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Occurrence and population dynamic of potential insect vectors of *Xylella fastidiosa* in citrus groves of central Greece

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Abstract

Citrus is a global fruit crop and one of the most destructive citrus diseases is the Citrus Variegated Chlorosis (CVC) caused by the bacterium *Xylella fastidiosa* Wells *et al*. The pathogen is usually transmitted by xylem sap-feeding insects. Given the tremendous impact that a *X. fastidiosa* CVC invasion could have in the Mediterranean and the consequent catastrophic scenarios, we offer robust data about the potential vector's bionomics in citrus groves. Throughout 2022, we sampled two citrus groves with sweet orange trees, *Citrus* × *sinensis* (L.) Osbeck, in central Greece plus a nearby natural area with pine trees, *Pinus halepensis* Miller, and lentisk shrubs, *Pistacia lentiscus* L., to study the presence, seasonal occurrence and population dynamic of potential insect vectors of *X. fastidiosa*. Additionally, another thirty-five citrus fields were single sampled in 2023 to gain more reliable results about the presence and relative abundance of the potential vectors of the bacterium in a wide area. Seven potential insect vectors were recorded. *Philaenus spumarius* L. and *Neophilaenus campestris* (Fallen) were the most frequently found and abundant species in all the citrus groves. Spittlebug nymphs were observed between late March and mid-May, whereas adults were present in spring, autumn and early winter. In the natural area, *N. campestris* observed from summer to early autumn on the pines, suggesting that pines are among the food plants for the species.

Key words: meadow spittlebug, Aphrophoridae, *Citrus* × *sinensis*, phytopathogenic bacterium.

Introduction

Citrus is one of the major fruit crops globally, and it is cultivated in more than 140 countries worldwide (FAO, 2023). Citrus has high economic importance in Europe, especially for the southern European countries. The production of citrus fruits in 2022 in Spain, Italy, Greece and Portugal was 5,558, 3,094, 1,180 and 452 thousand tons, respectively, while the cultivation area was approximately 298,280 ha, 145,930 ha, 42,980ha and 21,770 ha in the respective countries (European Commission, 2024). In Greece in 2022, 67% of the citrus cultivated area were sweet orange trees, *Citrus* × *sinensis* (L.) Osbeck, which was the main cultigen, 22% were small citrus fruit trees, *Citrus reticulata* Blanco, *Citrus* × *unshiu* (Yu.Tanaka ex Swingle) Marcow, *Citrus* × *clementina* Yu.Tanaka and 9% were lemon trees, *Citrus* × *limon* (L.) Osbeck. In the European Union, the respective cultivated areas were 53%, 30% and 15% (European Commission, 2024). Thus, it is evident that the cultivation of citrus is significant both for the national economy of southern European countries and for the income of many farmers.

Xylella fastidiosa subsp. *pauca* Wells *et al.* causes the Citrus Variegated Chlorosis (CVC), a severe citrus disease. CVC first appeared in Brazil in 1987 and rapidly invaded the country and neighbouring territories (Lee, 2015). Each year, the disease kills about 6 million trees in Brazil (Bove and Ayres, 2007), corresponding to a value of 120 million US$. *X. fastidiosa* is a xylem-inhabiting, insect-transmitted bacterium (Janse and Obradovic, 2010). Its host list includes 595 wild or cultivated plant species belonging to 85 families (EFSA, 2020). Apart from CVC, the bacterium causes many diseases on economic crops, such as Pierce's disease of grapevines, the phony peach disease, the plum leaf scald, the leaf scorch on almonds and the quick decline syndrome in olives (Janse and Obradovic, 2010; EPPO, 2018; Krugner *et al.*, 2019). The *X. fastidiosa* subsp. *pauca* ST53 is the one that caused the outbreak of quick decline syndrome of olives in southern Italy (Saponari *et al.*, 2019). In citrus, the bacterium gets into the xylem sap, driving it throughout the tree and causing tree decline and reduced production. *X. fastidiosa* colonises the xylem network within plants. The main function of xylem vessels is to transport water from the roots to leaves and when the bacteria colonise the plant, those vessels become blocked and the plant finally dies (EFSA, 2024). Pectic gels tyloses and *X. fastidiosa* biofilms contribute to these vessel occlusions (Roper *et al.*, 2007). The sweet orange varieties are more susceptible to *X. fastidiosa pauca* strain 9a5c and show severe symptoms of CVC (Coletta-Filho *et al.*, 2020). The symptoms of the infested trees are leaves with zinc deficiency-like interveinal yellowing of the upper leaf side and corresponding brownish gum-like material under side, defoliation, and twig dieback (Bove and Ayres, 2007). In addition, the diseased fruits are considerably smaller than the healthy ones and finally produced with hard rind (Bove and Ayres, 2007; Lee, 2015).

X. fastidiosa has several ways to pass from an infected plant to a healthy one such as grafting or interplant self-grafting (Picciotti *et al.*, 2021). However, the primary way of transmission is by xylem-sap-feeding Hemiptera insects and specifically sharpshooter leafhoppers (Cicadellidae, subfamily Cicadellinae), spittlebugs (Aphrophoridae and Cercopidae), and potentially cicadas (Cicadoidea) (Redak *et al.*, 2004). Those insects feed in xylem vessels, where *X. fastidiosa* infects plants (Purcell, 1989). Although CVC is vectored mainly by several species of sharpshooters in Brazil (Coletta-Filho *et al.*, 2020), Cornara *et al.* (2017a) showed that spittlebugs can transmit *X. fastidiosa* subsp. *pauca* ST53 to several hosts, including sweet oranges.

In recent years many studies focused on the biology and ecology of *X. fastidiosa* vectors in olive agroecosystem of south European countries due to the outbreak of the bacterium in Italy (Ben Moussa *et al.*, 2016; Cornara *et al.*, 2017b; Morente *et al.*, 2018; Dongiovanni *et al.*, 2019; Bodino *et al.*, 2019; 2020; Antonatos *et al.*, 2021). However, information available on spittlebug bioecology in European citrus agroecosystems is scarce. In summer, adult spittlebugs mass move from cultivated fields to natural areas where the climatic conditions are more favourable (Morente *et al.*, 2018; Bodino *et al.*, 2019; Antonatos *et al.*, 2021). Nevertheless, very few studies have been conducted concerning the host plant species and the population trend of spittlebugs in natural areas and, subsequently, about the role they have in the population dynamic of spittlebugs in the cultivated groves (Cornara *et al.*, 2021; Lago *et al.*, 2021; Mesmin *et al.*, 2022). Given the tremendous impact of a CVC outbreak on the Mediterranean basin, we need data about the vector's bioecology in both citrus groves and natural areas. Missing information is crucial for managing the spread of *X. fastidiosa*. The knowledge will help estimate the risk of outbreaks and shape effective control strategies. The present work aimed to: 1. improve the knowledge about the insect species, potential vectors of *X. fastidiosa*, inhabit citrus groves and neighbouring natural areas and estimate their mean relative abundance in citrus fields; 2. describe the seasonal occurrence and the population dynamic of nymphs and adults of *X. fastidiosa* potential vectors that inhabit citrus groves of central Greece and a neighbouring natural area, suggesting the importance that neighbouring natural ecosystems have in vector abundance. We have studied two fields in central Greece where different varieties of sweet oranges are produced and their neighbouring natural area for one year. To accomplish more reliable results about the presence of insect potential vectors of the bacterium we also conducted a single sampling in thirty-five citrus groves in central Greece during the 2023 spring.

Table 1. Mean monthly temperature and monthly precipitation of the sampling areas.

Month	Mean temperature (°C)	Total rain (mm)
January 2022	10.0	62.6
February 2022	11.9	32.0
March 2022	10.3	10.6
April 2022	17.0	14.0
May 2022	21.8	12.8
June 2022	27.6	11.6
July 2022	29.8	3.2
August 2022	29.0	4.0
September 2022	25.1	7.6
October 2022	21.0	23.6
November 2022	17.6	100.2
December 2022	15.0	16.2

The climatic data are from AMONI, Corinthos station of the meteorological stations network of the National Observatory of Athens

Materials and methods

Sampling groves

In 2022, samplings at regular intervals (10-15 days) conducted in two citrus groves in central Greece to study the occurrence and population dynamic of the potential vectors of *X. fastidiosa*. The first citrus grove was in Ancient Corinthos (37°54'34.15"N 22°52'08.09"E), and it managed with sweet oranges of the cultivar ‘Washington navel’. The second grove was in Kechries (37°53'24.08"N 22°59'38.14"E), cultivated with ‘Valencia’ sweet oranges. The sampling area of each grove was approximately 1 hectare. The ground vegetation of the first citrus grove consisted of spontaneous herbs mainly from the families Poaceae (~45%), Fabaceae (~20%), Asteraceae (~15%) and Oxalidaceae (~10%). In the second grove, the wild vegetation consisted of plants from the family Poaceae (~70%), mainly *Avena sterilis* L., Asteraceae (~15%) and Oxalidaceae (~5%). The two groves did not suffer from chemical insecticide distributions during the sampling period, but one mowing managed the dried herbs in late June once the vectors abandoned the habitat. We also sampled a neighbouring natural area bordering the east and south sides of the second citrus grove and studied the site role for spittlebugs' aestivation. We searched the vectors on spontaneous shrubs (*Pistacia lentiscus* L.) (Sapindales Anacardiaceae) and pine trees (*Pinus halepensis* Miller) (Pinales Pinaceae), the most abundant food plants. The mean monthly temperature and the monthly rain of the area are in table 1. The meteorological data were retrieved from the AMONI, Corinthos station of the meteorological network of the National Observatory of Athens (http://meteosearch.meteo.gr).

To increase the data robustness, we also sampled 35 "sweet orange" groves during 2023 in the regional unit of Corinthia (central Greece). No insecticide applications had been conducted and no mowing of the ground herbs had taken place in those groves during or prior the period of samplings. The groves located in the citrus producing areas of the regional unit namely Kechries, Corinthos, Ancient Corinthos, Zeugolatio, Vochaiko, Velo, Kokkoni, Krines, Poullitsa and Xylokeriza. Those areas usually have the typical Mediterranean climatic conditions (mild winter, dry and hot summer). We decided to get a single sampling from each grove in late April - mid-May (28/4/2023, 9/5/2023 and 17/5/2023) because, according to the results of 2022, spittlebug adults were present in the groves during that period. In those samplings we took one sample from the ground vegetation and another one from citrus canopy from each field. All grove coordinates and the sampling dates are in supplemental material table S1.

Sampling methods

Each citrus grove encompasses ten plots, each corresponding to 0.1 hectares and hosting approximately 40 trees. We used a quadrate (50 × 50 cm) to randomly frame the herbs and count the spittlebug nymphs. We received one sample per plot so that ten nymph samples were performed in each grove. The data recorded were the number and species of nymphs and the family of the host plant. When it was impossible to identify the species of nymph in the field, they were collected and transferred to the laboratory for identification. The data described the seasonal occurrence and abundance of nymphs and their host plant associations. The samplings for spittlebug nymphs were performed every 10-15 days from March to May of 2022.

Adult collections originated from the foliage of citrus trees and from the ground vegetation. The foliage of citrus trees and the ground vegetation of every plot was swept with a 38 cm wide entomological net. A sample from the foliage consisted of twelve sweeps on three trees (four sweeps per tree) within the plot. We sampled 30 trees in total per site and date. Each ground herbs sample accumulated ten consecutive sweeps per plot. To take the samples randomly we were moving through the plot following a 'Z' shaped course and we selected for sampling one every four trees on each side of 'Z' course. The starting point of 'Z' shaped course changed at each sampling.

Additionally, five samples were retrieved from pine trees, *P. halepensis*, and another five samples from *P. lentiscus*. A sample from the wild trees and shrubs consisted of twelve sweeps on the foliage of three plants randomly selected. The content of the sweeping net was emptied in a plastic bag, properly labelled and sealed. We froze all samples at −20 °C, later sorting and storing insects in 98% V/V EtOH/water in the laboratory until identification. Adult sampling occurred every 10-15 days from February to May and 15-20 days from June to December 2022.

The samplings from the 35 citrus groves conducted in 2023, one time per site. One sample was collected from the ground vegetation and another one from the citrus foliage.

Insect identification

A stereoscope was used for the taxonomic scrutiny of collected insects. The identification of spittlebug nymphs follows the key of Vilbaste (1982). Identification of *Philaenus signatus* Melichar nymphs was based on their host plant (*Asphodelus microcarpus* Salzmann et Viviani) since the nymphs are strictly monophagous (Drosopoulos, 2003). The adult recognition based on key publications and illustrations by Ossiannilsson (1981), Drosopoulos and Asche (1991), Holzinger *et al.* (2003), Biedermann and Niedringhaus (2009) and Lahbib (2022). Identification of *Philaenus spumarius* L. versus *P. signatus* female adults was based on their body length. According to Drosopoulos and Remane (2000) the body length of *P. spumarius* females is 6.35 ± 0.20 mm while that of *P. signatus* is 7.82 ± 0.25 mm. The obvious difference in body length (1.5 mm) allows a reliable identification between the two species.

Molecular methods were applied in order to conduct the identification of cicadas. Six adults (2 males and 4 females) with different appearances were left to dry at room temperature, and a part of their prothorax (specifically of the pronotum) was cut and removed for further genomic DNA extraction following the DNeasy Blood & Tissue Kit (QIAGEN) protocol, and finally producing a 50 μL DNA volume. We examined Cytochrome Oxidase I (COI) sequences, targeting the genes for species identification (Hebert *et al*., 2003; Ratnasingham and Hebert, 2007; EPPO, 2021).

The two sets of universal primers amplified partial mitochondrial gene sequences (mtDNA) via PCR: LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) as forward primer paired with the reverse primer HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) producing a 658 base pairs segment (Folmer *et al*., 1994) and the forward primer C1-J-2195 (5′-TTGATTTTTT-GGTCATCCAGAAGT-3′) paired with the reverse TL2-N-3014 (5′-TCCAATGCACTAATCTGCCATATTA-3′) and resulting in a 723 segment (Simon *et al*., 1994). Each PCR reaction mixture contained 5 μL of 10×PCR buffer, 1.5 μL of MgCl2 (50 mM), 0.5 μL of dNTPs (10 mM), 1 μL of each primer (10μM), 5 μL of template DNA (20-40ng), 0.5 μL of the thermostable Taq DNA polymerase (Platinum, Thermo Fisher Scientific) and molecular grade water (up to 50 μL). The reaction conditions for the first set of primers (LCO-1490/HCO-2198) consisted of an initial denaturation of 3min at 94 °C, followed by five cycles at 94 °C for 30 seconds, 45 °C for 30 seconds and 72 °C for 1 minute and by 35 cycles of 94 °C for 30 seconds, 51 °C for 1 minute and 72 °C for 1 minute and a final step of extension at 72 °C for 10 minutes. The thermocycling program for the second set of primers (C1-J-2195/TL2-N-3014) included an initial denaturation of 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 56 °C for 1 minute and 72 °C for 60 seconds and a final extension of 72 °C for 5 minutes.

Gel electrophoresis, 1.2% agarose, confirmed the template's amplification and size using 5 μL of the PCR products. NucleoFast 96 PCR Clean-up (Macherey-Nagel GmbH & Co. KG) purified the rest of the volume we sent to Macrogen Europe (The Netherlands) for automated bidirectional sanger sequencing analysis. Geneious Prime v2023.0.2 (www.geneious.com, Biomatters Ltd.) aligned the results for comparison in the GenBank sequences.

Statistical analysis

We used the nonparametric Wilcoxon's test, searching for significant differences in potential vector numbers between ground vegetation and citrus foliage of the 35 citrus groves. The same test searched for significant differences among the potential vectors in the ground vegetation and the citrus foliage. SPSS 21.0 (IBM, 2012) analysed all sampling units.

Results

Presence of insect vectors of *X. fastidiosa* in citrus groves and natural area

Seven insect species were recorded in total as potential vectors of *X. fastidiosa*. Six species were recorded in the two citrus groves of regular samplings in 2022, two in the natural area and five in the thirty-five citrus groves of single samplings in 2023.

Citrus groves - regular samplings in 2022

P. spumarius and *Neophilaenus campestris* (Fallen) (Hemiptera Aphrophoridae) were the potential insect vectors of *X. fastidiosa* in the citrus grove of Kechries. Adults thrive mainly in the ground vegetation under the

Table 2. Insect species, potential vectors of *X. fastidiosa*, found in ground vegetation, citrus' foliage and wild plants' foliage in the regional Unit of Corinthia.

		Regular samplings in two citrus groves (2022)		Regular samplings in natural area (2022)	Single samplings in 35 citrus groves (2023)
		Kechries	A. Corinthos		
Ground vegetation	*P. spumarius*	P (66)*	P (206)	N/S	P (210)
	N. campestris	P (104)	P (27)	N/S	P (91)
	P. signatus	A	P (7)	N/S	P (3)
	C. sanguinolenta	A	P (9)	N/S	P (10)
	C. viridis	A	A	N/S	P (2)
Citrus/wild plants foliage	*P. spumarius*	P (2)	P (8)	P (5)	P (22)
	N. campestris	P (5)	P (1)	P (77)	P (10)
	P. signatus	A	P (1)	A	A
	C. sanguinolenta	A	A	A	A
	C. viridis	A	A	A	A
	C. orni	A	P (N/R)	A	A
	T. plebejus	A	P (N/R)	A	A

P: Presence, A: Absence, N/S: No sampling, N/R: Not recorded.
* In the parenthesis is given the total number of individuals collected.

citrus trees, 66 and 104 *P. spumarius* and *N. campestris*, respectively while only two and five individuals of the respective species collected from the citrus foliage during the year. In the second citrus field of Ancient Corinthos, we collected four spittlebug species, namely *P. spumarius*, *N. campestris*, *P. signatus* (Hemiptera Aphrophoridae) and *Cercopis sanguinolenta* (Scopoli) (Hemiptera Cercopidae). The four taxa were relatively abundant in the spontaneous herbs, summing 206, 27, 7 and 9 individuals, respectively. The same were scarce: 8 *P. spumarius*, 1 *N. campestris*, 1 *P. signatus*, and 0 *C. sanguinolenta*, in the canopy. In the same citrus grove canopies, we collected *Cicada orni* L. and *Tibicen plebejus* (Scopoli) (Hemiptera Cicadidae) in summer (table 2).

Natural area - regular samplings in 2022

Pine tree collection from early May until mid-November gathered 73 *N. campestris*; *P. lentiscus* gave further 4. Moreover, five *P. spumarius* were on pine trees in late October and mid-November (table 2).

Thirty-five citrus groves - single samplings in 2023

Potential *X. fastidiosa* vectors in the spontaneous herbs of citrus groves -in citrus producing areas of the regional unit of Corinthia were 210 *P. spumarius*, 91 *N. campestris*, 10 *C. sanguinolenta*, 3 *P. signatus*, and 2 *Cicadella viridis* (L.) (Hemiptera Cicadellidae Cicadellinae). The citrus canopies hosted 22 *P. spumarius* and 10 *N. campestris* (table 2).

Nymph sampling

Host plants association and population dynamic of spittlebug nymphs

In the citrus grove of Kechries 42 and 65 nymphs belonged to *P. spumarius* and *N. campestris*, respectively, were collected corresponding to percentages of 39% and 61%. In the citrus grove of Ancient Corinthos, 114, 31 and 6 nymphs belonged to *P. spumarius*, *N. campestris* and *P. signatus*, respectively, were collected corresponding to percentages of 75, 21 and 4%. Poaceae hosted more than 50% of the *P. spumarius* nymphs and 100% of *N. campestris* nymphs in the Kechries citrus grove (figure 1). *Avena sterilis* was the most frequent spittlebug host plant in the grove. In Ancient Corinthos grove Fabaceae and Asteraceae hosted most of *P. spumarius* nymphs, while *N. campestris* nymphs used as host only Poaceae plants (figure 1). *Medicago polymorpha* and *Crepis* sp., Fabaceae and Asteraceae, respectively, hosted more nymphs. *Asphodelus* sp. only hosted *P. signatus* nymphs.

In the citrus grove of Kechries, both *P. spumarius* and *N. campestris* nymphs appeared in late March to disappear in mid-May. The highest record of *P. spumarius* nymphs was 6.0 nymphs/m^2 in 20/4/2022 while *N. campestris* population peaked at 13.6 nymphs/m^2 on 29/4/2022. In Ancient Corinthos, *P. spumarius* and *N. campestris* nymphs had a similar population trend. The *P. spumarius* nymphs, 16.4 nymphs/m^2, peaked on 20/4/2022 and the *N. campestris* population peaked at 4.0 nymphs/m^2 on 29/4/2022. A scarce population of *P. signatus* nymphs exists in April (figure 2).

Adult sampling

Occurrence and population dynamic of spittlebug adults

Figure 3 (ground vegetation) and figure 4 (citrus foliage) shows the seasonal appearances of spittlebug adults found in the two citrus groves. Adults of *P. spumarius* and *N. campestris* appeared in the two citrus groves in spring, autumn, and early winter. *P. spumarius* adults observed on the ground vegetation from late April until mid-May or early June in Kechries and Ancient Corinthos grove, respectively. *N. campestris* first appeared in late April and remained in both groves until the end of May in spontaneous herbs, with few captures in the citrus

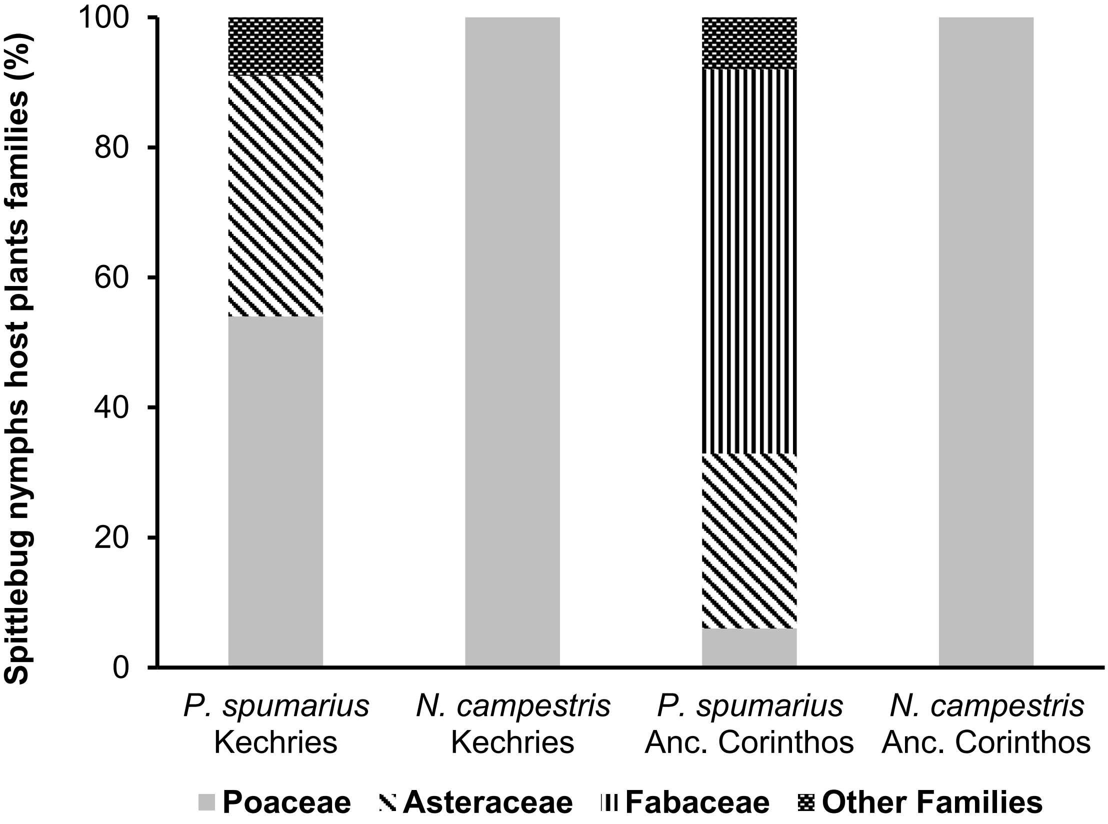

Figure 1. Host plants families of *Philaenus spumarius* and *Neophilaenus campestris* nymphs in Kechries and Ancient Corinthos citrus groves.

canopy in the two groves. During summer months, spittlebug species were absent from the citrus groves. Adults of *P. spumarius* reappeared again on ground vegetation in late October, while those of *N. campestris* in early November and remained during November and December in both groves. Kechries grove has a single record for *P. spumarius* on canopies during autumn. In Kechries citrus grove, the highest density of *P. spumarius* and *N. campestris* adults in ground vegetation was recorded on 10/5/2022 (2.5 and 4.2 adults/sample, respectively), while in the Ancient Corinthos grove, the population of both spittlebugs peaked in 23/5/2022 (9.9 and 1.5 adults/sample, respectively). Concerning citrus foliage, the highest population density of *P. spumarius* and *N. campestris* adults was recorded on 10/5/2022 (0.1 and 0.3 adults/sample, respectively) in the Kechries citrus grove. In comparison, in the Ancient Corinthos grove, the population of both spittlebugs in citrus foliage peaked on 23/5/2022 (0.5 and 0.1 adults/sample, respectively). In the grove of Kechries, *P. spumarius* and *N. campestris* accounted for 38.8% and 61.2% of the total number of spittlebug adults, respectively. In Ancient Corinthos, the contributions of *P. spumarius*, *N. campestris*, *P. signatus* and *C. sanguinolenta* were 82.8, 10.8, 2.8 and 3.6%, respectively. Molecular analysis resulted in two cicada species that inhabit the Ancient Corinthos citrus grove tree canopies in summer, namely *C. orni* and *T. plebejus*. The cicadas were mainly found on the thicker branches of the trees and not on the foliage. For this reason, a small number of cicadas were collected by the net. Thus, we do not present data about their seasonal abundance, accepting that the sweep net is not an appropriate collection tool.

N. campestris was the most abundant spittlebug in the natural area near the citrus grove of Kechries and collected almost exclusively (95%) from pine trees from mid-May until early November. Their population density in the foliage of pine trees remained relatively stable during that period, fluctuating mainly between 0.8 and 1.6 adults/sample while the *P. lentiscus* records only four adults in the same area and period. *P. spumarius* was absent from the area in summer, but we captured five adults on pines from October to November (figure 4).

Regarding the single samplings of the 35 citrus groves the ground vegetation hosts *P. spumarius* in 25, *N. campestris* in 12, *C. sanguinolenta* in 8, and *P. signatus* and *C. viridis* in one grove. Figure 5 summarizes the mean relative abundance of spittlebug adults in the 35 citrus groves. In citrus foliage, *P. spumarius* was observed in 9 groves and *N. campestris* in 5 groves, while the other three species were not found (supplemental material table S1). Higher population density of *P. spumarius* and *N. campestris* adults was recorded in the ground vegetation than in citrus foliage ($Z = -4.059$, $df = 1$, $P < 0.001$; $Z = -2.101$, $df = 1$, $P = 0.036$). Significant differences exist in the abundance of the five insect species recorded in ground vegetation. *P. spumarius* adults were more abundant than *N. campestris* ($Z = -2.707$, $df = 1$, $P = 0.007$), *C. sanguinolenta* ($Z = -4.605$, $df = 1$, $P < 0.001$),

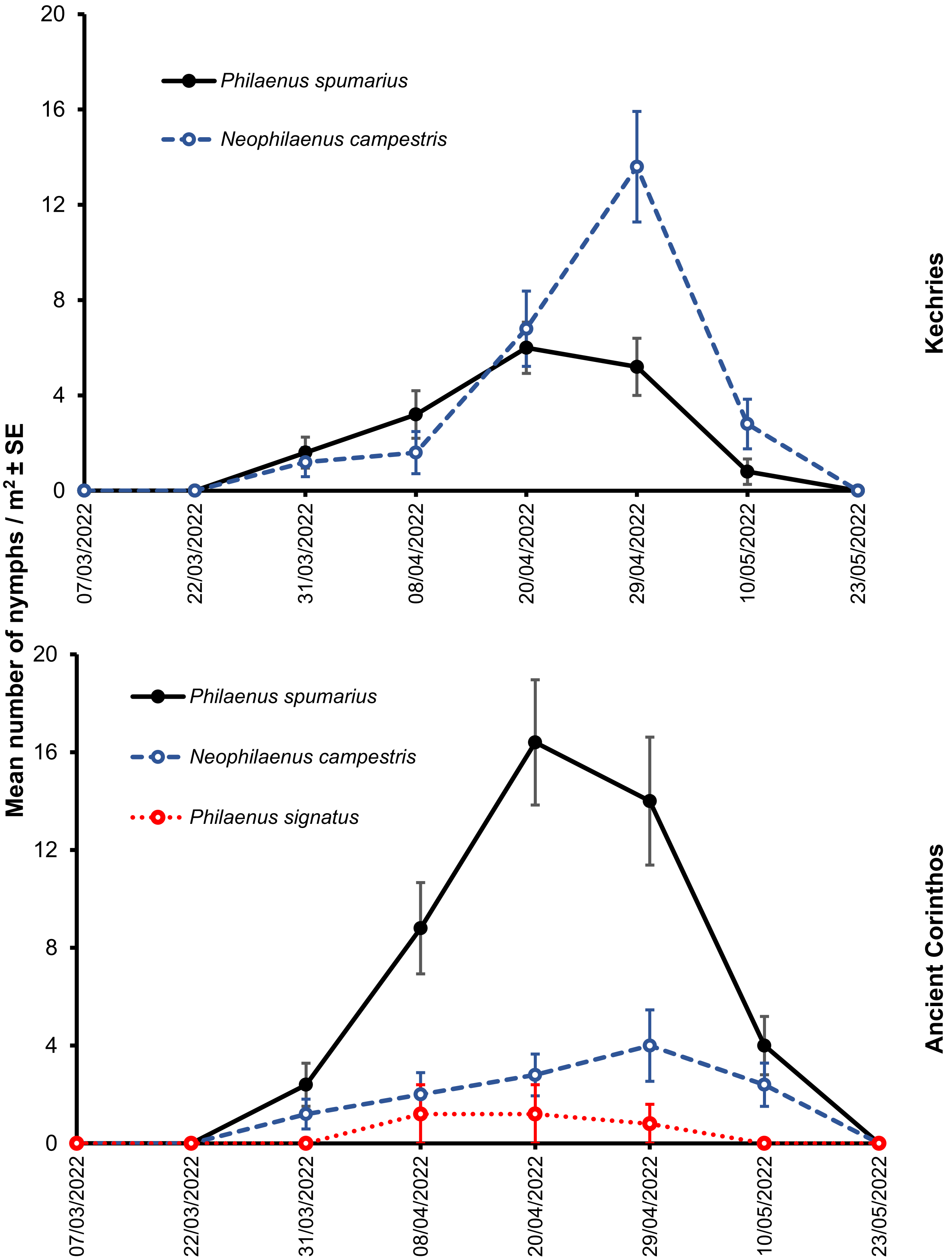

Figure 2. The mean number of spittlebug nymphs/m^2 ± standard error (SE) per sampling date in the sampled citrus groves.

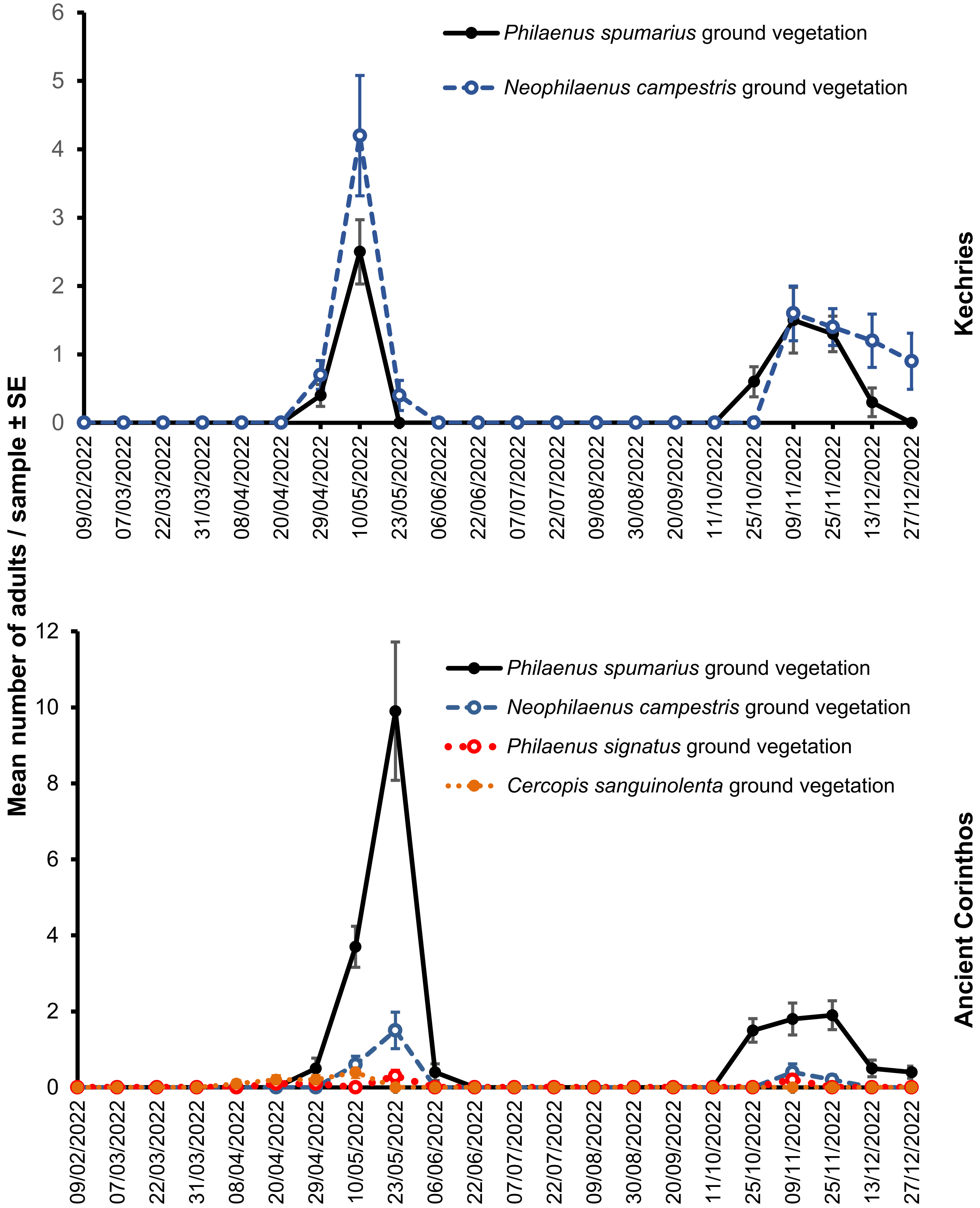

Figure 3. Mean number of adults/sample (± SE) of spittlebugs per sampling date in the ground vegetation of the sampled citrus groves.

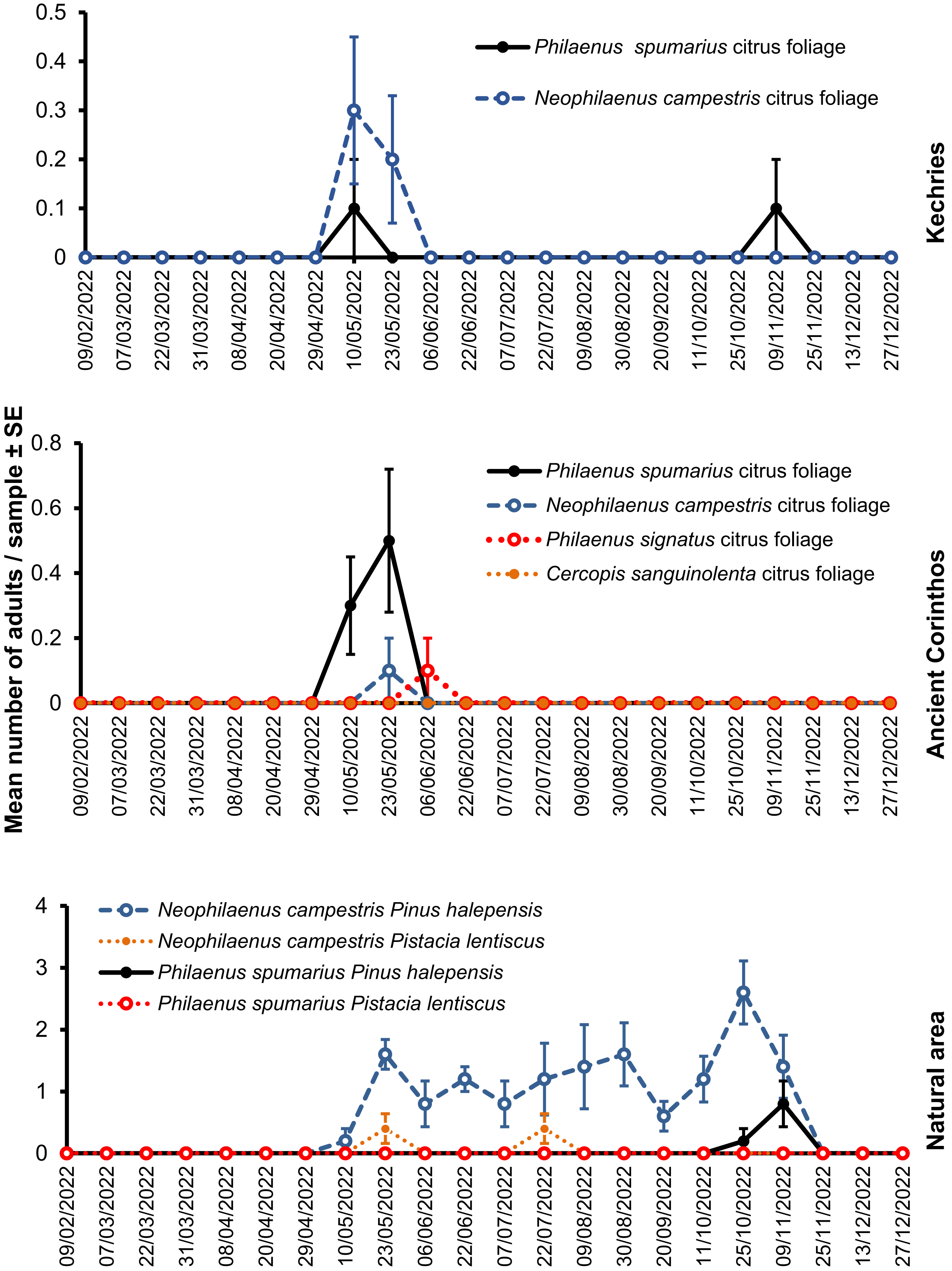

Figure 4. Mean number of adults/sample (± SE) of spittlebugs per sampling date in the foliage of plants in the sampled citrus groves and the natural area.

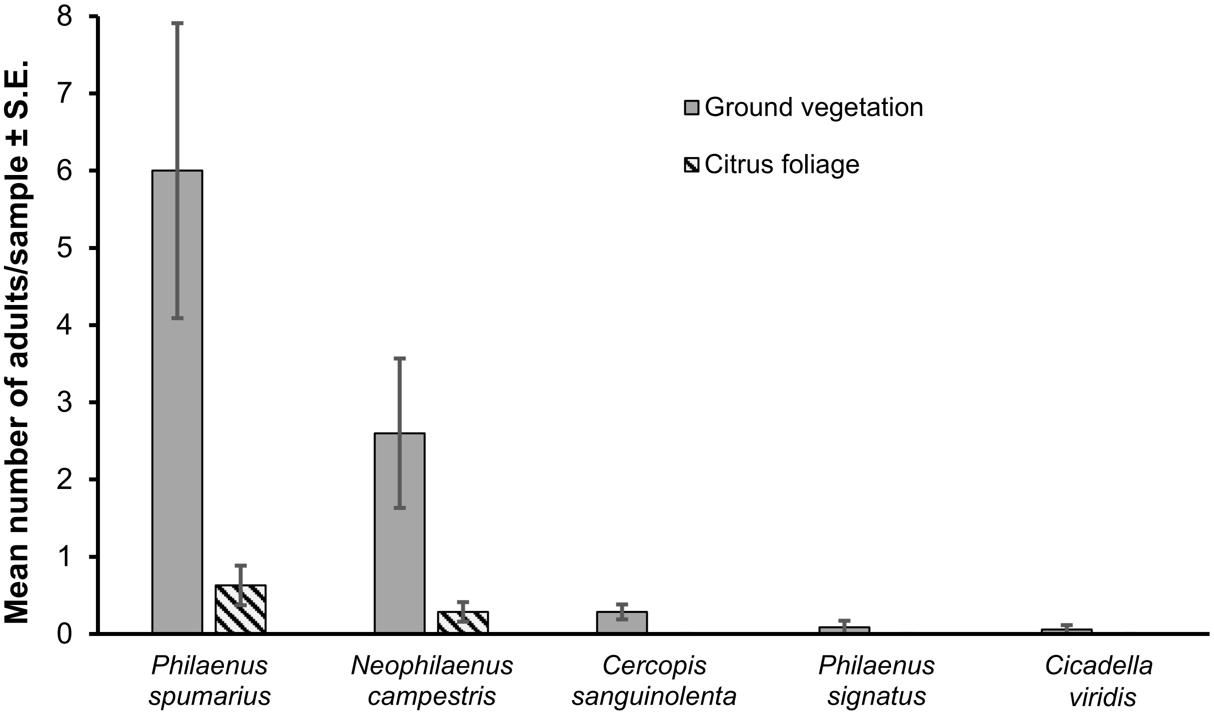

Figure 5. Mean relative abundance of adults/sample (± SE) for spittlebugs in the ground vegetation and the foliage of citrus plants in the 35 citrus groves

P. signatus (Z = −5.722, *df* = 1, P < 0.001) and *C. viridis* (Z = −5.756, *df* = 1, P < 0.001). Catches of *N. campestris* did not differ from those of *C. sanguinolenta* (Z = −1.447, *df* = 1, P = 0.148), while they were higher than those of *P. signatus* (Z = −3.369, *df* = 1, P < 0.001) and *C. viridis* (Z = −3.369, *df* = 1, P < 0.001). *C. sanguinolenta* adults were more abundant than *P. signatus* (Z = −2.395, *df* = 1, P = 0.017) and *C. viridis* (Z = −2.416, *df* = 1, P = 0.016). *P. signatus* did not differ from *C. viridis* (Z = −0.020, *df* = 1, P = 0.984). *P. spumarius* and *N. campestris* catches in the citrus foliage did not differ (Z = −1.153, *df* = 1, P = 0.249).

Discussion

Seven species characterized as potential vectors of *X. fastidiosa* existed in the citrus groves of central Greece. Four of them were spittlebugs belonging to the families Aphrophoridae and Cercopidae, one of them was a sharpshooter belonging to the family Cicadellidae and two were cicadas belonging to the family Cicadidae. However, the sharpshooter *C. viridis* and the spittlebug *P. signatus* appeared extremely rare. Year-round samplings in the citrus groves of Kechries and Ancient Corinthos recorded two and four spittlebug species. The most frequent species in citrus groves were *P. spumarius* and *N. campestris.* Those two species are the most common spittlebugs in Europe, and they have also been recorded in olive groves in Greece (Antonatos *et al.*, 2020; 2021; Theodorou *et al.*, 2021) and in other south European countries (Morente *et al.*, 2018; Dongiovanni *et al.*, 2019; Bodino *et al.*, 2020), as well as in vineyards of Italy (Bodino *et al.*, 2021) and California (USA) (Beal *et al.*, 2021). Spittlebug nymphs colonize the ground vegetation, which is significantly alike in the plant species between these agroecosystems. Thus, unsurprisingly, the same spittlebugs were recorded in groves with olives, citrus and grapes.

The nymphs appeared in the two groves in late March, as the weather conditions of the area influence the period of spittlebugs' appearance. The two groves are in central Greece, in the Mediterranean region, with mild winter and hot and dry summer climates. However, during 2022, a heavier and longer winter than those of previous years was recorded. According to the meteorological data, the mean temperature of March 2022 (10.3 °C) was 3.7 °C lower than that of the years 2018-2021 (14 °C) (meteosearch.meteo.gr). This lower temperature in March 2022 may explain the late Greece nymphs finding, compared to the 10-15 days earlier nymphs' observations in Italy and Spain the years before (Morente *et al.*, 2018; Dongiovanni *et al.*, 2019; Bodino *et al.*, 2019; 2021). Bodino *et al.* (2019), suggesting that the egg hatching depends on the cold temperatures experienced throughout the winter. *P. signatus* nymphs appeared in April on its only host plant, a species of *Asphodelus* that is not abundant in the area. *P. signatus* nymphs might not have been recorded earlier, probably due to the scarcity of the host plant, which has not entered the quadrate frame used in samplings.

In the region of Kechries, the nymphs of *N. campestris* were found to be the most popular among spittlebugs, while in Ancient Corinthos, those of *P. spumarius* were prevalent. Relative species abundance may depend on the different composition of plant species on the ground cover of the groves and the plant species that occurred in the neighbouring areas. In the Kechries citrus grove, the ground vegetation consisted of Poaceae plants at a ratio of 70%, while in the Ancient Corinthos grove, these plants were less abundant. It is well-documented that *N. campestris* prefer Poaceae as hosts, and in parallel it is seldomly found in plants of other families (Morente *et al*., 2018; Dongiovanni *et al*., 2019; Bodino *et al*., 2020; Antonatos *et al*., 2021). Moreover, the surrounding area of Kechries citrus grove was covered by a large number of pine trees, while in Ancient Corinthos, they rarely occurred. It is a fact that *N. campestris* aestivates on pine through the summer in Greece and other countries (Lago *et al.*, 2021; Cornara *et al.*, 2021), threatening the bordering orchards for infestation.

The adults of spittlebugs were observed twice in regularly sampled citrus groves. Firstly, they appear in spring and usually in May, then they disappear in the period of summer till early autumn (from June to mid-October), and finally they reappeared in late autumn and early winter, following the growth and abundance of spontaneous herb food plants. The late reappearance of adults in autumn may depend on scarce rain (7.6 and 23.6 mm, respectively) and the relatively high temperature (25.1 and 21.0 °C, respectively) in September and October. This fact unveils that these climatic conditions were unfavourable for the spittlebugs, while at the same time the appearance of the new vegetation was delayed.

In summer, especially in July, many cicadas from the species *C. orni* and *T. plebejus* were observed in the foliage of citrus trees in the citrus grove of Ancient Corinthos. Cicadas are xylem-sap feeders; therefore, their feeding behaviour characterizes them as potential vectors of *X. fastidiosa*. Data on the ability of cicadas to transmit *X. fastidiosa* to plant species are scarce and especially in citrus are absent. There are two records of successful transmission; Paiaõ *et al.* (2002) reported that *Dorisiana viridis* (Olivier) (Hemiptera Cicadidae) can transmit the bacterium to coffee plants and Krell *et al.* (2007) stated that the transmission of the bacterium through *Diceroprocta apache* (Davis) (Hemiptera Cicadidae) to grapevines was possible. However, Cornara *et al.* (2020) reported that they were unable to transmit *X. fastidiosa* either to grapevines with *Platypedia minor* Uhler (Hemiptera Cicadidae) or to olive plants with *C. orni*. Thus, they concluded that the cicada species have no or negligible role in the natural spread of *X. fastidiosa*.

Adults of *N. campestris* were initially observed in the neighbouring citrus grove natural area in Kechries from mid-May till late November. Their population density in pine trees remained relatively stable during this period. In November, they returned to the groves aiming to lay their eggs in plant debris near herbaceous plants, which would serve as hosts for the nymphs the following spring. According to our data, the pine trees must be among the food plants of the species and at the same time they act as a shelter for *N. campestris* during summer. Our results are in accordance with Lago *et al.* (2021) and Cornara *et al.* (2021), who also stated that this species moves and settles on pine trees during late spring and summer. The few adults observed in *P. lentiscus* must have occurred there accidentally. Thus, the wide distribution of pine trees in the Mediterranean area is an advantage for the species. On the contrary, adults of *P. spumarius* miss the wild plants and pines that are not their aestivation sites. However, a few non-aestivating adults were on pine trees in November, possibly due to mass movements from their aestivation sites to the cultivated fields.

The citrus crop is an irrigated cultivation in Greece during summer. Therefore, the weeds *Cynodon dactylon* L. (Poaceae), *Sorghum halepense* L. (Poaceae), *Cyperus* sp. (Cyperaceae) and *Setaria* sp. (Poaceae) were present under the citrus trees in Kechries, while only *C. dactylon* was raised in the citrus grove of Ancient Corinthos during summer. However, no spittlebugs existed in those plants at that time. Our results indicate that conditions in cultivated groves such as high temperature, high solar radiation, low humidity, etc., makes the environment unfavourable for the spittlebugs during summer, fact that may trigger the mass movement behaviour.

The adults of all spittlebug species were rarely found on citrus tree foliage, showing a preference for the herbaceous plants in the ground. In the samplings conducted in 2023, adults of *P. spumarius* and *N. campestris* were found in the citrus foliage only when increased adult densities in the ground vegetation were observed (supplemental material table S1). In the present work we did not search whether or not spittlebug adults can feed with citrus. However, the presence of adults in several cases in the foliage of citrus trees may imply that they can feed at least occasionally with citrus. Moreover, according to Cornara *et al.* (2017a), *P. spumarius* can transmit *X. fastidiosa* to sweet oranges in experimental conditions and this is an additional indication that, at least the meadow spittlebug, can feed with citrus. Thus, we can conclude that spittlebugs may not be very efficient vectors of the bacterium in the citrus fields due to the low densities of adults found in the tree canopies. However, since the transmission of the bacterium via the spittlebugs is possible, in the case of outbreaks and especially when there are no plenty host plants for spittlebugs other than citrus, the implementation of environmentally friendly methods to control spittlebug nymphs, such as mowing or tillage in spring, should be considered (Cornara *et al*., 2018; Dongiovanni *et al*., 2018; 2019).

Conclusion

The present study reports the occurrence and the population dynamic of *X. fastidiosa* potential vectors in citrus groves and a natural area. The spittlebugs *P. spumarius* and *N. campestris* were the most frequent species in the citrus groves of central Greece. Furthermore, we recorded five other potential vector species (*C. sanguinolenta*, *P. signatus*, *C. viridis*, *C. orni* and *T. plebejus*). Nymphs of spittlebugs were observed between late March and mid-May, whereas adults were present in spring, late autumn and early winter. During the summer months, the adults of *N. campestris* moved massively towards pine trees, which they act as a shelter for the hot period of the year. Most spittlebug adults were recorded in ground vegetation, while low densities were in canopies. The low occurrence of spittlebugs on citrus foliage indicates that their effectiveness in transmitting the bacterium may be limited, fact that is an advantage in preventing the rapid spread of the disease over wide areas. However, control measures for spittlebugs must run in case of bacterium outbreaks.

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