOH 70% at -20 °C until the assessment of its infectivity. In the 2017 EPG experiments, there were four treatments with termination of probing during different EPG waveforms: pathway (C); xylem contact (Xc); xylem ingestion (Xi; from five to 15 min); and first interruption during xylem activity (N) (either during Xc or Xi) (Table 2A). After each replicate, the probed olive portion (ca. 1 cm) was marked with tape; the plants were stored in an insect-proof air-conditioned chamber at 27 ± 2 °C 40% HR and watered once a week. The recipient plants were tested three months after the EPG-assisted IAP; inoculation of bacterial cells into the xylem was assessed by qPCR on either the probed part of the seedling (2 cm portion, both stem and at least one leaf petiole) or a portion three to four cm distal to the former (2 cm portion, both stem and at least one leaf petiole). Moreover, in 2017 we had an additional treatment: some of the insects were given a one-hour EPG-assisted IAP (insects tethered and connected to the EPG) on the receptor test plant without artificially interrupting the probe during a certain waveform

Table 1 Acquisition behavior

| Acquisition | WDI*** | WDEI*** | NWEI*** |
|-------------|--------|---------|---------|
| Treatment   | n Replicates§ | Ps positive§§ | AAP | Succ pr | Unsucc pr | np | C | Xc | Xi | N | R |
| C           | 30     | 0       | 1h     | 1      | 0          | 28.5 | 13.7 | 1.5 | 14.8 | 2.3 | 1.2 |
| Xc          | 34     | 0       | 1h     | 1      | 0          | 14.5 | 4.56 | 1.5 | 4.93 | 0.19 | 1.2 |
| 1h          | 30     | 1       | 3h     | 1      | 3          | 6.05 | 3.94 | 1.1 | 61.4 | 0.2 | 0.8 |
| 3h          | 37     | 1       | 3h     | 1      | 3          | 47   | 42   | 11  | 16  | 12  | 1 |

§=number of replicates per each treatment; §§=number of spittlebugs that acquired Xylella fastidiosa; §§§=WDI, WDEI and NWEI calculated only for the spittlebugs that acquired (and retained) the bacterium.

Table “Acquisition” (on the left) summarizes the experimental design, the treatments, and the number of replicates, together with the number of spittlebugs that acquired the bacterium for each treatment. The tables on the right, report three EPG non-sequential variables (WDI, WDEI and NWEI) calculated for the two spittlebugs that acquired X. fastidiosa. WDI: waveform duration per individual. WDEI: waveform duration per event per individual. NWEI: number of waveform events per individual. The variables are described in Table 4. Time is expressed as minutes.
(Table 2A). The plants were maintained at the same conditions described above for the recipient plants used for waveform interruption IAP; plant status was ascertained by qPCR three months after the IAP, testing a portion of ca. 10 cm including the 5 cm stem/leaves exposed to P. spumarius probing. The sample was not split in two (probed and distal parts) as for the waveform interruption treatments, since the spittlebugs were allowed to make multiple probes. For the 2017 experiment, we performed 25 replicates for each waveform interruption treatment and 30 for the 1 h IAP, which included both infective and non-infective P. spumarius. Table 2A reports results only from replicates where spittlebugs were positive for X. fastidiosa by qPCR (with a Ct approximately ranging from 26 to 32). None of the recipient plants that were exposed to spittlebugs found to be non-infective according to qPCR results tested positive for X. fastidiosa.

In 2018, treatments in the inoculation tests were either interrupting the probe during the waveform of interest or giving the spittlebug an IAP of three hours on the olive recipient plant. The waveform interruption treatments were: pathway (C); xylem contact (Xc); xylem ingestion (Xi; from five to 15 min), either with or without xylem activity interruptions (N); interruption during xylem activity (N); and resting (R; from one to two minutes) (Table 2B). For the 2018 inoculation experiment, there were 30 to 60 replicates for each of the waveform-interrupted treatments and 90 replicates for the 3 h IAP. In Table 2B, we reported only

| Treatment | Sequence of events | Ps positive | Inoculation |
|-----------|--------------------|-------------|-------------|
| A)        |                    |             |             |
| Inoculation 2017 (olive) | | | |
| Waveform interruption | | | |
| C | C | 3 | 0 |
| Xc | C–Xc | 9 | 0 |
| Xi | C–Xc–Xi | 6 | 0 |
| N | C–Xc–N or C–Xc–Xi–N§ | 12 | 0 |
| 1 h | NA | 12 | 2 |
| B)        |                    |             |             |
| Inoculation 2018 (olive) | | | |
| Waveform interruption | | | |
| C | C | 17 | 0 |
| Xc | C–Xc | 16 | 0 |
| Xi | C–Xc–Xi | 21 | 0 |
| N | C–Xc–N or C–Xc–Xi–N§ | 15 | 0 |
| R | C–Xc–Xi–R | 19 | 0 |
| 3 h IAP §§ | NA | 63 §§ | 3 §§ |
| C)        |                    |             |             |
| Inoculation 2018 (oleander) | | | |
| Waveform interruption | | | |
| C | C | 8 | 0 |
| Xc | C–Xc | 1 | 0 |
| Xi | C–Xc–Xi | 8 | 0 |
| N | C–Xc–Xi–N | 7 | 0 |
| R | C–Xc–Xi–R | 1 | 0 |
| Xe | C–Xc–Xi or C–Xc–Xi–Xe | 5 | 3 |

“Sequence of events” stands for the sequence of behaviors performed by the insect before interrupting the probe (not shown for the 1-h and 3-h IAP). “Ps positive” stands for the number of replicates carried out with infective spittlebugs (as determined by qPCR) for each treatment. “Inoculation” indicates the number of plants inoculated with X. fastidiosa by the qPCR positive P. spumarius per each treatment. NA (not applicable) is used for the sequence of events of the treatments 1 h and 3 h, since the insects were given access to the plant without interrupting the probe after a precise event/sequence of events

§ = probe interrupted after the first N occurred; §§ = the spittlebugs performed from 1 to 3 xylem interruptions N; §§§ = calculated by pooling together the inoculation results from the 3 h IAP inoculation experiment (49 infective spittlebugs) and the comparison infective vs non-infective (14 infective spittlebugs)
the plants exposed to infective *P. spumarius* according to qPCR (with Ct values approximately ranging from 24 to 33). None of the plants exposed to non-infective spittlebugs (as determined by qPCR) tested positive for *X. fastidiosa*. In addition to olive, in 2018 we performed also EPG-assisted waveform interruption inoculation tests on 4-month-old oleander plants. As shown by Cornara et al. (2017b), *X. fastidiosa* inoculation rate to oleander by *P. spumarius* is greater than to olive, despite oleander being a very poor host for the spittlebug. Furthermore, *P. spumarius* on oleander performs single or repeated unconventional and “occasional” EPG signals different from the stereotypically repeated patterns (C, Xc, Xi, R, N) far more frequently than in olive and other plants (vetch, grapevine, cherry) (Cornara et al. 2018b; Markheiser et al. 2019). These unconventional EPG patterns, occurring from seconds to few minutes after the insect has reached the xylem vessel, include a spikelet burst similar to the B1s waveform described for sharpshooters (Backus et al. 2005, 2009; Joost et al. 2006) (Fig. 1f and Fig. 2a and b) and a voltage drop similar to N, but occurring during an initial resting phase alternated with low-frequency Xi (frequency ≤ 0.1 Hz) (Fig. 2c). Here, we grouped these two EPG patterns under a single treatment, provisionally termed Xe.

Therefore, for the waveform interruption inoculation tests on oleander, we added the treatment Xe to those described for olive (Table 2C); Xe was not produced by spittlebugs on olive during the waveform interruption experiments carried out either during 2017 or 2018.

For the 2018 EPG-assisted inoculation tests, and insect and plant maintenance, we followed the same protocol described above for the 2017 experiments. The waveform interruption recipient plants (both olive and oleander) were tested three months after the EPG-assisted inoculation tests; the presence of bacterial cells in the recipient plants was assessed by qPCR on either the probed part or a portion three to four cm distal to the probed part. For the olive recipient plants where *P. spumarius* had an EPG-assisted 3-h IAP on, we tested a portion of ca. 10 cm

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**Fig. 2** Xe waveform. **a** and **b** coarse structure of Xe (simple spikelet burst) in oleander following pathway C and xylem contact Xc, coarse structure; **c** coarse structure Xe (voltage drop) in olive following a resting phase alternated with very low-frequency xylem ingestion (≤ 0.1 Hz) Xi/R; **d** fine structure of the spikelet burst in **a**, **e** fine structure of the spikelet burst in **b**, **f** fine structure of the drop in **c**. Spikelet bursts in **a**–**c** are indicated with black arrows. Time (s) is reported on the x axis; voltage (V) is reported on the y axis.
including the 5 cm stem/leaves exposed to *P. spumarius* probing; as for the 1-h IAP of 2017, we did not split the sample in two.

For qPCR on insects and plants, we followed the protocols described by Harper et al. (2010) and Loconsole et al. (2014), respectively.

As explained above, during both 2017 and 2018, we added to the waveform interruption experiments further treatments, namely 1 h IAP (2017) and 3 h IAP (2018), without interruption of the probe during specific waveforms. We decided to use relatively short IAPs for two main reasons: (i) as remarked by Wayadande and Nault (1993), long feeding periods result in more switching from one behavior to another, making it difficult to know which behavior(s) is/are associated with pathogen inoculation; (ii) several indirect evidences suggest that *X. fastidiosa* inoculation might occur during the initial steps of the probe (Jackson et al. 2008; Daugherty and Almeida 2009; Backus et al. 2009; Backus 2016).

### Comparison of infective versus non-infective *Philaenus spumarius* probing behavior

To compare the feeding behavior of infective versus non-infective *Philaenus spumarius*, adult females were used. The insects were given a 10-day AAP on five infected olive seedlings inside a Bugdorm-2 Insect tent (https://shop.bugdorm.com). Following the AAP, the spittlebugs were moved to healthy vetch plants until the EPG-assisted IAP was considered infective if at least one of the two samples per each replicate (either the insect or the periwinkle recipient plant) tested positive for *X. fastidiosa* by qPCR.

**EPG procedure and data analysis**

For running the EPG tests, the insects were: (1) starved for one hour inside an aerated Petri dish; (2) slightly stunned by exposure to 4°C for ca. 30 s.; (3) immobilized with a cased diaphragm pump (Dymax 5, Charles Austen Pumps Ltd, Byfleet, Surrey, England/UK); and (4) tethered according to the protocol described by Cornara et al. (2018b). Briefly, the tip of an 18 µm gold wire, 3 cm long, was placed on the insect pronotum and glued with a double layer of silver conductive glue (Ted Pella, no. 16034; Pelco® Colloidal Silver, Ted Pella, Redding, CA, USA). The tip of the wire was bent in order to create a loop that enhanced the resistance of the connection. The other end of the wire had been attached previously with silver paint to a copper electrode measuring 3 cm in length × 1 mm in diameter. Thereafter, the electrode was plugged into the EPG probe, with the insect left hanging over the plant without touching it for ca. ten minutes before placing it on the plant. The soil copper electrode (10 cm long × 2 mm wide) of the EPG device was then inserted into the pot substrate. The system was assembled inside a Faraday cage, in an acclimatized room (25 ± 2°C), and under artificial light (20 W, 1200 Lm (lumen)). Probing and feeding behavior was recorded with a Giga 4-DC EPG device (EPG Systems, Wageningen, The Netherlands) with 1 gigaohm input resistance. Output from the EPG at 100 × gain was digitalized at a rate of 100 samples per sec. per channel and recorded using Stylet+ software (EPG Systems, Wageningen, The Netherlands). Substrate voltage was adjusted following the calibration instructions of the DC EPG equipment so that EPG signals fit into the +5 V to −5 V window provided by the software Stylet+. For the acquisition experiment (Table 1) and the comparison between infective and non-infective *P. spumarius* (Table 3), several sequential and non-sequential variables were calculated. The variables calculated and the abbreviations used in Tables 1, 2, and 3 are described in Table 4. EPG variables were calculated with an Excel Workbook developed purposely for *P. spumarius* waveforms by Antonio J. Alvarez (Universidad de Almeria, Spain) (Cornara et al. 2018b). Differences in probing and feeding behavior between infective and non-infective spittlebugs were assessed by Mann–Whitney *U* test. Statistical analysis was performed with the software R 3.5.2 (R Core Team 2015).

### Results

**Feeding behavior associated with the acquisition/retention of *X. fastidiosa* by *P. spumarius***

Two *P. spumarius* individuals only, one given 1-h AAP and the other given a 3-h AAP tested positive for *X. fastidiosa*...
by qPCR (Ct = 31.67 and 31.34) (Table 1); no transmission to periwinkle occurred. None of the spittlebugs whose probe was interrupted during pathway (C) or xylem contact (Xc) phases acquired and retained the bacterium. The extremely low acquisition rate did not permit any statistical inference; nevertheless, we analyzed the sequence of events and

**Table 3** Comparison of infective versus non-infective *P. spumarius* probing behavior

|                | Infective (n = 14) | Non-infective (n = 35) | Mann–Whitney |
|----------------|-------------------|------------------------|-------------|
|                | Min  | Max | Mean | SE (±)   | Min | Max | Mean | SE (±) | W  | p   |
| **WDI**        |      |     |      |         |      |     |      |         |     |     |
| np*            | 12.3 | 79.4 | 47.36 | 6.15    | 0.7 | 114.8 | 32.06 | 5.19    | 340 | 0.036 |
| C              | 1.1  | 47.2 | 11.16 | 4       | 1.1 | 33.7  | 8.26  | 1.29    | 228 | 0.707 |
| Xc             | 0.1  | 7.3  | 2.914 | 0.51    | 0.4 | 11.9  | 2.72  | 0.4     | 281 | 0.425 |
| Xi*            | 32.9 | 163.2 | 89.88 | 10.01   | 47.6 | 163.7 | 116   | 4.94    | 136 | 0.016 |
| N              | 0    | 2.5  | 0.63  | 0.21    | 0   | 6.4   | 1.25  | 0.24    | 171 | 0.100 |
| R              | 0    | 99.3 | 28.68 | 8.48    | 0   | 74.6  | 20.97 | 3.5     | 260.5 | 0.731 |
| **WDEI**       |      |     |      |         |      |     |      |         |     |     |
| np*            | 5.9  | 79.4 | 16.82 | 5.02    | 0.7 | 29.06 | 8.28  | 1.18    | 350 | 0.020 |
| C              | 0.52 | 5.721| 1.84  | 0.47    | 0.5 | 11.23 | 1.81  | 0.35    | 229.5 | 0.731 |
| Xc             | 0.1  | 7.3  | 1.64  | 0.48    | 0.3 | 11.9  | 1.57  | 0.37    | 283 | 0.400 |
| Xi             | 1.73 | 81.6 | 22.79 | 6.89    | 2.57 | 155.1 | 22.28 | 4.91    | 229 | 0.723 |
| Xi < 10 min    | 1.73 | 6.13 | 3.91  | 0.42    | 0   | 11.22 | 3.43  | 0.37    | 294 | 0.278 |
| Xi > 10 min    | 0    | 157.9 | 45.82 | 13.33   | 0   | 155.1 | 45.72 | 7.48    | 227 | 0.690 |
| N              | 0    | 0.62 | 0.22  | 0.05    | 0   | 0.4   | 0.2137| 0.018   | 241 | 0.928 |
| R              | 0    | 14.8 | 3.55  | 1.04    | 0   | 11.4  | 2.47  | 0.4     | 282 | 0.412 |
| **NWEI**       |      |     |      |         |      |     |      |         |     |     |
| np             | 1    | 9    | 4.00  | 0.65    | 1   | 11    | 4.17  | 0.49    | 245.5 | 0.991 |
| C              | 1    | 10   | 5.57  | 0.86    | 1   | 14    | 5.82  | 0.63    | 241 | 0.929 |
| Xc             | 0    | 5    | 2.07  | 0.35    | 1   | 5     | 2.17  | 0.19    | 231 | 0.747 |
| Xi             | 2    | 19   | 8.21  | 1.52    | 1   | 36    | 11.03 | 1.53    | 204 | 0.363 |
| Xi < 10 min    | 1    | 19   | 6.50  | 1.48    | 0   | 36    | 8.43  | 1.58    | 234.5 | 0.815 |
| Xi > 10 min*   | 0    | 4    | 1.71  | 0.28    | 0   | 6     | 2.6   | 0.22    | 152.5 | 0.032 |
| N*             | 0    | 8    | 1.86  | 0.60    | 0   | 21    | 4.8   | 0.88    | 146 | 0.027 |
| R              | 0    | 18   | 6.36  | 1.47    | 0   | 34    | 9.03  | 1.54    | 215 | 0.505 |
| **Sequential variables** |      |     |      |         |      |     |      |         |     |     |
| np to Xc       | 2    | 84   | 16.15 | 6.02    | 1.2 | 45.4  | 9.91  | 1.57    | 253.5 | 0.546 |
| np to Xi       | 2.4  | 85.3 | 17.08 | 5.89    | 1.5 | 46.5  | 11.87 | 1.6     | 253.5 | 0.851 |
| C to Xc        | 0.5  | 8.2  | 3.18  | 0.76    | 0.6 | 22.6  | 5.27  | 0.91    | 165.5 | 0.150 |
| C to Xi        | 1    | 12.7 | 4.99  | 0.98    | 1.1 | 24.4  | 7.22  | 1.01    | 187 | 0.199 |
| np to Xi > 10  | 6.6  | 68   | 25.13 | 4.42    | 1.5 | 118   | 31.46 | 5.49    | 209.5 | 0.891 |
| C to Xi > 10   | 2.2  | 63.8 | 17.79 | 4.49    | 1.1 | 117.4 | 26.68 | 5.38    | 187 | 0.671 |
| Time to the 1st probe* | 0.5  | 79.4 | 12.08 | 5.60    | 0.05 | 43.7 | 4.65  | 1.47    | 346.5 | 0.024 |
| Time to the 1st probe with Xi | 1.11 | 79.4 | 13.98 | 5.61    | 0.4 | 44.66 | 8.08  | 1.58    | 209.5 | 0.314 |
| Time to the 1st probe with Xi > 10 | 1.11 | 66.2 | 17.13 | 5.06    | 0.4 | 110.3 | 16.52 | 4.13    | 238 | 0.395 |
| **Others variables** |      |     |      |         |      |     |      |         |     |     |
| Succ pr        | 1    | 5    | 2.00  | 0.28    | 1   | 5     | 2.05  | 0.18    | 238.5 | 0.879 |
| Unsucc pr      | 0    | 7    | 1.78  | 0.57    | 0   | 10    | 2.03  | 0.41    | 234 | 0.799 |
| Tot pr         | 1    | 8    | 3.78  | 0.64    | 1   | 11    | 4.08  | 0.48    | 239.5 | 0.902 |
| Frequency Xi   | 2.78 | 6.68 | 4.40  | 0.30    | 0.23 | 0.65 | 0.43  | 0.01    | 247.5 | 0.956 |

WDI: waveform duration per individual. WDEI: average waveform duration per event per individual. NWEI: number of waveform events per individual. Sequential variables are variables related to a succession of events/behaviors. The EPG variables are explained in Table 4. Time is expressed as minutes

*Variables significantly different between infective and non-infective spittlebugs as indicated by Mann–Whitney U test
calculated non-sequential variables for the two replicates that acquired the bacterium, in order to have preliminary indications about the feeding activities associated with the acquisition and retention of *X. fastidiosa* (Table 1). Considering the acquisition that occurred in the 1 h treatment, the spittlebug performed a single probe of 31.2 min, of which 14.8 min spent in xylem ingestion and xylem interruption activities (2.3 of the 14.8 min of Xi were spent in N; 12 N waveforms were performed), and 1.2 min in resting. The spittlebugs that acquired the bacterium in the 3 h treatment performed a long xylem ingestion phase (122.8 min), with a single xylem interruption, and a resting phase that lasted 0.8 min.

**Feeding behavior associated with the inoculation of *X. fastidiosa* by *P. spumarius***

Considering the waveform interruption experiments, no inoculation to olive was obtained in 2017 and in 2018 by interrupting the probes during the occurrence of the patterns C, Xc, Xi (whether or not containing from one to three interruptions N), N, or R (Table 2A and B). On oleander, five *P.
spumarius positive for X. fastidiosa to qPCR produced the pattern Xe (namely one of these five spittlebugs performed a voltage drop, and 4 spittlebugs performed each a spikelet bursts). Three of these five spittlebugs, i.e., one performing a drop and two producing spikelet bursts, inoculated X. fastidiosa to the receptor plant (Table 2C). Both the probing and the distal parts of each of the inoculated oleanders were positive for the bacterium by qPCR, indicating that bacterial cells were released into the xylem. The voltage drop performed by the spittlebug that successfully inoculated the recipient plant occurred 7 min after the beginning of the probe and 3 min after the first contact with xylem. The two spikelet bursts in the spittlebugs that inoculated the plants occurred two and two and a half minutes after the beginning of the probe, and 0.5 and 1 min after the xylem contact. No inoculation to oleander occurred with the other patterns tested (Table 2C).

Considering the 1-hour IAP on olive, the two spittlebugs (out of the 12 infective insects) that were able to infect the plants were the only ones that produced an Xe pattern (one voltage drop occurring 6 min after the onset of the probe and 5 min after the contact with xylem; one spikelet burst performed 2.5 min after the beginning of the probe and 2 min after the xylem contact). Finally, considering the 3-h IAP, one inoculation out 49 infective P. spumarius was obtained. The inoculative spittlebug was one of the only three insects (out of the 49 infective) producing Xe (a drop in R occurring 3 min after the onset of the probe, 1 min after the first contact with xylem); the other two, producing spikelet bursts, did not transmit X. fastidiosa to the host plant.

**Comparison of infective versus non-infective Philaenus spumarius probing behavior**

We included in the analysis only clear recordings (without noise or unclear signals) performed by P. spumarius that: (i) remained on the plant for the 3 h of EPG without breaking the wire and escaping or falling off of the host; (ii) were alive and active at the end of the IAP on periwinkle; and (iii) probed the tissue at least once during the recording. By these criteria, 49 P. spumarius females, 14 infective and 35 non-infective were selected for statistical analysis. Nine out of 14 P. spumarius positive to the bacterium by qPCR transmitted X. fastidiosa to the periwinkle recipient plants. Two out of the 14 infective spittlebugs inoculated the fastidious bacterium during the EPG-assisted three-hour IAP to olive; the limited number of inoculations did not permit any statistical inference. Looking at the behavioral patterns displayed during the probes, the waveform Xe was performed only by the two insects that inoculated X. fastidiosa to olive (one spittlebug producing a drop and one a spikelet burst both occurring ca. 4 min after the beginning of the probe and 3 min after the xylem contact). A third spittlebug performing a spikelet burst did not inoculate the bacterium.

Sequential and non-sequential variables calculated for the infective and non-infective spittlebugs are reported in Table 3. Infective P. spumarius spent significantly longer time in non-probing (W = 340, p = 0.036) and shorter time in xylem ingestion (W = 136, p = 0.016) activities compared to non-infective spittlebugs. Furthermore, we observed also that the average duration of the single non-probing events in infective insects was almost twice the value recorded for non-infective ones (W = 350, p = 0.020). Moreover, infective P. spumarius performed significantly fewer sustained xylem ingestion events, i.e., xylem ingestions longer than 10 min (W = 152.5, p = 0.032) and interruptions of the xylem activity (waveform N) (W = 146, p = 0.027) than non-infective. Finally, infective spittlebugs required longer time to perform the first probe compared to individuals not carrying the bacterium (W = 346.5, p = 0.024).

**Discussion**

The data presented here can guide further attempts to determine the vector feeding behaviors necessary for P. spumarius transmission of X. fastidiosa to plants. Our principal conclusions were that: i) spittlebug acquisition rate appeared to be extremely low, and bacterial cells binding to the foregut might occur in a time as short as 15 min spent by the insect performing xylem ingestion, or other activities interspersed with xylem ingestion (interruption or resting); ii) inoculation of bacterial cells into the host plant xylem by P. spumarius was associated with an early and very occasional waveform that we provisionally termed Xe (that occurred ca. 2 to 7 min after the onset of the probe). The common feeding behavioral patterns, i.e., C, Xc, Xi, N, and R, which the spittlebugs stereotypically repeat during most of the probes, were not associated with bacterial cell delivery to the host plant. Our hypothesis is that Xe waveform likely represents egestion of fluids regulated by the pre-cibarial valve fluttering following a possible lack of insect phagostimulation. However, the low inoculation rate displayed by P. spumarius during our experiments makes it difficult to draw a definitive conclusion about the exact behavior associated with bacterial cell inoculation, and more research efforts are needed; iii) probing and feeding behavior of infective P. spumarius differed from the one of the non-infective spittlebugs. The EPG analysis showed that infective P. spumarius had more difficulties than non-infective ones in feeding on a non-infected host plant.

**Feeding behavior associated with acquisition/retention of X. fastidiosa**

The interaction between two main factors makes X. fastidiosa acquisition and retention within the vector foregut a relatively rare event: First, the bacterium is unevenly distributed within
the plant; thus, for acquisition (uptake) to occur the insect should probe from one of the vessels colonized by the bacterium (Hopkins 1981; Newman et al. 2003; Cardinale et al. 2018); second, the xylem sap flows within the insect foregut at an extremely high velocity, generating turbulence, thus hindering the bacterial cell attachment (Purcell et al. 1979; Dugravot et al. 2008). Therefore, even if the insect lands on an infected plant, and probes a vessel containing *X. fastidiosa* cells, most of the cells up taken would be swallowed without being retained in the pre-cibarium (Retchless et al. 2014). However, it is expected that long access periods could increase the probability of vector–pathogen encounters, overall increasing the acquisition rate (Almeida 2016). Our data suggest that *X. fastidiosa* acquisition and retention by *P. spumarius* do not necessarily require very long probe, and likely occur during xylem ingestion (waveform Xi) from infected vessels; a xylem ingestion as short as 15 min is sufficient for successful binding. Therefore, acquisition and successful retention might occur during the xylem ingestion, with cell binding during the simultaneous collapse of the cibarial diaphragm and closure of the pre-cibarial valve sealed by the bell-like invagination (Ruschioni et al. 2019), or during activities interspersed with xylem ingestion, namely xylem interruption N (a single interruption could be sufficient) or resting. The extremely low acquisition rate we observed for the meadow spittlebug is consistent also with previous data by Cornara et al. (2016). However, our experiments did not permit any statistical inference or draw conclusions about the precise behavior(s) or sequence of events leading to *X. fastidiosa* acquisition. Furthermore, the low acquisition rate could have been influenced by the relatively low bacterial population within our olive source plants, given the positive correlation between *X. fastidiosa* population within the infected plant and the transmission efficiency ((Hill and Purcell 1997). Nevertheless, high *X. fastidiosa* population lead to symptoms development, and the vectors tend to discriminate against symptomatic plants (Marucci et al. 2005; Miranda et al. 2013; Zeilinger and Daugherty 2014; Del Cid et al. 2018), thereby considering our scenario, consisting of infected but non-symptomatic plants bearing a bacterium population still too low to cause severe symptoms and consequent reduction in host plant attractiveness, the most epidemiologically realistic for inferences on acquisition dynamic. However, further experiments either with olive or with other host plants should be conducted to deepen our knowledge about the mechanism of acquisition of *X. fastidiosa* by *P. spumarius*, and about how and where bacterial cells do initially bind to the spittlebug foregut.

**Feeding behavior associated with the inoculation of *X. fastidiosa***

*X. fastidiosa* cell delivery into the xylem vessels by *P. spumarius* was associated with the occurrence of a pattern that we provisionally called Xe, as demonstrated by: (i) the successful inoculation to oleander plants only when the probe was interrupted in correspondence of this particular pattern; (ii) the only inoculations to olive occurred during IAPs where the spittlebugs engaged in Xe; and (iii) the lack of inoculation with the other behavioral patterns tested either on olive or on oleander. In other words, whenever there was infection of test plants, spittlebugs always made at some point an Xe waveform on receptor test plants. The spittlebug performed this specific behavior 2 to 7 min after the beginning of the probe and 0.5 to 5 min after the first contact with the xylem. Furthermore, in all the observed cases in olive, the pattern Xe was always preceded by waveforms C (pathway), Xc (xylem contact), and Xi (xylem ingestion activity); in oleander, Xe was preceded by the sequence of events C–Xc in two out of three inoculative probes and by C–Xc–Xi in the other positive case. The pattern Xe was never preceded by the xylem interruption N waveform. No inoculation occurred when infective spittlebugs probe was interrupted during waveforms C, Xc, or Xi, neither in olive nor in oleander. Under the term Xe, we grouped two apparently different EPG signals, a voltage drop occurring during a period where resting (R) alternates with low-frequency Xi (frequency ≤ 0.1 Hz) (Fig. 2c, f) and a “simple” spikelet burst (Figs. 1f and 2a, b, d, e). The common element between the two signals is the presence of spikelet bursts (indicated with arrows in Fig. 2a–c), characterized by highly variable frequency (3 to 10 Hz) and amplitude (4 to 25%). During voltage drops, spikelet bursts were repetitive and no longer than 1–2 s, while the duration of the “simple” spikelet bursts ranged between 6 and 17 s. This similarity suggests that the inoculation of *X. fastidiosa* cells into the plant by *P. spumarius* could be associated with the spikelet bursts occurring when the insect stylets are located in a xylem vessel, after having built the salivary sheath, penetrated through the plant tissues reaching a xylem vessel, and after a first tasting of the host plant suitability through the pre-cibarial chemosensilla. According to Joost et al. (2006) and Backus et al. (2009), spikelet burst (termed B1s in these and in further works on sharpshooters performed by Backus and colleagues) represents an insect internal activity, possibly streaming potentials (Walker 2000) caused by pre-cibarial valve movements denoted as fluttering. This behavior is interspersed during the probe and occurs frequently during the pathway phase before reaching the xylem vessel; its occurrence may therefore be associated with movements of the pre-cibarial valve during tasting of the host plant (Backus et al. 2009; Backus and McLean 1982; Backus 1985). For a thorough review of the different waveform subtypes in sharpshooters, refer to Backus (Backus 2016). As shown in our experiment, at least for *P. spumarius*, the occurrence of spikelet bursts when the insect stylets are located in a xylem vessel, thus putative pre-cibarial valve fluttering within the xylem vessel pushing
bacterial cells out of the food canal possibly helped by the tension of the xylem fluid while the insect is feeding, may lead to X. fastidiosa inoculation. Pre-cibarial valve involvement in X. fastidiosa inoculation has also been proposed by other authors (Purcell et al. 1979; Almeida and Purcell 2006). Spikelet bursts are also major components of the X waveform found to be associated with the inoculation of the Maize Chlorotic Dwarf Virus (MCDV, Waikavirus), a semi-persistent virus sharing with X. fastidiosa the characteristic of being foregut-borne ((Childress and Harris 1989; Ammar and Nault 1991; Wayadande and Nault 1993). Wayadande and Nault (1993) suggested that the biological meaning of the X waveform is egestion (sensu Harris (1977); termed extravasation by McLean and Kinsey (1984)), the delivery of plant fluids present within the food canal anterior to the cibarial pump back to the stylets and then into the plant, occurring when the plant fluid itself fails to induce phagostimulation (McLean and Kinsey 1984).

Theoretically, valve fluttering occurring during the voltage drop inside a resting/low-frequency Xi phase (when stylets are inside the xylem), even if shorter than “simple” fluttering (not occurring during a drop), would generate a force sufficient to egest bacterial cells from the foregut to the plant. Indeed, during the resting phase, insect cibarial (and pre-cibarial) muscles are either not contracting, or contracting at a very low frequency (<0.1 Hz) (Cornara et al. 2018b). Theoretically, the slower a muscle contracts, the greater the internal tension, thus the greater the force it can generate (Malone et al. 1999; Sutton and Burrows 2018). Therefore, if the fluttering occurs after resting, the force generated (likely by the pre-cibarial valve) would be sufficient to propel bacterial cells toward the xylem vessels even if the behavior is performed for a short period. Therefore, the Xe waveform may represent the opening and likely fluttering of the pre-cibarial valve that propels the bacterial cells toward the xylem vessel possibly helped by the negative tension of the xylem sap.

Considering the spittlebugs that had 3 h of IAP without interruption of the probe (both the 49 infective spittlebugs in the inoculation experiment and the 14 individuals in the behavioral comparison; Table 2B), this behavior (Xe) was performed by six out of the 63 infective individuals, leading to successful inoculation in three cases. Therefore, Xe represents a relatively occasional/relatively rare behavior (on olive), given that in our experiment, ca. 9.52% (six out of 63, data not shown in the table) of the infective individuals performed it, and only a half of these individuals (three out of 63, ca. 4.76% of the infective spittlebugs) inoculated the bacterium. This inoculation rate is consistent with data on P. spumarius bacterium inoculation to grape, with one out of 30 plants infected by single insects given an IAP of either 1.5 or 4.5 h (Cornara et al. 2016). The association of X. fastidiosa inoculation by the meadow spittlebug with a relatively infrequent/occasional behavior is also consistent with the occasional transmissions to grapevine during spittlebugs sequential daily transfer to healthy recipient plants reported by Severin (1950) (infection rate ranging from 5 to 16%). In fact, transmission rate of X. fastidiosa by P. spumarius is much more inefficient than the rate of transmission of other foregut-borne plant pathogens such as Beet yellows virus (which is close to 50% by a single aphid) (Jiménez et al. 2018). Therefore, data presented here, supported by the observations by other authors described above, suggest that P. spumarius likely inoculates X. fastidiosa during the pattern Xe occurring just few minutes after the beginning of the probe, and that, at least on suitable plants as olive, this behavior is a relatively rare event different from the patterns stereotypically repeated by the insect during most of the probes (namely C, Xc, Xi, N, R). Overall, considering not only the Xe behavior, but also the sequence of events preceding it, we propose that the behavior leading to X. fastidiosa inoculation into the host plant by P. spumarius is egestion driven by pre-cibarial valve fluttering resulting from a failure of insect phagostimulation following the tasting of the host plant xylem sap, possibly helped by xylem fluid tension while the insect is feeding. Moreover, as discussed in the materials and methods section, such unusual behavior occurs more frequently in oleander than in olive (observed by Cornara et al. 2018b and Markheiser et al. 2019). Cornara et al. (2017b) reported that P. spumarius transmission rate to oleander is far greater than to olive, although all the insects on the former host died within 24 h from caging. Therefore, transmission is apparently enhanced if the spittlebug is forced to feed on an unsuitable substrate, possibly because lack of phagostimulation (or feeding deterrence) and subsequent egestion would be more likely to occur on a less—or not—acceptable host. This hypothesis is also supported by increased rate of transmission by Homalodisca virtipennis Germar (1821) (Hemiptera: Cicadellidae) caged on grapevines treated with the insecticide pymetrozine (Bextine et al. 2004). Therefore, the behavior associated with X. fastidiosa inoculation could be triggered by conditions of the host plant unfavorable for the insect; the identifications of such factors, whether related to the host plant, to the vector, or to the interactions between the two elements, deserve further investigation. Furthermore, the frequency of Xe may also increase because of the presence of the bacterium in the foregut, but this needs further investigation.

**Comparison of infective versus non-infective* Philaenus spumarius* probing and feeding behavior**

Plant pathogens influence the transmission process, i.e., the recruitment of the vector on the infected plants for acquisition and the successive dispersal for inoculation, via effects on plant or vector phenotypes that modify the nature and the
frequency of the interactions between them (Mauck 2016; Mauck et al. 2018). To be categorized as parasite manipulation, a documented effect of a plant pathogen on its vector should: 1) enhance or create conditions expected to enhance transmission and 2) be under genetic control of the pathogen (Mauck et al. 2019). Vector transmission may be enhanced by the pathogen through indirect effects, i.e., effects on host derived sensory cues (Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004; Mauck et al. 2010; Shapiro et al. 2012), or direct effects on insects behaviors such as probing and host searching/dispersal (Stafford et al. 2011; Ingwell et al. 2012; Moreno-Delafuente et al. 2013; Martini et al. 2015). The same effects can be induced by highly divergent pathogens sharing the same mechanism of transmission (Mauck 2016; Stafford et al. 2011; Lefevre and Thomas 2008). According to Moreno-Delafuente et al. (2013), persistent circulative viruses are more likely to influence vector behavior given that the vector–pathogen relationship lasts for the entire insect life span, although semi-persistent viruses effects on vector behavior have been documented (Lu et al. 2017; Pereira et al. 2019). Mauck et al. (2019) suggest proteins encoded by pathogens to facilitate interaction with their vectors following acquisition may be co-opted to induce behavioral changes that enhance transmission. X. fastidiosa fulfill both the previously mentioned “requirements,” being persistent in its vectors (Severin 1950; Purcell and Finlay 1979), and encoding proteins necessary for interacting with the insect vector (Killiny and Almeida 2014). Additionally, the fact that X. fastidiosa exploits the cuticle of its vectors as a substrate for multiplication suggests a parasitic relationship, with a negative impact of the bacterium on the insect (Labroussaa et al. 2017). As observed in our experiment, the probing and feeding behavior of infective P. spumarius females significantly differs from that of non-infective ones. The main affected behaviors were non-probing and xylem ingestion, with an overall longer time spent in non-probing and a shorter time spent in xylem ingestion by infective insects. Particularly, P. spumarius carrying X. fastidiosa showed evident difficulties in performing sustained xylem ingestion (ingestion longer than ten minutes), with fewer events compared to healthy insects. Furthermore, infective spittlebugs showed a duration of individual non-probing events twice that of non-infective insects, fewer xylem interruptions N, and longer time before probing the host plant for the first time compared to insects not carrying the bacterium. Taken together, these observations suggest difficulties in feeding caused by the presence of X. fastidiosa within the foregut, similarly to what has been recently hypothesized by Ranieri et al. (2019), possibly caused by a mechanical obstruction of the food canal. However, a biological effect caused directly by X. fastidiosa on the insect aimed at creating a favorable environment for the bacterium within its vector cannot be ruled out. Indeed, longer non-probing alternated with short xylem ingestion; thus, longer period with almost no muscle contraction, sap flow or turbulence, would represent a perfect condition for bacterial cells to bind, multiply, and colonize the foregut. Such manipulation could either affect vector fitness or be conducive for transmission. Indeed, as observed for example in mosquitoes bearing the malaria Plasmodium, the vector could respond to difficulties in feeding by increasing the number of probes (Lefevre and Thomas 2008). Since, as observed in this study, inoculation of bacterial cells by the meadow spittlebug can occur just a few minutes after the beginning of the probe and is possibly associated with an occasional event (Xe), an increased number of probes could theoretically increase the probability of the inoculation behavior to occur and thus the overall inoculation rate.

Conclusions and further perspectives

Recent researches on vector–pathogen relationship disruption (Killiny et al. 2012; Labroussaa et al. 2016), bacterium biological control (Baccari et al. 2018), and sources of resistance (Gianpetruzzi et al. 2016) offer promising perspectives for a sustainable and effective X. fastidiosa disease control. However, with regard to the European outbreaks of the bacterium, these perspectives are limited by our lack of knowledge about several pivotal aspects of the epidemics, especially concerning the spittlebug–bacterium interaction and the spittlebug-mediated transmission mechanism. Here, we began to shed some light on X. fastidiosa transmission dynamics by P. spumarius, opening at the same time new challenging questions. For example, the identification of conditions triggering the putative egestion behavior associated with bacterial cell inoculation would have interesting implications on sustainable control strategies. The X. fastidiosa inoculation behavior should also be characterized on other vector–host plant–strain combinations. Furthermore, an in-depth characterization and description of the waveform Xe and its subpatterns is absolutely needed.

Considering the relatively low acquisition and inoculation rates displayed by P. spumarius, an effective control of the meadow spittlebug populations could result in a significant reduction in the risk of X. fastidiosa spread. Indeed, according to Irwin and Ruesink (1986), vector intensity depends on vector propensity (innate ability of the vector to transmit a certain pathogen) and vector activity (number of insect vectors alighting on the host plant for a certain period of time); therefore, a reduction in vector activity would lead to a decrease in vector intensity. However, several aspects related to vector ecology should be investigated in order to develop a sustainable long-term X. fastidiosa management strategy: (i) vector population abundance within the orchard; (ii) factors driving vector
host selection and within-host plant preference; (iii) vector aggregation and dispersal dynamics; and (iv) influence of landscape on vector population dynamics (Santoiemma et al. 2019; Bodino et al. 2019).

Finally, other challenging questions come from our finding about differences in probing and feeding behavior between infective and non-infective P. spumarius: we discussed above how these differences could be beneficial to the bacterium and detrimental for the spittlebug. However, we recognize the limits of our experimental approach. (We used only females, monitored for a relatively short period (3 h) and with the bacterium acquired from the infected plant.) This does not permit drawing conclusions about pathogen manipulation exerted by the bacterium on the spittlebug. More research efforts should be put in place to thoroughly characterize the intimate X. fastidiosa–P. spumarius interaction. First, possible plant effects on the behavioral manipulation should be excluded by artificial acquisition of the bacterium; second, observations should be extended to males and to the entire adult life span, also increasing the duration of the IAP; third, it should be verified if such behavioral effect is under genetic control of X. fastidiosa.

Author contribution

DC and AF conceived research, DC and MoM conducted experiments, DC, MaM and EG analyzed the data, DC wrote the manuscript, MoM, MaM, EG, AM, MS and AF reviewed and edited the manuscript, and AM, MS and AF secured funding. All authors read and approved the manuscript.

Acknowledgements We are deeply thankful to Enzo Manni and Federico Manni (Coop. ACLI-Racale) for the use of the rearing and transmission facilities, and helpful discussions about sustainable containment strategies of X. fastidiosa in Salento (Apulia, South Italy). We acknowledge Francesco Palmisano, Crescenzia Dongiovanni, and Giulio Fumarola (CRSFA-Basile Caramia) for plants rearing and support in field activities. We also acknowledge Giuseppe Altamura and Vincenzo Cavalieri (IPSP-CNR Bari) for technical support in laboratory analysis. An additional thank to Alexander Purcell, Adam Zeilinger, Nicola Bodino and Anna Markhaiser for helpful discussions on early experimental scheme and data analysis. This work has been financially supported by European Union Horizon 2020 research and innovation program under Grant Agreements No. 727987 XF-ACTORS (Xylella Fastidiosa Active Containment Through a multidisciplinary-Oriented Research Strategy).

Data availability statement Additional data will be furnished by the authors upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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