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ORIGINAL CONTRIBUTION

Transmission of *Xylella fastidiosa* by naturally infected *Philaenus spumarius* (Hemiptera, Aphrophoridae) to different host plants

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Abstract

The recent establishment of *Xylella fastidiosa* subspecies *pauca* in the southern Italian region of Apulia threatens agricultural crops and the environment. Olive is an important and widespread ancient crop in Italy and, so far, the most impacted host. The meadow spittlebug *Philaenus spumarius* (Hemiptera, Aphrophoridae) has been identified as a vector of *X. fastidiosa* in southern Italy; this species is one of the most common potential vectors in Europe. To generate disease management strategies, data on *X. fastidiosa* transmission by *P. spumarius* are necessary. Therefore, we carried out transmission experiments by using field-collected spittlebugs in 2014 and 2015 (5 and 11 collection dates, respectively), and transferring groups of insects immediately on to recipient plants. Various host plant species were tested: olive, oleander, sweet orange, grapevine and the stone fruit rootstock GF677 (*Prunus persica* × *Prunus amygdalus*). *Xylella fastidiosa* was detected in all the host plants after insect plant access except for grapevine; infections to sweet orange and stone fruit were not systemic. In 2015, estimates of insect *X. fastidiosa* infectivity were obtained; the number of PCR-positive *P. spumarius* on each plant was positively correlated with the plant infection status. The proportion of *P. spumarius* infected with *X. fastidiosa* ranged from 25% to 71% during the entire survey period. The number of *X. fastidiosa* cells detected in *P. spumarius* heads ranged from 3.5×10 to 4.0×10^2 (CFU equivalents), which is lower than that reported for leafhopper vectors in the Americas. These data show that field-collected *P. spumarius* have high rates of *X. fastidiosa* infection and are competent vectors.

Introduction

Pathogen introduction is often regarded as the main driver of emerging infectious diseases, although an arthropod-borne pathogen accidentally introduced in a new region requires a vector for establishment (Fereres 2015). The introduction of *Xylella fastidiosa* into Europe (Martelli et al. 2016) is an example of a biological invasion that required endemic competent vectors for establishment (Almeida and Nunney 2015). In 2013, olive trees (*Olea europaea*) on the west coast of the Salento Peninsula (Apulia, Italy) showing leaf scorch and dieback, that is an unknown disease named 'olive quick decline syndrome' (OQDS), were found to be infected with *X. fastidiosa* subspecies *pauca* (Saponari et al. 2013). *Xylella fastidiosa* has been

detected in olive in the USA (Krugner et al. 2014; *X. fastidiosa* subsp. *multiplex*), Argentina (Haelterman et al. 2015; *X. fastidiosa* subsp. *pauca*) and more recently in Brazil (Coletta-Filho et al. 2016; *X. fastidiosa* subsp. *pauca*). There are limited data on *X. fastidiosa* infecting olives, but evidence indicates that pathogen genotype defines pathogenicity. While *X. fastidiosa* is associated with but does not cause disease in olives in the USA (Krugner et al. 2014), Koch's postulates have been fulfilled in Italy (Saponari et al. 2016); pathogenicity data are not available from Brazil or Argentina.

Only one genotype, based on multilocus sequence typing, has been recovered from olive trees and other hosts in Italy (Loconsole et al. 2016). The genotype is an identical match to *X. fastidiosa* subsp. *pauca* strain ST53, reported from Costa Rica in 2014 (Nunney et al. 2014; Giampetruzzi et al. 2015). In Italy, ST53 has been detected in cherry (*Prunus avium* L. 1755), myrtle-leaf milkwort (*Polygala myrtifolia* L.), coastal rosemary (*Westringia fruticosa* Willd.), acacia (*Acacia saligna* Labill.) and Spanish broom (*Spartium junceum* L.). To date, the host range of this genotype includes 22 plant species (Loconsole et al. 2016). It has been demonstrated that the meadow spittlebug *Philaenus spumarius* was a vector of *X. fastidiosa* in Italy using periwinkle as an indicator plant Saponari et al. (2014); it has also been demonstrated that *P. spumarius* transmits *X. fastidiosa* from infected to uninfected olive trees (Cornara et al. 2016a).

Unfortunately, this vector species is ubiquitous and highly polyphagous; the nymphs can feed on huge varieties of herbaceous plants, while the adults disperse to an even higher number of plant species, including many trees and shrubs (Weaver and King, 1954; Delong and Severin 1950). In the contaminated area, olive is the predominant host in the cultivated areas, although other perennial hosts have been identified, mainly in non-cultivated areas, such as gardens and natural landscape. However, since both *X. fastidiosa* and *P. spumarius* have a wide host range (EFSA 2015), and plant community composition is important in the epidemiology of *X. fastidiosa* -associated diseases, we conducted studies in order to (i) evaluate the transmission of *X. fastidiosa* by *P. spumarius* to different host plants species and cultivars; and (ii) assess *P. spumarius* natural infectivity in olive orchards infected by *X. fastidiosa*.

Material and Methods

Plants

Plants used for the transmission experiments included different species and cultivars: (i) olive (*Olea europaea*) cv. Coratina, cv. Ogliarola (two autochthonous olive cultivars widely grown in the Apulia region) and olive seedlings obtained from seeds of the cv. Cima di Mola; (ii) oleander (*Nerium oleander*); (iii) sweet orange (*Citrus sinensis*) cv. Madame vinous; (iv) grapevine (*Vitis vinifera*) cv. Cabernet Sauvignon; (v) stone fruit rootstock GF677 (hybrid *Prunus persica* × *Prunus dulcis*), commonly used for stone fruits breeding; and (vi) periwinkle (*Catharanthus roseus*). For the two olive cultivars and grapevines, experimental plants consisted of self-rooted cuttings; for the stone fruit rootstock GF677, they consisted of micropropagated plantlets, whereas seedlings were used for sweet orange cv. Madame Vinous and olive. All the plants were grown in a greenhouse located on the premises of the Campus of the University of Bari (in the pest-free area). Plants of 15–20 cm with young vegetating apexes were then transferred in a screenhouse in the infected area where the transmission tests were carried out.

Transmission tests

We collected adult *P. spumarius* every 2 weeks from May to October, from infected olive orchards in Gallipoli (Apulia, Italy), by sweeping net and mouth aspirator. Insects were collected either from canopy or suckers of olive trees with severe symptoms of desiccation and dieback. Two distinct olive groves severely affected by OQDS (with 100% of symptomatic trees), one in 2014 and another one in 2015, were selected for insect collection; in both olive groves, the presence of *X. fastidiosa* in olive trees (ca. 200) was confirmed by real-time quantitative polymerase chain reaction (qPCR) (data not shown). Spittlebugs were temporarily stored in groups of five individuals in aerated plastic vials and brought to an insect-free transfer room. We caged five randomly selected spittlebugs on each recipient plant. Cages were made with fine mesh net sealed at the superior rim of the pot with two rubber bands. Transmission tests took place in an insect-proof screenhouse. A 4-day and 7-day inoculation access period (IAP) and 7-day IAP were used in 2014 and 2015, respectively. For each treatment (i.e. host plant), five replicates were performed; five plants of the same species/cultivar for each date with five insects per plant, except for periwinkle, for which two replicates per date were used. At the end of the IAP, insects were removed from the plants and stored in 75% ethanol. *P. spumarius* was identified according to Ossiannilsson (1981). Plants were treated with a systemic insecticide (Imidacloprid, Confidor 200 SL), stored in an insect-free screenhouse and tested by

qPCR. In 2015, the presence and the population size of *X. fastidiosa* in *P. spumarius* were determined by qPCR in insects surviving the IAP. All the protocols are described below.

Details of transmission assays for 2014 and 2015, including insect collection dates, plant species and number of tested plants, and insect and plant infection status after experiments, are available in Tables S1 and S2.

Xylella fastidiosa detection in plants and insects

Plants were tested for the presence of *X. fastidiosa* by qPCR, using the assay developed by Harper et al. (2010). In 2014, all plants were sampled and tested 3, 6 and 14 months after the end of the last transmission tests (19 September 2014). Similarly, plants exposed to infective *P. spumarius* in 2015 were sampled and tested 3 and 6 months after the last transmission experiment (14 October 2015). Samples consisted of 4–6 leaves representative of the entire plant (collected from the bottom to the top of the main stem). Recipient plants that tested positive were resampled to assess the bacterial spread and the host colonization following the plant growth. Specifically, from each recipient qPCR-positive plant, leaves from the portion exposed to insect feeding and from the new growth were collected and tested separately (table 1).

Spittlebugs were tested individually for the presence of *X. fastidiosa.* Briefly, the head of each insect was excised, the DNA extracted using a CTAB-based protocol (Loconsole et al. 2014) and SYBR-Green-based qPCR assays performed using the primers targeting the conserved hypothetical HL protein (Francis et al. 2006); post-amplification melting curve analysis was carried out to assess the specificity of the amplification products. In 2015, at each date of collection, absolute quantification of the bacterial population harboured in the head of each *P. spumarius* was performed on five randomly selected qPCR-positive spittlebugs. Quantification cycle (Cq) value was plotted against a standard curve generated using serial 10-fold dilutions ranging from 10 to 10^7 CFU/ml of a bacterial suspension of Salentinian strain (CoDiRO) of *X. fastidiosa*.

Statistical analysis

IBM SPSS Statistics 20 (SPSS 2012) was used for statistical analysis. To test for differences in transmission rate among species/cv used as recipient plants, and for differences related to the period when the transmission tests were carried out, data were analysed with the GENMOD procedure using the binomial distribution and the logit as link function in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). A likelihood ratio test was used to determine whether the host species or period when transmission test was performed affected ($P < 0.05$) transmission rate. The statistical significance ($P < 0.05$) of the likelihood ratio was determined by a chi-square test and least square means were used to determine significant differences ($P < 0.05$) among treatments (Agresti 2007). Hosts to which there was no detected transmission were excluded from statistical analysis. The 2014 and 2015 data sets were analysed independently. For olive seedlings, oleander and citrus, we assessed possible differences in transmission rate related to the different IAP (i.e. 4 and 7 days) through one-way ANOVA. Furthermore, for 2015, olive cv. Ogliarola and olive seedling, oleander and periwinkle tested plants, we evaluated the effect of the number of PCR-positive *P. spumarius* in each group (or plant) on the plant infectious status (0 = negative; 1 = positive) through binary logistic regression.

Table 1 Detection of *Xylella fastidiosa* in leaf samples of the recipient plants. Samples were collected at the insect feeding site and on the new growth developed after the transmission experiment. Transmission experiments of 2014 and 2015

Species	No. of total positive plants	No. of positive samples for the corresponding plant part*	
		(a)	(b)
Olea europaea cv. Coratina	8	8	8
Olea europaea cv. Ogliarola	24	24	24
Olive seedling	30	30	30
Prunus persica × *Prunus dulcis* (hybrid GF677)	1	1	0
Nerium oleander	37	37	37
Citrus sinensis cv. Madame Vinous	10	10	0
Vitis vinifera cv Cabernet Sauvignon	0	0	0
Catharanthus roseus	9	9	9

*Part (a) refers to the basal part of the plant (ca. 15 cm) including the leaves exposed to the insect feeding; part (b) refers to the plant parts above 15 cm including the new growth developed after the transmission experiment.

Results

Transmission tests

In 2014, transmission rates differed significantly among host species ($P < 0.05$), although grapevines and the GF677 rootstocks were not included in the analysis as there was no detection of *X. fastidiosa* in those hosts (fig. 1). Transmission rate was highest for oleander (72.00 ± 8.00), decreasing significantly ($P = 0.0484$) for olive seedling (44.00 ± 13.27) and olive cv. Coratina ($P = 0.0060$) (32.00 ± 14.97) with no significant differences between them ($P = 0.3836$), being lowest for sweet orange (16.00 ± 16.00). Lastly, there was no difference in overall transmission rate related to the period when transmission tests were carried out in 2014 ($P = 0.356$), except for that performed in August that showed a significantly lower value than those sampled in April ($P = 0.0026$) or September ($P = 0.0242$). In 2015, treatments with *X. fastidiosa* infection were statistically different ($P < 0.05$). Transmission to GF677 rootstock, with only one plant infected in mid-June 2015, was significantly lower than to olive cv. Ogliarola ($P = 0.0012$), olive seedlings ($P = 0.015$), oleander ($P < 0.0001$) and periwinkle ($P = 0.0010$), but not differed for sweet orange ($P = 0.1295$). For those hosts, transmission rate was significantly higher for oleander (72.00 ± 8.00) and periwinkle (50.00 ± 11.79), decreasing significantly for olive cv. Ogliarola (43.64 ± 10.02) and olive seedling (42.22 ± 8.46) with no significant differences among them ($P \geq 0.05$), being lowest ($P < 0.0015$) for sweet orange (12.00 ± 5.33). There were no significant differences ($P \geq 0.05$) in transmission rate related to the period when experiments were carried out, except for samples taken in mid-May and early June that showed a significantly lower transmission rate ($P < 0.05$). The increment in IAP from 4 days in 2014 to 7 days in 2015 had no effect on infection rate for olive seedlings ($P = 0.8855$), oleander ($P = 0.7473$) or sweet orange ($P = 0.6320$).

Periodic diagnostic tests performed after both sets of transmission experiments showed that in both oleander and olives, bacterial spread occurred through the entire plants, including colonization of the new growth (table 1, Figure S1). For sweet orange, the bacterium was consistently detected only in the leaves and stems of the portion exposed to the infective insects, that is the 15 basal centimetre exposed to insect feeding. None of the samples (leaves and stem portions) collected from the new growths were positive for *X. fastidiosa*. Infection of the GF677 rootstock occurred only in one out of 65 plants exposed during 2014 and 2015. Similarly to sweet orange, the bacterium was detected only in the portion exposed to the infective spittlebugs; additional tests failed in detecting the bacterium in the new growth.

Infectivity of *Philaenus spumarius* used for the transmission tests

In 2015, a total of 1242 specimens used for transmission tests conducted from May to October were tested for the presence of *X. fastidiosa*; statistical difference among sampled population was observed (Student's $t = 8.02$, d.f. = 10, P-value <0.0001). We detected PCR-positive *P. spumarius* in early May, soon after adult emergence in late April (Cavalieri, personal observation). Infectivity reached values higher than 50% from June throughout August, with a peak of about 70% of PCR-positive specimens in late June. Thereafter, a decreasing trend was observed from late August to October (fig. 2). The number of PCR-positive *P. spumarius* individuals per plant was a valid explanatory variable for plant infection status calculated in 2015 for olive cv. Ogliarola, olive seedling, oleander and periwinkle ($Exp(B) = 2.007$; $P < 0.001$). *Xylella fastidiosa* populations in the head of *P. spumarius* ranged from 3.4×10 to 4.0×10^2 CFU/insect head (mean = 183.2 ± 31.38 SE, n = 55).

A different survival rate was recorded after the IAP on the host plants ($\chi^2 = 181.99$, 5 d.f., $P < 0.001$) (Figure S2). While the percentages of insects

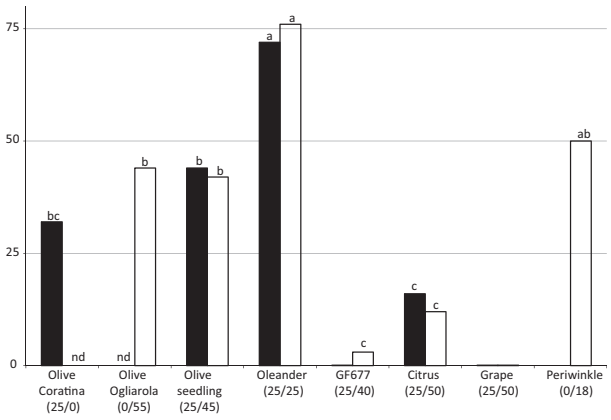

Fig. 1 Proportion of *Xylella fastidiosa* -positive plants infected with field-collected *Philaenus spumarius* adults in 2014 (black bars) and 2015 (white bars). Data from different sampling periods within the same year are pooled. Numbers in parenthesis indicate the total number of tested plants in each of the 2 years; 'nd' indicates that the treatment was not tested. For each year, bars with the same letter are not different according to Fisher's least significant difference test ($P < 0.05$).

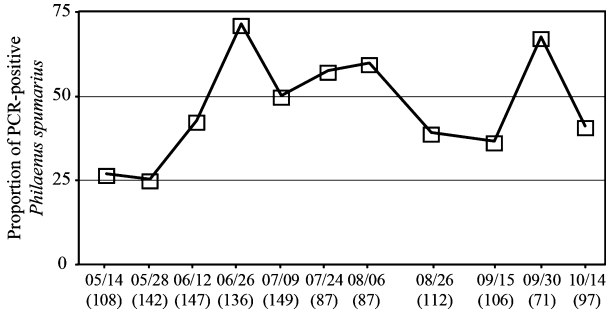

Fig. 2 Proportion of field-collected adult *Philaenus spumarius* that tested positive for *Xylella fastidiosa* (qPCR) in 2015. Values in parenthesis under collection dates indicate the number of insects tested. Insects were used for transmission experiments on different plants, but combined here based on collection date as individuals were collected as a single cohort from olive fields.

surviving the 7-day IAP were similar for the recipient olives, grapevines, stone fruit rootstocks GF677 and periwinkles (from 63% to 71% of survived insects), lower percentages, 8 and 58%, were recovered on oleander and citrus plants, respectively.

Discussion

Field-collected *P. spumarius* transmitted *X. fastidiosa* to plants under greenhouse conditions during all tested periods in 2014 and 2015. The proportion of *X. fastidiosa* -infected vectors ranged from 25% to ca. 70% during May–October 2015. *Xylella fastidiosa* is persistent in spittlebugs (Severin 1950); therefore, infection rates would be expected to increase overtime as *P. spumarius* has one generation per year only (Weaver and King 1954). However, it can be noted that infection rates were already high (25%) shortly after adult emergence at the end of spring. Freitag and Frazier (1954) assessed by transmission tests natural infectivity of three main vectors of *X. fastidiosa* in California, that is *Draculacephala minervae*, *Xyphon fulgida* and *Graphocephala atropunctata*, finding out that sharpshooters collected from grapevine and a wide variety of wild plants were naturally infective all over the year and were able to sustain primary spread of *X. fastidiosa* from wild infected plants bordering vineyards. On the contrary, in Florida, according to Adlerz and Hopkins (1979), *Oncometopia nigricans* and *Homalodisca vitripennis* were active all the year round, but only found infected at the time they visited vineyards, thus suggesting that in Florida the disease spreads mainly within vineyard (secondary spread). The epidemiology of the olive disease in Apulia seems to be explained by a secondary spread pattern, similar to the one observed in Florida, as the first infective *P. spumarius* of the season are found on olive plants (Cornara et al. 2016a), and the proportion of positive spittlebugs is much higher while collecting insects from olive plants. Interestingly, while Adlerz and Hopkins (1979) found an extended period, from 6 to 10 weeks, between the first flights of *O. nigricans* to the vineyard and the first transmission of the bacterium, in the olive orchards we were able to detect *X. fastidiosa* in *P. spumarius* very soon after the adults emerged and visited olives. The meadow spittlebug natural infectivity rate is high, also compared to the ones recorded on five species of sharpshooters collected throughout the year from coffee plantations of the State of Paranà, Brazil (Silva et al. 2007) (about 50% when analysed in groups of three to five insects). Therefore, in the case of olive quick decline syndrome in Apulia, we observe a high percentage of naturally infected vectors insects. Although *X. fastidiosa* transmission efficiency by *P. spumarius* remains to be estimated in detail, the large proportion of infected insects suggests that the vector population would have to be suppressed to very low numbers prior to having any impact on pathogen spread (Purcell 1981). Spittlebugs were collected from olive trees for this study, and they likely acquired *X. fastidiosa* from olive trees, but it is possible that some spittlebugs may have acquired the bacterium from any other surrounding plant in orchards during any period prior to testing. The ecology of pathogen acquisition, specifically which plant species serve as sources as seasons progress, will have to be studied in detail to determine how to best decrease natural infectivity rates.

Xylella fastidiosa transmission, as measured by the detection of the pathogen in recipient plants as done here, is the outcome of vector–plant–pathogen interactions. Transmission rates have been shown to be impacted by vector species, host plant, pathogen strain (Redak et al. 2004; Lopes et al. 2009), as well as insect tissue preference on source plants (Daugherty et al. 2010). Therefore, results here represent a composite of these interactions; in addition, qPCR was used for bacterial detection, which unlikely cell culturing, allows for the detection of DNA from dead cells. Regardless, *X. fastidiosa* was not detected in grapevines, and it was detected only once on the stone fruit rootstock GF677 and in one-third of sweet orange plants. In both GF677 and sweet orange, *X. fastidiosa* was recovered from the portion of the plant exposed to insects during inoculation, but not from new growth, suggesting lack of systemic movement of the pathogen. A more detailed study on the potential host range of the ST53 genotype present in

Italy demonstrated that in citrus initial multiplication of the bacterium may occur soon after the inoculation, but further colonization and systemic spread do not occur (Saponari et al. 2016). In addition, a field survey on citrus plants in areas with high prevalence of *X. fastidiosa* in olive found no infections in 350 tested plants (Martelli et al. 2016). These results indicate that citrus may support early and localized multiplication of *X. fastidiosa*, but this plant is not a systemic host of ST53 and, therefore, it is very unlikely to represent a source of inoculum for the vector or, at least, it is likely to be a very inefficient source plant. Similar cases have been previously described for *X. fastidiosa* (Purcell and Saunders 1999). The olive cultivars Ogliarola and Coratina, as well as the olive seedlings, were equally susceptible to *X. fastidiosa* infection by spittlebugs. Oleander and periwinkle were similarly susceptible. It has to be noted that transmission experiments were carried out under 'no-choice' conditions (insects were caged on to a single plant and forced to feed on it) and, therefore, the results might not reflect the likelihood of infection of the test plant species under field conditions, as they do not take into account insect feeding preferences.

The positive correlation between the number of *X. fastidiosa* PCR-positive insect vectors and infected plants was similar to that previously found with sharpshooter vectors. Daugherty and Almeida (2009a) demonstrated that the number of PCR-positive individuals on grapevines was correlated with plant symptom development, suggesting that the number of inoculation events would enhance pathogen spread within plants. Although not tested in either study, it is plausible that the number of inoculation events is a determinant of successful infections, especially under field conditions.

Interestingly, transmission to oleander was the most efficient, but survival of vectors on these recipient plants was by far the lowest. While studying temperature-dependent transmission, Daugherty et al. (2009b) suggested that high temperature stress may have increased transmission of *X. fastidiosa* by *Graphocephala atropunctata*. In our work, feeding on a non-host plant may have stressed the insects and consequently altered their feeding behaviour in a way that maximized transmission efficiency, for example, by decreasing the duration of each feeding bout, thus increasing the number of probes and, ultimately, inoculation events.

The number of *X. fastidiosa* cells estimated to colonize *P. spumarius* was one to two orders of magnitude smaller than those obtained for sharpshooter leafhopper vectors (Killiny and Almeida 2009; Rashed et al. 2011). Another recent study with *P. spumarius* also found *X. fastidiosa* populations in individuals as being lower than in leafhoppers (Cornara et al. 2016b). This observation is interesting because *X. fastidiosa* is persistent in both spittlebugs and leafhoppers (Severin 1949, 1950), but so far, two independent studies have estimated bacterial populations to be relatively low in spittlebugs.

In conclusion, this work demonstrates that *P. spumarius* collected from olive orchards in Apulia have high rates of *X. fastidiosa* infection and that these individuals transmit the bacterium to host plants from May to October. These data coupled with our previous findings show that infective *P. spumarius* can be collected on olive trees in late spring, whereas all the individuals previously collected on herbaceous plants within and outside the olive orchard do not harbour the bacterium (Cornara et al. 2016a), suggesting that vector control should be done (i) at the ground level (i.e. by mechanical weeding in winter and early spring) to suppress spittlebug nymphs on the herbaceous hosts, before they acquire the bacterium, and (ii) by insecticide spray applications to kill the *P. spumarius* adults that move on perennial trees (i.e. olives) or shrubs, where they feed and can acquire and spread the bacterium. However, considering that *P. spumarius* adults persist in and around the olive orchards all over the season, and their natural infectivity rates were high (at least 25%) throughout the entire season, repeated insecticide applications would be needed to protect olive trees from infectious spittlebugs and to reduce vector adult population. Thus, for a sustainable vector control strategy, major efforts should be done in controlling *P. spumarius* at the larval stages and soon after the emergence of the adults, trying to hamper the acquisition of *X. fastidiosa* from infected plants. Moreover, the high rate of vector infectivity suggests that an integrated approach should be implemented to control pathogen spread in the landscape, which, besides the suppression of vector populations, should include the removal of *X. fastidiosa*-infected plants that represent the source of inoculum for the vector.

More in-depth investigations on vector population level in and around olive orchards, and on vector transmission efficiency to olives, are necessary in order to estimate the level of vector suppression needed for an effective control strategy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Experimental plants used for the transmission tests.

Figure S2. Proportion (%) of insects surviving the 7-day inoculation access period (IAP) on the different recipient plant species

Table S1. Transmission experiments 2014. For each species/cultivar and date, the number of infected out of tested plants, are reported.

Table S2. PCR detection of *Xylella fastidiosa* in test plants and insects from transmission tests performed in 2015.