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Natural areas as reservoir of candidate vectors of *Xylella fastidiosa*

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Abstract

Spittlebugs have been identified as the main European vectors of the bacterium *Xylella fastidiosa* Wells *et al*. in different pathosystems. However, our knowledge about bacterium epidemiology in Apulian (Southern Italy) olive orchards is currently limited to the late spring secondary (olive-to-olive) spread mediated by the spittlebug *Philaenus spumarius* (L.). Summer dispersal toward wild trees and shrubs is a common trait observed in several spittlebug species that could have important impacts on *X. fastidiosa* epidemiology. Here, we report preliminary data on presence and abundance of spittlebugs during the year in four different habitats interspersed with cultivated orchards within a natural area in Apulia. In two surveyed habitats, a pine forest and a lake shore, the latter dominated by elm plants, spittlebug adults peaked during the period June-September, with maximum values in July-August on elm. Three spittlebug species, *Neophilaenus campestris* (Fallen), *P. spumarius* and *Philaenus italosignus* Drosopoulos et Remane were evenly collected on pine plants, while the latter was the most abundant on elms. A few individuals were collected on elm throughout the end of the year, while on pines spittlebugs were only collected in summer. The possible implications of our findings for *X. fastidiosa* epidemiology in both cultivated orchards and natural habitats, together with future research needs are discussed.

Key words: spittlebugs, survey, pine forest, lake shore, dry grasslands, oak forest.

Introduction

Many pathogens responsible for some of the most important diseases in humans, animals and plants are routinely described as "vector-borne". A vector can be defined as: i) a host within a multi-host transmission cycle; or ii) the most mobile host in a transmission cycle of two or more hosts (Wilson *et al*., 2017). Understanding the dynamics and drivers of a vector's dispersal throughout its life cycle is crucial to predict and eventually control the spread of a plant pathogen (Fereres and Moreno, 2009). Spittlebugs (Rhynchota Aphrophoridae) are the only confirmed vectors in Europe of the bacterium *Xylella fastidiosa* Wells *et al*., whose introduction in the Old World has caused economic and social turmoil (Cornara *et al*., 2019; Saponari *et al*., 2019). Our knowledge of *X. fastidiosa* epidemiology in Europe is currently limited to few information, e.g. a secondary spread (olive-to-olive) of the bacterial strain ST53 within Apulian (Southern Italy) olive orchards, mediated by the meadow spittlebug *Philaenus spumarius* (L.) (Rhynchota Aphrophoridae) in late spring (Cornara *et al*., 2017). In Apulian olive orchards, adults of *P. spumarius* disperse toward olive plants soon after their emergence on ground cover plants, mediating the secondary transmission of the bacterium olive-to-olive, with olive serving as both source and reservoir plant for *X. fastidiosa* (Cornara *et al*., 2017). The factors driving *P. spumarius* dispersal toward olive, together with cues used to search for, detect, and recognize host plants, are currently under debate (Germinara *et al*., 2017; Avosani *et al*., 2020). In Apulia, adults are abundant on olive canopies in May-June, during the phenological stage of inflorescence emergence. Thereafter, when olive is no longer suitable possibly because of changes in plant physiology and chemistry driven by the severe summer climatic conditions (high temperature and drought stress), *P. spumarius* disperses toward suitable wild trees and shrubs surrounding the olive orchard (Drosopolous *et al*., 2010; Cornara *et al*., 2018; Bodino *et al*., 2020). These plants seem to be important reservoir hosts for the spittlebug, sustaining the population and providing food and shelter during seasonal periods with unfavorable climatic conditions for feeding on plants such as annual weeds and olive trees. Indeed, at the end of summer, spittlebugs move back to ground cover within the olive orchards to oviposit (Bodino *et al*., 2020). Besides the meadow spittlebug, summer dispersal toward oversummering hosts has been reported for other spittlebug species (Drosopoulos *et al*., 2010; Cornara *et al*., 2018a; Morente *et al*., 2018; Bodino *et al*., 2019; 2020; Lago *et al*., 2020). Wild trees and shrubs play therefore a crucial role in the life cycle of spittlebugs and, consequently, a possible crucial role in *X. fastidiosa* epidemiology, as both hosts for the vector and potential sources/reservoirs of the bacterium (EFSA, 2020). However, our knowledge about dispersal toward oversummering hosts is currently limited to summer collection of spittlebugs on plants typical of the Mediterranean vegetation within or bordering the surveyed olive orchards. Investigation focused on shrubs and trees of natural areas interspersed with cultivated orchards (i.e. in farmlands), targeting spittlebugs presence, abundance and population dynamic, is missing. Furthermore, compared to fruit crops, very little is known about *X. fastidiosa* on forest trees (Desprez-Loustau *et al*., 2021). The *X. fastidiosa* subspecies *multiplex* is the one most often associated with forest trees (Nunney *et al*., 2013) where it is the causal agent of a disease known as Bacterial Leaf Scorch (BLS), whose incidence and impact in Europe is largely unknown (EFSA, 2019). Strains of the subspecies *multiplex* emerged from a clade with limited genetic diversity and likely originating in California (USA) are currently present in France, Spain and Italy (Tuscany region) (Denancé *et al*., 2017; Marchi *et al*., 2018; Landa *et al*., 2020). According to a recent bioclimatic suitability model, subspecies *multiplex* represents a threat to the whole of Europe (Godefroid *et al*., 2019).

In this study we described the spittlebug abundance on wild trees and shrubs during the year, using data collected during a survey aimed at characterizing the arthropod fauna of an Italian protected natural area (Alta Murgia National Park, AMNP) located in Apulia Region. The study area is about 60-100 km north-west of the front of *X. fastidiosa*-infected area. Our data, even if preliminary, might be of help for the scientific community working on the bacterium spread.

Materials and methods

Study areas

The AMNP is a Special Protection Area (SPA) in the Site of Community Importance (SCI) called "Murgia Alta" IT9120007 (Commission Implementing Decision EU, 2019). AMNP has a Mediterranean climate, commonly with mild, moist winters and hot-dry summers (Cotecchia, 2014), even though extreme climatic conditions have become more frequent in recent years (ARIF, 2019). The present state of the territory is the result of natural and human processes occurring over the centuries, like grazing, deforestation and agriculture, creating semi-natural dry grasslands of high naturalistic importance (Mattia and Fracchiolla, 2010). The more intensive human activities, in the last thirty years, have modified the natural landscape affecting the availability of habitats, their quality and their overall biodiversity (Mairota *et al*., 2015).

The surveyed area (figure 1) covers a surface of circa 500 km^2 and is surrounded mainly by cereals, legumes, olive and almond orchards. Four habitats were investigated (table 1) (European Commission, 2013; Perrino and Wagensommer, 2013):

1) Dry grasslands (afterwards also referred as DGs or DG1/2/3): three sites characterized by "Eastern sub-Mediterranean dry grasslands (*Scorzoneratalia villosae*)", "Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea*" with few native shrubs species such as wild pear (*Pyrus spinosa* Forssk.).

2) Oak forests (afterwards also referred as OFs or OF1/2/3): three sites characterized by forests of Downy oak (*Quercus pubescens* Willd.), a deciduous species belonging to the "Eastern white oak woods". It included clearings with thermo-xerophilous annual meadows.

3) Pine forests (afterwards also referred as PFs or PF1/2/3): three sites characterized by reforestation of conifers mainly composed by Aleppo pines (*Pinus halepensis* Mill.). It included clearings with thermo-xerophilous annual meadows.

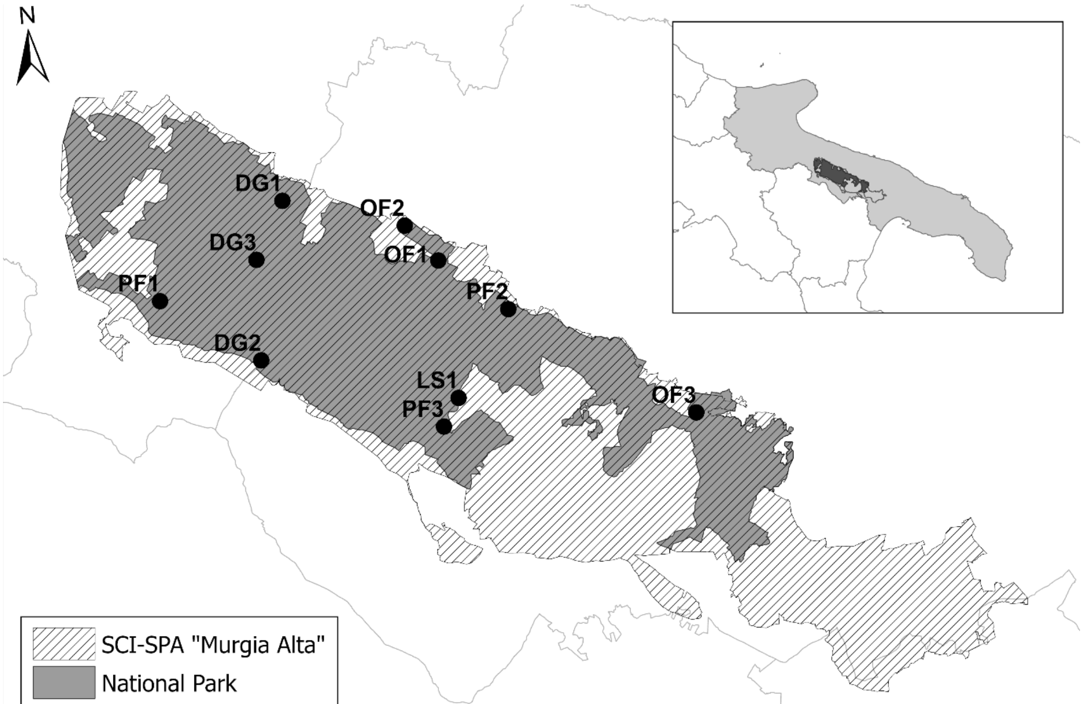

Figure 1. Map of the sampling sites in the Alta Murgia National Park (DG1/2/3 = dry grasslands; OF1/2/3 = oak forest; PF1/2/3 = pine forest; LS1 = lake shore).

Table 1. Sampling sites, habitat features and geographical details.

Code	Site	Habitat	Latitude N	Longitude E	Elevation (m a.s.l.)
DG1	Castel del Monte	Dry grassland	41°04'54.69"	16°17'09.69"	540
DG2	Rocca del Garagnone	Dry grassland	40°57'56.88"	16°15'09.11"	528
DG3	Monte Savignano	Dry grassland	41°02'24.16"	16°15'21.92"	539
OF1	Bosco Scoparella	Oak forest	41°01'41.86"	16°25'56.51"	250
OF2	Lama delle Grotte	Oak forest	41°03'21.72"	16°24'09.55"	379
OF3	Monte Cucco	Oak forest	40°54'00.44"	16°40'09.71"	428
PF1	Foresta di Acquatetta	Pine forest	41°00'55.91"	16°09'33.09"	595
PF2	Bosco Rogadeo	Pine forest	40°59'17.17"	16°29'46.04"	379
PF3	Pulicchio di Gravina	Pine forest	40°54'16.84"	16°25'9.37"	555
LS	Laghetto San Giuseppe	Lake shore	40°55'33.51"	16°26'26.16"	515

4) Lake shore (afterwards referred as LS): a permanent or semi-permanent pond belonging to "Natural euthrophic lakes with *Magnopotamion* or *Hydrocharition-type* vegetation", which showed significant variation in level related to the rainfall and evapotranspiration regime. Most of the trees near the lake are elms (*Ulmus minor* Mill.).

Sampling

The survey was carried out monthly from August 2016 to July 2017. Insects were collected from trees and shrubs using entomological umbrella through frappage method (hitting the canopy with three strokes and collecting the insects falling on a cloth spread beneath the canopy with a mouth aspirator). The collections were carried out between 10:00 and 16:00 in a serene weather day (no heavy rain, hailstorm, snow or strong wind occurred during the sampling periods) in the late days of each month.

We selected the dominant tree species for each habitat type: trees of Almond-leaved pear (*P. spinosa*) were sampled in DGs, Downy oak (*Q. pubescens*) in OFs, Aleppo pine (*P. halepensis*) in PFs and elm (*U. minor*) in LS. One sample per experimental site and sampling date was performed, consisting of collection from five trees through frappage, storing and recording separately the specimens collected from each of the trees. Insect collection was carried out on the same tree specimens at each sampling round during the survey period. These insects were stored in plastic tubes filled with 70% of ethanol and kept at 4 °C until identification. Xylem feeders were identified at species level according to the EPPO standard PM 7/141 (1) (EPPO, 2020). Temperatures of the sampling sites were recorded by Acrotec Foundation (CIMA Research Foundation, Savona, Italy) at five different meteorological stations placed throughout the Park (supplemental material table S1, figure S1), located in a range of 4 to 8 km from each of the sampling sites.

Data analysis

We explored the effects of habitat and temperature factors on species abundance (i.e. sum of insect abundances per sampled tree and shrub, per sampling round) with a generalized linear mixed-effects model (GLMM; Poisson distribution with a log link function). We built a single model using as the response variable the pooled abundances of all species, since models built for single species were underdispersed. The explanatory variables were habitat type (DG, LS, OF and PF), mean monthly temperature per site per sampling round (for each site we used the mean monthly temperature recorded by the meteorological station closest to the site in a range of four to eight km) and the interaction between these two factors. These variables showed no collinearity. We accounted for the nested design of the study by including site identity (N = 10), and replicate within each site (N = 50) as random factors. All the analyses were performed in R (R Core Team, 2020). We ran the model using 'lme4' package (Bates *et al*., 2017). We calculated marginal and conditional coefficients of determination (R^2) computed with lognormal method for GLMM using the 'MuMIn' package (Barton, 2016). We checked the model for overdispersion (scale parameter = 0.92) using the 'blmeco' package (Korner-Nievergelt *et al*., 2015) and for residual distribution using the 'DHARMa' package (Hartig, 2017). There was no evidence of either spatial or temporal autocorrelation of model residuals (analyses performed using the 'ncf' and 'acf' packages, respectively; Bjørnstad, 2016).

Results

We counted a total of 211 xylem-sap feeder individuals (mean per sampled tree = 0.35, SD = ± 1.20). *Philaenus italosignus* Drosopoulos et Remane (Rhynchota Aphrophoridae), *Neophilaenus campestris* (Fallen) (Rhynchota Aphrophoridae) and *P. spumarius* were the most represented species, with 131 (62.1%), 52 (24.6%) and 25 (11.8%) individuals collected, respectively. Two other xylem-sap feeder species were occasionally found: *Aphrophora alni* (Fallen) (Rhynchota Aphrophoridae) and *Cercopis vulnerata* Rossi (Rhynchota Cercopidae) (supplemental material table S2). No sharpshooters (Rhynchota Cicadellidae Cicadellinae) were present at the sampling sites. We found higher relative abundances of spittlebugs in LS and PF habitats (supplemental material table S3), especially during the warmer months, i.e. June-September (figure 2). On elms of LS habitat almost exclusively *P. italosignus* individuals were collected, while on Aleppo pines of PF habitat the three main spittlebug species were more evenly found throughout May-September (figure 2). Few spittlebugs were collected overall in both DG and OF habitats. LS habitat was the only site where spittlebugs were present up to December, although just a few specimens were collected after the summer peak (figure 2).

GLMM analysis showed that species abundance was positively correlated with the increase in temperature (figure 3). This trend was particularly evident in LS habitat (table 2).

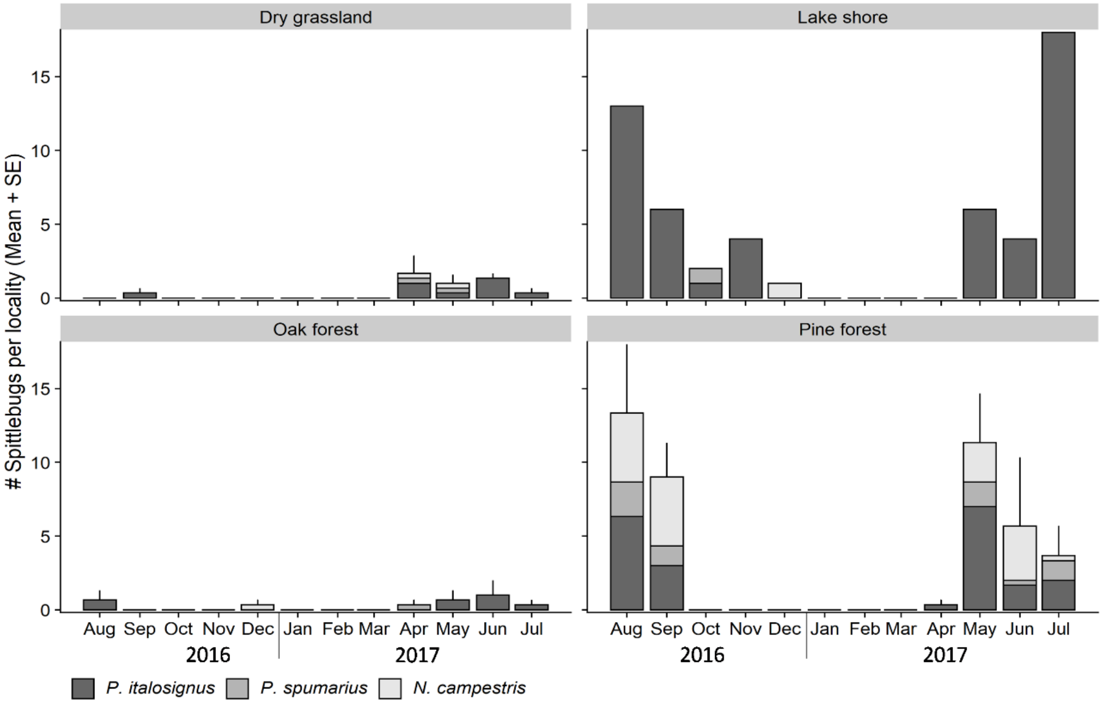

Figure 2. Monthly abundance of spittlebug adults (*P. italosignus*, *P. spumarius*, *N. campestris*) (mean no. individuals per locality ± SE) for each habitat; standard error bars refer to the total number of individuals collected; missing standard error in LS (lake shore) because a single locality was sampled.

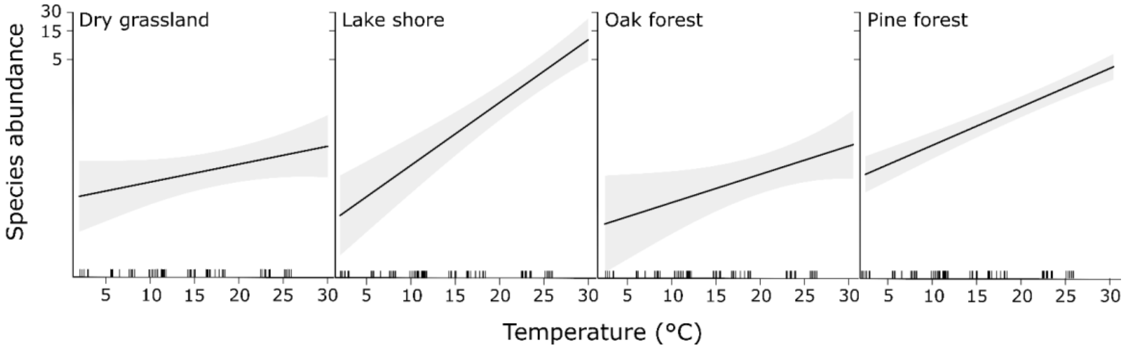

Figure 3. Mean specimens abundance (per sampling round) in response to site/sample specific temperature in the different habitat types. Plots include model estimate (black line) and 95% confidence interval (shading).

Table 2. Results of the GLMM (family: Poisson, link-function: log) testing the response of species abundance to temperature, habitat type and their interaction. Dry grassland was used as a baseline for the other three habitats and their interactions with temperature. Marginal $R^2 = 0.56$; conditional $R^2 = 0.62$.

Variable	Estimate	SE	*z*-value	*p*-value
Intercept	−3.71697	0.75035	−4.954	<0.0001
Lake shore	−1.01011	1.11806	−0.903	0.36629
Oak forest	−1.06881	1.26552	−0.845	0.39836
Pine forest	0.72853	0.83533	0.872	0.38313
Temperature	0.06741	0.03931	1.715	0.08641
Lake shore × temperature	0.16951	0.05175	3.276	0.00105
Oak forest × temperature	0.03949	0.06367	0.620	0.53509
Pine forest × temperature	0.07764	0.04226	1.837	0.06618

Discussion

Spittlebugs dispersal toward oversummering hosts is a crucial and largely unexplored step related to *X. fastidiosa* epidemiology in Europe. Changes in plant physiology and chemistry and climatic conditions can affect spittlebug dispersal directly and indirectly (Drossopoulos *et al.*, 1988a; 1988b; Drake, 1994). Host acceptability and feeding rate of xylem-sap feeders largely depend on xylem nutrient content, particularly amino acids, whose concentration and availability can be strongly affected by high temperature and plant drought stress (Horsfield, 1977; Andersen *et al.*, 1992; Brodbeck *et al.*, 1993; Krugner and Backus, 2014). Wild trees and shrubs are abler to cope with water stress than cultivated plants (Zhao *et al.*, 2010) and represent the preferable oversummering hosts for spittlebugs, particularly in Mediterranean climate characterized by hot and dry summer. Consistently with this hypothesis, in the present study we observed a strong positive correlation between temperature and spittlebugs abundance on wild woody plants in all the monitored habitats. However, such correlation cannot be considered conclusive, but rather suggestive of a temperature-related dispersal, since climatic data were downloaded from meteorological stations located in a range of four to eight km from each sampling site instead of being measured at the site. Nevertheless, as evident in supplemental material figure S1, mean monthly temperatures were rather similar all over the area. Overall, the exact host-related factors influencing spittlebugs attraction and the subsequent dispersal deserve further investigation.

The correlation temperature/abundance of spittlebugs was statistically highly significant in the lake shore where elm plants were monitored. Also, a positive correlation close to statistical significance was observed in the pine forest, where Aleppo pines were sampled. Elm is characteristic of humid areas and was reported by Pavan (2000) as an oversummering host of *P. spumarius*, possibly migrating from a nearby vineyard. The meadow spittlebug has been also indicated as vector of the Elm Yellow phytoplasm (Matteoni and Sinclair, 1988), a statement that appears questionable given the insect feeds predominantly on xylem (Malone *et al.*, 1999; Cornara *et al.*, 2018b). In our study, the majority of individuals collected on elm were *P. italosignus*, a species sympatric with *P. spumarius* as adult, while the juveniles develop exclusively on lily plants (*Asphodelus* spp.) (Drosopoulos and Remane, 2000; Drosopoulos, 2003; Panzavolta *et al.*, 2019). Recently, *P. italosignus* received attention because it was demonstrated to transmit *X. fastidiosa* to various host plants under laboratory conditions (Cavalieri *et al.*, 2019). Although this spittlebug species appears to have no role in the epidemiology of *X. fastidiosa* in Southern Apulia olive orchards, it could be relevant in other pathosystems (Cornara *et al.*, 2019; Cavalieri *et al.*, 2019; Panzavolta *et al.*, 2019). *N. campestris*, *P. spumarius* and *P. italosignus* were found on Aleppo pine trees in the pine forests throughout the summer. Mediterranean coniferous trees may better tolerate water stress than dicot trees, possibly because of their vascular anatomy (Sperry *et al.*, 2006; Hacke *et al.*, 2015). Drought tolerance, perhaps leading to possible less drastic variations in plant physiology and chemistry during the summer period, could be the reason why pine forests might be areas attracting oversummering spittlebugs, as reported by other authors for *N. campestris* (Lopes *et al.*, 2014; Morente *et al.*, 2018; Lago *et al.*, 2020) and *Philaenus* spp. (Drosopoulos *et al.*, 2010). Spittlebug abundance on both elms and pines peaked during the June-September period, with greatest values in July-August on elms. In contrast with pines, spittlebugs were collected from elms continuously until the end of the year, although in far lower numbers compared to the June-September period. The presence of spittlebugs in the lake shore area during autumn could be related to the location of this habitat within a landscape dominated by both arable land and semi-natural prairies (*Onopordion illyrici*), and also with a small pine reforestation area present in the vicinity (circa 200 m). Thus, the proximity of herbaceous habitats favourable to oviposition, given the presence of the nymphal host-plants (*Asphodelus* spp.; data not shown), could have favoured the colonization of elm trees by *P. italosignus* during autumn (Maryanska-Nadachowska *et al.*, 2012; Panzavolta *et al.*, 2019). In the other two habitats, dry grassland with *P. spinosa* and oak forest with *Q. pubescens*, few individuals were collected. The low spittlebug presence in the dry grassland could be due to the low preference of spittlebugs for *Pyrus* spp. plants (N. Bodino personal observation) together with the high levels of summer water stress and poor understory not supporting the development of high levels of resident nymphal population. Therefore, the few individuals collected on *P. spinosa* plants could just be transients migrating to oversummering areas in surrounding habitats. Regarding the low spittlebug captures in oak forests, the oak species present was *Q. pubescens*, a woody species on which spittlebugs have been rarely found (N. Bodino personal observation). *Q. pubescens* may represent a suboptimal woody host for spittlebugs in dry periods, possibly because less able than other Mediterranean plants to overcome severe water stress, resulting in changes in xylem vessels density and amino acids content (Gričar *et al.*, 2017; 2019). Previous studies carried out in olive agroecosystems, both in Apulia Region and in other Mediterranean areas, showed that *Quercus* spp. may represent important hosts for spittlebugs during summer months (Drosopoulos, 2003; Morente *et al.*, 2018; Bodino *et al.*, 2019; 2020). However, these studies sampled spittlebugs mainly in the surroundings of olive groves, in an agricultural landscape, and mainly on *Quercus ilex*, a species presenting less hardened leaf tissues than *Q. pubescens*.

Exploitation of and movement among different plants and habitats by spittlebug adults may have important ramifications for a hypothetical scenario of *X. fastidiosa* outbreak. On the one hand, pine forests may host and shelter populations of infectious spittlebugs coming from cultivated areas during the unfavourable summer periods, with insects going back to the crops in late summer potentially as a source of inoculum. On the other hand, humid areas like the lake shore could be an ecological sink for *X. fastidiosa*, with possibly infective vectors migrating toward these habitats for oversummering and remaining there until the end of their life cycle. In this latter case, late-summer infections caused by adults migrating back to crops after oversummering in humid areas would be of marginal importance. However, these scenarios are completely speculative and focused research is needed to clarify the epidemiological importance of each type of oversummering habitat. In addition, detailed and large-scale observations on the entire spittlebug life-cycle and population dynamic in both cropping systems and natural areas, coupled with dispersal trials to quantify movement within and between ecological compartments, are crucial to set-up sustainable long-term management of *X. fastidiosa* mediated diseases.

Indeed, common pests control practices based on reducing insect population levels under acceptable thresholds are likely of limited use for containing the spread of vector-borne pathogens transmitted to the host plants in few minutes, as *X. fastidiosa* was recently proposed to be (Cornara *et al.*, 2020).

Conclusions

Our findings, consistently with other studies (Santoiemma *et al.*, 2019), highlight the importance of the whole landscape composition on spittlebug population structure. Natural and semi-natural areas interspersed with cultivated orchards could act as vector reservoirs, sustaining their populations during the summer and possibly acting as both recipient and source/reservoir areas for *X. fastidiosa*. Further research efforts are needed for: i) understanding the role natural areas may play in *X. fastidiosa* epidemiology, i.e. coupling observations on host plant susceptibility to different bacterial strains and bacterial colonization of host tissues, with both field and laboratory transmission experiments determining host competence and transmission efficiency; ii) determining the host-related factors driving spittlebugs host-selection and dispersal; iii) analysing the spatial scale at which natural areas may affect behaviour and movement of spittlebugs.

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