Seasonal Fluctuations of Sap-Feeding Insect Species Infected by Xylella fastidiosa in Apulian Olive Groves of Southern Italy

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Abstract

A study on seasonal abundance of Auchenorrhyncha species and their infectivity by Xylella fastidiosa in the Apulia region of Italy was conducted to identify ideal periods for monitoring and adoption of potential control measures against insect vectors. Adult populations of Auchenorrhyncha species were monitored monthly over a 2-yr period from five olive groves. A total of 15 species were captured, identified, and tested for presence of X. fastidiosa by polymerase chain reaction (PCR). For three species, Philaenus spumarius L., Neophilaenus campestris (Falle`n), and Euscelis lineolatus Brulle´, positive reactions to X. fastidiosa were obtained, on average, in 16.3, 15.9 and 18.4% of adult insects, respectively. Philaneous spumarius was the dominant species (39.8% of total Auchenorrhyncha captured) with the highest adult abundance in summer months. Adult P. spumarius and N. campestris were first detected between March and May in both years, and all insects tested during these periods (year 1: n¼42, year 2: n¼132) gave negative reactions to X. fastidiosa by PCR. Similarly, first adults of E. lineolatus that appeared from October to November (year 1: n¼20, year 2: n¼15) tested negative for presence of X. fastidiosa. Given the lack of transstadial and transovarial transmission of X. fastidiosa and considering that P. spumarius is univoltine, control measures against nymphal stages of P. spumarius should be investigated as means of population suppression to reduce spread of X. fastidiosa in olive groves.

Key words: Philaenus spumarius, Auchenorrhyncha, vector, plant disease, PCR

Xylella fastidiosa is an economically important bacterial pathogen of several commercial crops. Among the diseases caused by X. fastidiosa, particularly important are Pierce's disease of grapevine (Davis et al. 1978), citrus variegated chlorosis (Chang et al. 1993), phony peach (Wells et al. 1983), plum leaf scald (Raju et al. 1982), and leaf scorch of coffee (Li et al. 2001), pecan and almond (Mircetich et al. 1976, Sanderlin and Heyderich-Alger 2000). Pathogenicity varies according to the bacterial strain and host plant species. The bacterium invades xylem vessels and blocks transport of water and soluble mineral nutrients. Affected plants typically show symptoms of drying, leaf scorching, wilting of the foliage and, in the case of grapevines, plant death (Janse and Obradovic 2010).

In California, X. fastidiosa subspecies multiplex has been found naturally infecting olive trees. Furthermore, a strain belonging to subspecies fastidiosa was shown to infect olive trees under experimental conditions (Krugner et al. 2014). In southern Italy, X. fastidiosa subspecies pauca has been found in olive trees affected by a disease named "olive quick decline syndrome" (OQDS), which include symptoms of leaf scorching and branch dieback (Saponari et al. 2013). Quarantine measures aimed to counteract its spread in the Euro-Mediterranean area have been implemented since July 2014 (European Food Safety Authority [EFSA] 2015).

Xylella fastidiosa is a vector-borne pathogen transmitted by several species of Auchenorrhyncha, mainly in the Cicadellidae (sharpshooters) and Aphrophoridae (spittlebugs). Being a xyleminhabiting bacterium, the vector range of X. fastidiosa is restricted to xylem fluid-feeding insects. When acquired by insects, bacteria attach to and multiply in the food canal (precibarium) and pumping chamber (cibarium) in the foregut of the insect vector (Hill and Purcell 1995). There is no transstadial or transovarial passage of the bacterium. Transmission is characterized by the lack of a latent period and only few bacterial cells are required for the transmission. When acquired by adult insects, X. fastidiosa is noncirculative, propagative, and transmitted persistently (Purcell and Finlay 1979, Hill and Purcell 1995).

A list of all known vectors of X. fastidiosa was reported by Redak et al. (2004), although the following cicadellids seem to be the most important in the Americas: Homalodisca vitripennis (Germar), Draeculacephala minerva Ball, and Graphocephala atropunctata (Signoret) in California (Hill and Purcell 1995, Hail et al. 2010) and Bucephalogonia xanthophis (Berg), Acrogonia citrina (Marucci and Cavichioli), Dilobopterus costalimai Young, and Oncometopia facialis (Signoret) in Sao Paulo state, Brazil (~ Lopes and Krugner 2016). The Italian species, Cicadella viridis

L. (Hemiptera: Cicadellidae) and Philaenus spumarius L. (Hemiptera: Aphrophoridae), were considered potential vectors of the bacterium in Europe according to trophic characteristics (Janse and Obradovic 2010), but a list of potential vectors could be extended to all xylem fluid-feeding insects (Purcell 1989).

Recent investigations in the Apulia region of Italy showed that P. spumarius, Neophilaenus campestris (Falle`n) (Hemiptera: Aphrophoridae), and Euscelis lineolatus Brulle´ (Hemiptera: Cicadellidae) were able to acquire and retain the bacterium, and may be targets to monitor both the geographical distribution and population dynamics of X. fastidiosa (Elbeaino et al. 2014). Among these three species, only P. spumarius was reported as a vector of X. fastidiosa in Italy (Saponari et al. 2014). Considering that containment of X. fastidiosa spread is mainly based on vector control, further investigations are needed to identify other possible species capable of acquiring and transmitting the bacterium and to determine the optimal seasonality of vector control strategies. Therefore, in this study, a 2-yr investigation was performed to determine seasonal abundance of Auchenorrhyncha species in Apulian olive groves and prevalence of X. fastidiosa in insects. These data are required to develop an effective vector control strategy.

Materials and Methods

Capture and Identification of Insects

From October 2013 to September 2015, Auchenorrhyncha insects were collected monthly from four olive groves located in the Lecce province of Apulia and known to have high incidence of X. fastidiosa. As negative controls for the bacteria detection assays described below, additional insects were captured from an olive grove located in Bari province, where X. fastidiosa was known to be absent based on preliminary surveys. A circular sweep net (38 cm diameter) was used to collect insects from 10 randomly selected locations per grove. In each location, samples were collected from an olive tree and ground vegetation under the tree canopy. Trees were sampled by shaking four branches, from four different sides of the tree in the sweep net, whereas samples from the ground vegetation were collected by 10 sweeps. Captured insects were collected on site using an aspirator, placed in vials containing 70% ethanol, and transported to the laboratory for identification. All adults of Auchenorrhyncha species collected were identified. The taxonomic classification of captured insects was based on Ribaut (1952), Della Giustina (1989), and Holzinger et al. (2003). For species identification, male genitalia was dissected and kept in KOH (10%) for 24h, and then mounted on glass slide in Faure's liquid and observed under stereoscopic microscope. Due to the multiplication and accumulation of X. fastidiosa cells in the foregut (Janse and Obradovic 2010), only the head of each adult specimen was detached from the body, as described by Bextine et al. (2004), and used for the DNA extraction method described below.

Extraction of Xylella fastidiosa DNA

Insect heads were macerated individually using a sterile pestle in a tube containing 200ll of extraction buffer (Triton X 1%, Tris-HCl 20mM, EDTA 2mM). After the homogenization, 180ll of suspension were transferred to a new tube and left to incubate at 60C for 30min to allow complete lysis of cells. Two hundred microliters of chloroform-isoamyl alcohol (24:1) were added to the suspension and centrifuged at 12,000g for 10min. DNA was precipitated by adding 1vol of cold isopropanol and incubated overnight at 20C. After a new centrifugation at 20,000g for 20min, the pellet was washed with 70% ethanol, dried, and finally resuspended in 20ll of sterile water.

DNA Amplification

DNA extracts from insect samples were tested by PCR with RST31 and RST33 specific primers targeting the RNA polymerase sigma-70 factor genomic area and generating an amplicon of 733bp (Minsavage et al. 1994). DNA extracts from midveins of X. fastidiosa-infected olive leaves were used as positive controls. Each PCR mixture contained template DNA at 10ng/ll, 1.25 U of Go Taq polymerase (Promega, Madison, WI), 1 Go Taq Flexi DNA Buffer, 0.2mM dNTPs, 0.3lM forward and reverse primers, all in a final reaction volume of 25ll. Samples were put in the thermocycler (IQ5 Thermocycler, BioRad, USA) with the following program: initial denaturation at 94C for 4min, 35 cycles at 94C for 30s, 55C for 30s, and 72C for 40s, and a final extension at 72C for 7min. Products were visualized by ethidium bromide staining after electrophoresis in 1.2% agarose gel.

Data Analysis

Olive orchards were selected based on three characteristics: short distance from each other, same olive tree variety, and absence of insecticide applications. Data on insect abundance and prevalence of X. fastidiosa-infected insects from each orchard were combined for analysis. Species dominance was characterized according to the categories described by Tischler (1949): eudominant, more than 10% of the total number captured; dominant, between 5 and 10%; subdominant, between 2 and 5%; rare, between 1 and 2%; subrare, less than 1%. Mean separation for species abundance comparison was made with one-way analysis of variance (ANOVA) followed by

Tukey's honestly significant difference (HSD) test.

To compare abundance of captured species and elucidate relationships with rate of infection along a single vegetative season (12 mo), the mean of each monthly collection (M_c) and X. fastidiosa-infection (M_i) were calculated applying the following formulas:

where N_1 and X_1 were, respectively, the number of individuals captured and the number of individuals infected per species per month in the first year, while N_2 and X_2 were the number of individuals captured and the number of individuals infected of the same species and same month in the second year of study. In addition, the total monthly collection of insects (M_cT), which was the sum of M_c per each species, was calculated. The correlation between seasonal abundance (M_c) and incidence of infected specimens (M_i) for species found to harbor the bacterium was evaluated on the basis of Pearson's (multiple) correlation coefficient (r). Data analysis described above and graphs were treated using the statistical software STATISTICA (version

7.0.61.0).

Results

Identification and Abundance of Insects

Overall, 2,398 adults of Auchenorrhyncha were captured. Fifteen species were identified, belonging to two infraorders (Cicadomorpha and Fulgoromorpha) and five different families (i.e., Aphrophoridae (two species), Cercopidae (one species), Cicadellidae (10 species), Issidae (one species), and Flatidae (one species); Table 1). The Tischler's abundance classification in classes of abundance revealed three species as eudominant, i.e., P. spumarius (39.8%), Agalmatium flavescens (Olivier) (Hemiptera: Issidae) (15.1%), and Thamnotettix zelleri (Kirschbaum) (Hemiptera: Cicadellidae) (11.9%); three as dominant, i.e., N. campestris (8.6%), E. lineolatus (8.1%), and Anoplotettix putoni Ribaut (Hemiptera: Cicadellidae) (7.2%); one as subdominant, i.e., Synophropsis lauri (Horvart) (Hemiptera: Cicadellidae) (3.6%); three as rare, i.e., Exitianus capicola (Stal) (Hemiptera: Cicadellidae) (1.6%),€ Allygus modestus Scott (Hemiptera: Cicadellidae) (1.2%), and Fieberiella florii (Stal) (Hemiptera: Cicadellidae) (1.1%), while the remainder of€ the captured species were classified as subrare with less than 1% abundance (Table 1). Philaenus spumarius was clearly the most captured species in the olive groves (Fig. 1).

Occurrence of Auchenorrhyncha was mainly concentrated in the period from March to October with the only exception being E. lineolatus, which was prevalent from November to February. During two years of the study, the highest number of Auchenorrhyncha adults captured was in September ($M_cT¼256$), whereas May was the month with the highest species richness (10 out of 15 species collected; Table 2).

Detection and Seasonal Fluctuations of X. fastidiosa Infection in Captured Insects

Only three of 15 species collected were found positive for presence of X. fastidiosa by PCR: P. spumarius, N. campestris, and E. lineolatus. In the X. fastidiosa-infected areas, a total of 225 out of 2,398 adults (9.3%) of these three species were positive for presence of X. fastidiosa. In contrast, none of 460 adults of nine species, including P. spumarius, N. campestris, and E. lineolatus, captured from the X. fastidiosa-free area in Bari were positive by PCR. In the X. fastidiosa-infected areas, PCR assays showed that X. fastidiosa was detected in adults of P. spumarius, N. campestris, and E. lineolatus, starting from June until January, whereas all insect samples collected from February to May were negative for presence of X. fastidiosa (Fig. 2).

Results of PCR assays are reported in Table 3, subdivided into three periods per year based on presence or absence of infected individuals: Period 1. October to January, Period 2. February to May, and Period 3. June to September. The first period was characterized by increasing occurrence of X. fastidiosa-infected E. lineolatus adults, while presence of X. fastidiosa-infected P. spumarius and N. campestris decreased steadily, being almost negligible in winter. In the first year, 50 out of 154 adults (32.5%) of P. spumarius, N. campestris, and E. lineolatus collected during Period 1 tested positive for X. fastidiosa. Incidence of infection for E. lineolatus was 23 of 76 (30.2%), for N. campestris 16 of 37 (43.2%), and for P. spumarius 11 of 41 (26.8%). Similarly, during the same time period in the second year , 26 of 118 (22%) adults of the same three species tested positive for X. fastidiosa with 11 (42.3%) being E. lineolatus that was the most abundant (49.1% of the total adults) species. Incidence of X. fastidiosa infection in N. campestris and P. spumarius was, respectively, 28.0% (7 of 25) and 22.8% (8 of 35) (Fig. 2A, B).

The second period (February to May) was characterized by absence of adult individuals until April, when there was a rapid increase in the number of newly emerged adults. None of the insects collected during this period (P. spumarius, n¼115, N. campestris, n¼59, and E. lineolatus, n¼46) were found positive for

Table 1. Classification and total number of captured species during the 2-yr survey

Infra order	Superfamily	Family	Species	Na	P (%)b
Cicadomorpha	Cercopoidea	Aphrophoridae	Philaenus spumarius (L.)	955	39.8
			Neophilaemus campestris (Falle`n)	207	8.6
		Cercopidae	Cercopis sanguinolenta (Scopoli)	12	0.5
	Cicadoidea	Cicadellidae	Allygus modestus Scott	28	1.2
			Anoplotettix putoni Ribaut	172	7.2
			Fieberiella florii (Stal)€	26	1.1
			Synophropsis lauri (Horvart)	86	3.6
			Euscelis lineolatus Brulle´	195	8.1
			Conosanus obsoletus (Kirschbaum)	13	0.5
			Exitianus capicola (Stal)€	39	1.6
			Thamnotettix zelleri (Kirschbaum)	286	11.9
			Psammnotettix spp.	2	0.1
			Anaceratagallia spp.	13	0.5
Fulgomorpha	Fulgoroidea	Issidae	Agalmatium flavescens (Olivier)	363	15.1
		Flatidae	Metcalfa pruinosa Say	1	0.04
		Total		2,398	

a N: number of individuals captured.

b P (%): Percentage of individuals of each species among the total number of insects collected.

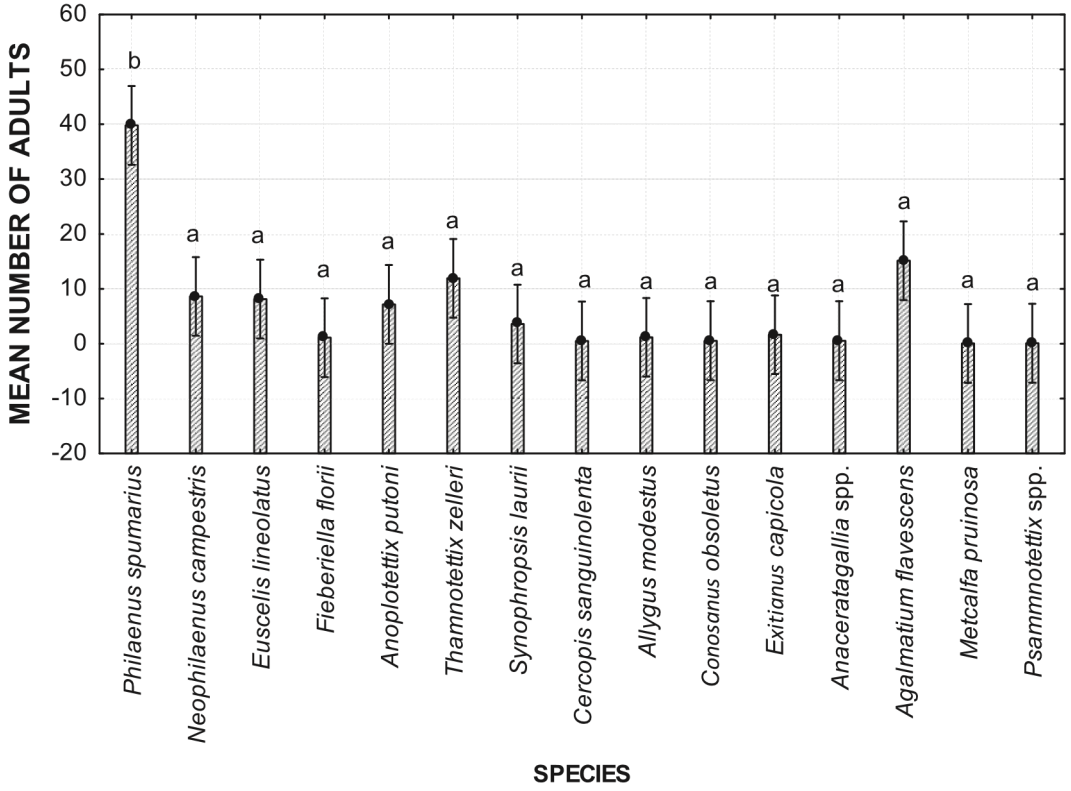

Fig. 1. LS mean of total number of individuals captured for each species during two years of study. Different letters above each bar indicate significant differences using one-way ANOVA with Tukey's HSD test (F¼8.05, df¼14, P<0.001). Vertical bars denote 0.95 confidence intervals.

Table 2. Average monthly abundance of Auchenorrhyncha species captured during a 2-yr survey

Species	M_{ca}											
	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.
P. spumarius	25.5	5.0	3.5	4.0	0	0.5	25.0	32.0	55.0	77.5	101.0	148.5
N. campestris	16.5	6.5	5.0	3.0	0	0	0	29.5	23.0	2.5	4.5	13.0
E. lineolatus	4.5	13.0	24.0	25.5	13.0	6.5	2.0	1.5	7.5	0	0	0
F. florii	2.5	0	0	0	0	0	0	1.5	1.5	1.5	1.5	4.5
A. putoni	0	0	0	0	0	0	0	56.0	24.0	6.0	0	0
T. zelleri	0	0	0	0	0	13.5	117.0	10.5	2.0	0	0	0
S. laurii	0	0	0	0	0	0	0	0	0	11.0	10.0	22.0
C. sanguinolenta	0	0	0	0	0	0	0	1.5	0	0	1.0	3.5
A. modestus	0	0	0	0	0	0	0	5.5	4.0	2.5	1.5	0.5
C. obsoletus	0	1.0	11.0	0	0	0	0	0	0	0	0	0
E. capicola	0	0.5	18.5	0	0	0.5	0	0	0	0	0	0
Anaceratagallia spp.	0.5	0	0	0	0	0	0	2.5	0	2.5	1.0	0
A. flavescens	16.0	5.0	0.5	0	0.5	0	0.5	26.0	33.0	20.0	16.0	64.0
M. pruinosa	0	0	0	0	0	0	0	0	0	0	0.5	0
Psammnotettix spp.	0	0	1.0	0	0	0	0	0	0	0	0	0
Total (M_cT)	65.5	31.0	63.5	32.5	13.5	21.0	144.5	166.5	150	123.5	137	256

[a] M_c: Mean of captured adults per month.

X. fastidiosa by PCR (Fig. 2). Finally, the third period (June to September) was characterized by a dominant presence of P. spumarius, with 445 of 507 (87.7%) and 319 of 358 (89.1%) individuals captured in the first and second year, respectively, with about 20% of these insects infected with X. fastidiosa (Fig. 2A).

A positive correlation between seasonal abundance of insects and relative incidence of X. fastidiosa was noticed for P. spumarius and E. lineolatus, for which the number of infected adults increased as abundance increased. Specifically, in January the number of E. lineolatus captured and incidence of infected insects were at maximum with M_c¼25.5 and M_i

¼11 (Table 3), which was supported by the high coefficient of correlation (r¼0.90; Fig. 3C). Similarly, the highest number of infected P. spumarius individuals coincided with greatest abundance in September with M_c ¼148.5 and M_i ¼21 (Table 3), resulting in a high coefficient of correlation (r¼0.92; Fig. 3A).

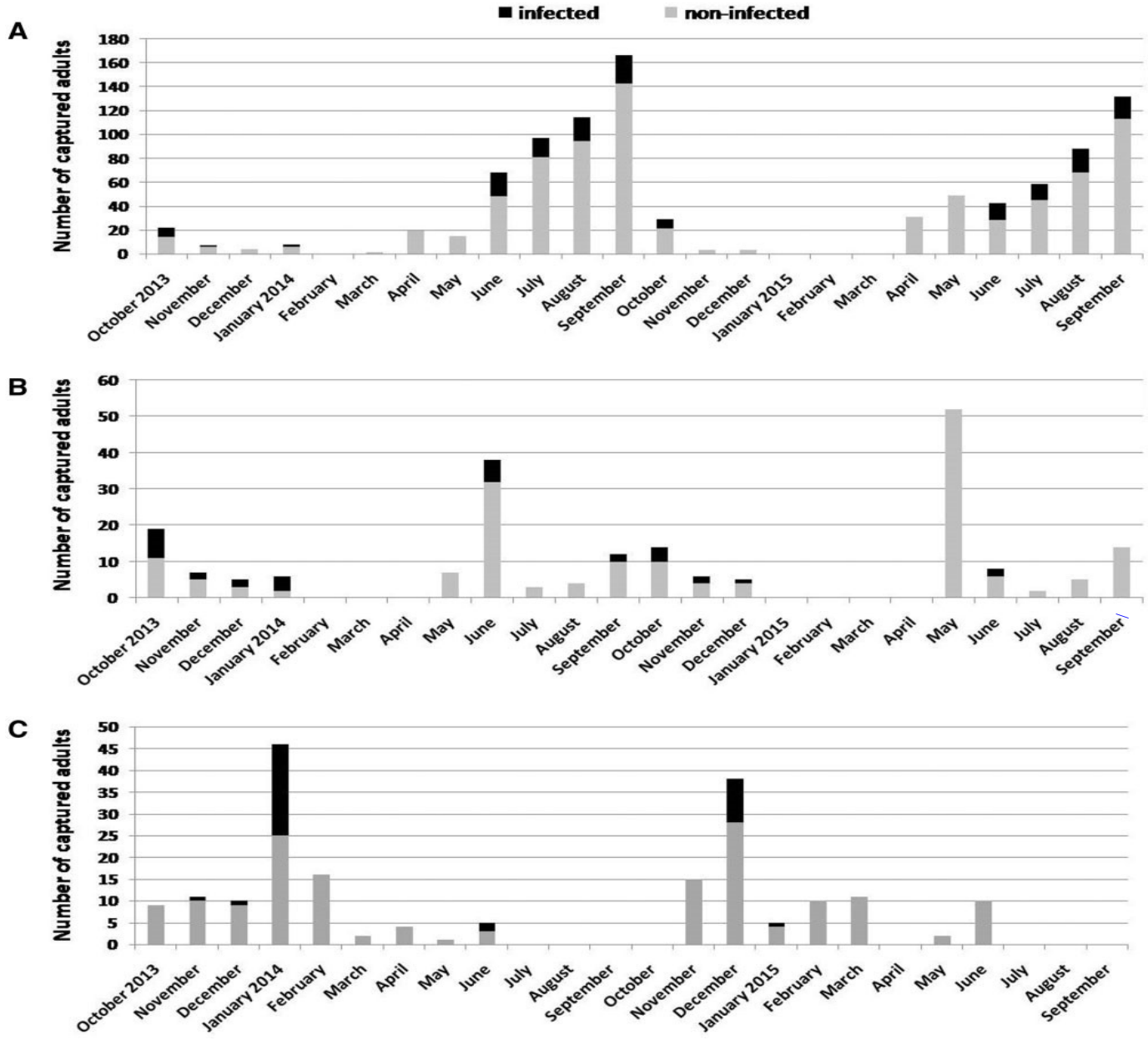

Fig. 2. Seasonal population dynamics of Xylella fastidiosa-infected and noninfected adults of Philaenus spumarius (A), Neophilaenus campestris (B), and Euscelis lineolatus (C) in olive orchards.

Unlike P. spumarius, the peak of N. campestris population was in May with M_c ¼29.5, when incidence of infected adults was null, while highest incidence of infected adults occurred in October (M_i ¼6). Moreover, the number of infected adults of N. campestris was clearly independent from abundance of this species, as supported by the low coefficient of correlation (r¼0.42; Fig. 3B). Population density was high in May (M_c ¼29.5) and June (M_c ¼23), decreased steadily from July to August and then increased again in September (M_c ¼13) and October (M_c ¼16.5; Table 3).

Discussion

Results of the present study provided new insights to X. fastidiosa epidemiology in the Apulia region of Italy. This study describes seasonal abundance of Auchenorrhyncha species and temporal dynamics of X. fastidiosa infection of insects in olive groves. The surveys determined that among 15 species of Auchenorrhyncha captured during the two years of study, P. spumarius was the most abundant species in olive groves in Lecce province. Adult P. spumarius began to molt to the adult stage between April and May and were

Table 3. Average monthly incidence of X. fastidiosa-infected Auchenorrhyncha specimens collected during a 2-yr survey

Month	P. spumarius		N. campestris		E. lineolatus	
	M_{ca}	M_{ib}	M_c	M_i	M_c	M_i
Oct.	25.5	8.0	16.5	6.0	4.5	0
Nov.	5.0	0.5	6.5	2.0	13.0	0.5
Dec.	3.5	0	5.0	1.5	24.0	5.5
Jan.	4.0	1.0	3.0	2.0	25.5	11.0
Feb.	0	0	0	0	13.0	0
Mar.	0.5	0	0	0	6.5	0
April	25.0	0	0	0	2.0	0
May	32.0	0	29.5	0	1.5	0
June	55.0	17.0	23.0	4.0	7.5	1.0
July	77.5	14.5	2.5	0	0	0
Aug.	101.0	20.0	4.5	0	0	0
Sept.	148.5	21.0	13.0	1.0	0	0

[a] M_C: Mean of captured individuals.
[b] M_i: Mean of infected individuals.

particularly abundant in summer. In contrast, E. lineolatus was the most abundant species during winter (November to February) with very few adults captured later in the season (i.e., June), which suggests that individuals of this species overwinter as adults in olive groves.

Over the 2-yr study, X. fastidiosa was detected by PCR in P. spumarius, N. campestris, and E. lineolatus, corroborating results previously reported by Elbeaino et al. (2014). Detection of X. fastidiosa coincided with presence of adult P. spumarius, N. campestris, and E. lineolatus, with incidence of X. fastidiosa-infected specimens highly correlated with seasonal abundance, except for N. campestris. One hypothesis to explain the lack of correlation between N. campestris abundance and peak in X. fastidiosa incidence in insects is that adult N. campestris prefers grasses as a feeding host plant, which were mostly absent in the studied environment throughout the summer. Consequently, host plants used for feeding also could have influenced correlation between the infection incidence and population size since adults of N. campestris may not frequently come in contact with infected plants during the summer.

Detection of infected specimens of P. spumarius from June to January was not surprising. Adults likely move from herbaceous plants and shrubs in late spring to olive trees in summer, from which acquisition of X. fastidiosa may occur and persist until insect death (Hill and Purcell 1995). Therefore, incidence of X. fastidiosa-positive insects was directly influenced by the life cycle of each species. None of the few adult P. spumarius and N. campestris captured in April and May were found infected. Likely, these individuals belonged to a new (first) adult generation because no adults of these species were captured in the previous period. In contrast, E. lineolatus captured in October and November were not infected by X. fastidiosa, which suggest that adults were collected prior to coming in contact with X. fastidiosa-infected olive trees.

Philaenus species, including P. spumarius, are vectors of X. fastidiosa in California (Severin 1950), but little is known about its role in Pierce's disease epidemics. In southern Italy, P. spumarius has been considered the most important vector of X. fastidiosa in olive groves. However, given similar trophic characteristics of Philaenus sp. and Neophilaenus sp., further studies are needed to evaluate transmission efficiency of X. fastidiosa to olive by these species. While N. campestris must be considered a candidate vector species, Euscelis spp. are phloem-fluid feeding insects unlikely to transmit a

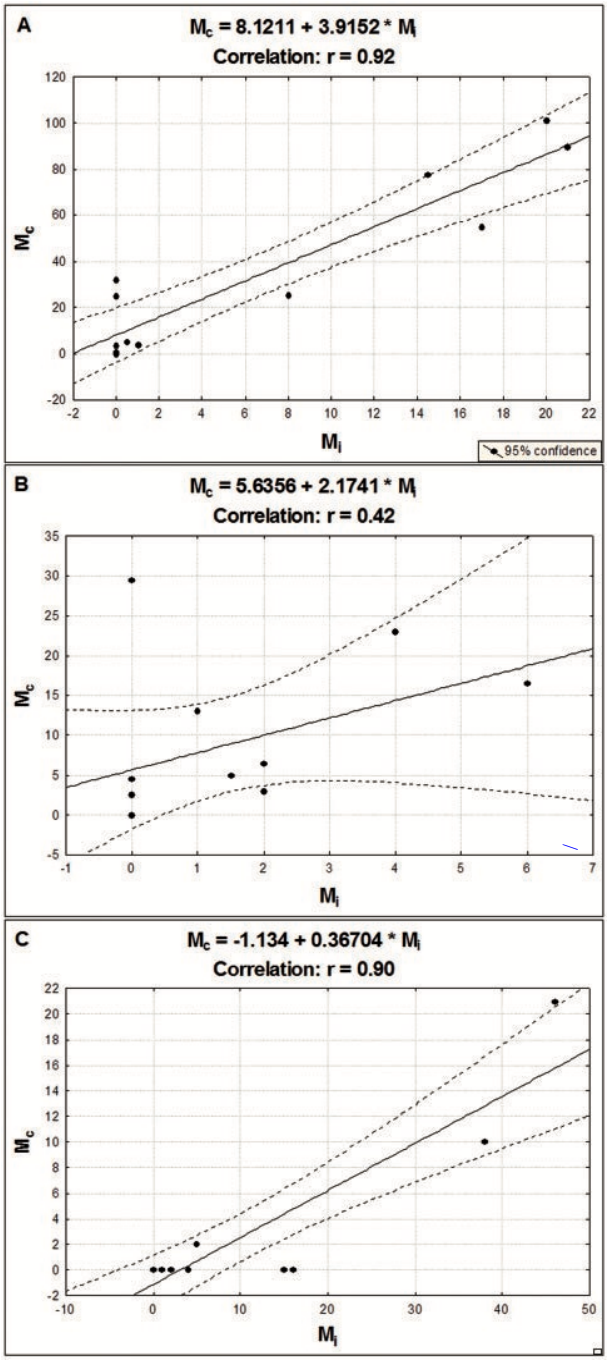

Fig. 3. Relationship between seasonal abundance (M_c) of Xylella fastidiosa-infected insects (overall abundance) and the relative incidence of infected specimens (M_i) along a single season. (A) Philaenus spumarius.
(B) Neophilaenus campestris. (C) Euscelis lineolatus.

xylem-limited bacterium. For instance, E. maculipenis DeLong and Davidson failed to transmit X. fastidiosa to grapevines in California (Severin 1949). However, considering the high numbers of X. fastidiosa-infected adults, it is clear that individuals of E. lineolatus acquire bacteria from infected host plants and retains X. fastidiosa with unexpectedly high efficacy for a phloem fluid-feeding insect. Although transmission of X. fastidiosa by phloem fluid-feeding insects has not been demonstrated, our study clearly confirms that these insects can come in contact with xylem vessels and become infected (Pompon et al. 2011). One possible explanation of why these insects cannot successfully inoculate the

bacterium into xylem vessels may be related to specific feeding behaviors (e.g., ingestion, egestion, salivation) that are performed in phloem but not in xylem tissue (Backus et al. 2012).

Our findings suggest that preventive control measures against juvenile stages of the univoltine vector, P. spumarius, may reduce spread of X. fastidiosa by reducing the number of adult insects. Since nymphs are stationary and primarily use ground vegetation as feeding hosts, managing alternative host plants in olive groves by mechanical and/or chemical practices and/or use of insecticides may help suppress P. spumarius populations. If adopted, treatments should target nymphal populations present between March and mid-April, or when signs of nymph development are visible (foamy substances present on the herbaceous plants). However, P. spumarius is a polyphagous insect that feeds and reproduces on plants in many habitats, including cultivated and noncultivated hosts near olive groves. Little is known about mobility of adult P. spumarius and potential to colonize olive groves. Therefore, additional research is needed to identify host plants of P. spumarius to guide landscape management strategies targeting key reproductive and feeding hosts. In addition, it is important to know which environmental conditions influence movement of vectors from other plants (in particular from herbaceous plants) to olive trees. Considering the complex olive–X. fastidiosa–insect pathosystem, a study on efficacy of different control approaches is warranted, taking particular consideration of the role of natural predators in regulating vector population density.

Species diversity and abundance of insect vectors in X. fastidiosa-affected regions are expected to vary due to a myriad of biotic and abiotic factors. Consequently, sampling methods and monitoring techniques may need to be adjusted according to habitat characteristics and vector behaviors.

In conclusion, this study provided the first information on seasonal abundance of potential vectors of X. fastidiosa in the Apulia region, as well as seasonal dynamics of X. fastidiosa incidence in insects inhabiting olive groves. In addition, the results provide insight to the design and potential adoption of monitoring and control measures for insect vectors.

Acknowledgments

We are grateful to Gianfranco Cataldi and Giuseppe Cavallo for their technical assistance during field collection. We thank Valedete Sefa for her help with insect identification.

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