UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE E TECNOLOGIE PER L'AGRICOLTURA, LE FORESTE, LA NATURA E L'ENERGIA

Corso di Dottorato di Ricerca in

SCIENZE DELLE PRODUZIONI VEGETALI E ANIMALI - XXIX Ciclo

Investigations on the transmission of the bacterium *Xylella fastidiosa* by insect vectors

(Settore Scientifico-disciplinare AGR/12)

Tesi di dottorato di: Dott. **ISSAM EDDINE BEN MOUSSA**

Coordinatore del corso

Prof. STEFANIA MASCI

Tutore

Prof. LEONARDO VARVARO

Prof. ANNA MARIA D'ONGHIA

Co-tutore

Prof. Caroline Roper

Dott. Franco Valentini

Prof. Stefano Speranza

A.A. 2016/17

Dedicace

To my beloved parents, my darling sister

and to Safsoufa

AKNOWLEDGEMENT

It is my pleasure to express my heartfelt thanks to all the people who helped me to accomplish my PhD thesis and those who had great contribution in my life.

First of all, I would like to thank the coordinator of the PhD program, Pr. Stefania Masci and the coordinator of IAMB's IPM department, Dr. Anna Maria D'ONGHIA for giving me the opportunity to work on this interesting subject, their direction and for their guidance.

Furthermore, I would like to gratefully and sincerely thank my supervisor Pr. Leonardo Varvaro and my advisors Dr. Valerio Mazzoni, Dr. Franco Valentini and Dr. Stefano Speranza for their assistance, patience and their critical comments in reviewing my paper.

Special thanks to my co-supervisors during my training at the university of California Riverside, Pr. Caroline Roper, Pr. Elaine Backus and Dr. Rodrigo krugner for their collaboration, for giving me valuable remarks and most importantly for their friendship.

My gratitude for all my friends who helped me to achieve this end and supported me during critical moments.

My deepest thanks to my beloved parents who raise me up, educated me and still continue to take care of me.

Table of contents

List of figures

List of tables

Introduction

Olive (*Olea europaea* L.) is a typical Mediterranean crop. The olive was one of the first species to be cultivated, and even during the Bronze Age, it represented economic wealth for many Mediterranean societies. The olive has contributed to the development of all the cultures in the Mediterranean Basin. Man has learnt to utilize every part of the plant: the foliage for animal feed, the fruit for table oil and lighting and the wood for fuel.

Among cultivated species, olive is the sixth most important oil crop in the world, due to the beneficial nutritional properties of its oil and high economic value. The Mediterranean basin is the traditional area of cultivation and has 95% of the olive production of the world. Average olive oil production in the EU in recent years has been 2.2 million tonnes, representing around 73% of world production. Spain, Italy and Greece account for about 97% of EU olive oil production. Italian olive cultivation covers approximately 1 700 000 ha, 80% of which located in southern Italy, where Apulia represents the most important region, with about 370,000 ha.

Xylella fastidiosa is an economically important pathogen of several commercial crops, olive included, on which it induces leaf scorch symptoms and dieback. After the first report of this bacterium on olive in California, quick decline of aged olive trees has also taken place for the first time in the Mediterranean basin, over a large area of Apulia (southern Italy). The outbreak has caused some panic and concern among olive growers because the disease was spreading very rapidly, posing an economic and environmental threat to an entire region where olive production is the staple agricultural activity and centuries-old olive trees form an inestimably valuable part of the landscape and natural heritage. Several investigations to ascertain the causes of this severe disease were performed and still are underway. As first results, symptomatic trees were found to be infected by several fungal species belonging to the genus *Phaeoacremonium* and

Phaemoniella. In addition, PCR assays on diseased trees gave positive reactions to *X. fastidiosa*. Consequently, the disease was called "Olive Quick decline Syndrome" (OQDS) since many pathogens were associated with it.

Even though the exact etiological role of *X. fastidiosa* in the “rapid decline” disease in Italy is still not fully demonstrated, the quick dissemination of the bacterium in Apulia region prompted an investigation to prevent its spread. *Xylella fastidiosa* is a vector-borne pathogen transmitted by several species of Auchenorrhyncha, belonging mainly to Cicadellidae (sharpshooters) and Aphrophoridae (spittlebugs). Therefore, considering that containment of *X. fastidiosa* spread is mainly based on vector control, the overall goal of this thesis was to study the transmission of the bacterium in the region and determine effective control measures.

To this aim, the main objectives of this thesis were: (i) to identify potential insect vector(s) of *X. fastidiosa* in Apulia region and to study their seasonal dynamics in relation with disease diffusion; (ii) to evaluate the possibility to utilize these insects as indicators of the presence of the bacterium in apparently uncontaminated areas so called " Spy Insect approach"; (iii) to explore the effect of *X. fastidiosa* endoglucanases on the acquisition, retention and transmission of the bacteria by an efficient insect vector, as a biological control measure.

Chapter I: Literature review

I.1. Background on *Xylella fastidiosa*

I.1.1. History

In the 1880's a 'mysterious' vine disease destroyed *ca*. 14,000 ha of grapes (*Vitis* spp.) and *ca.* 50 wineries had to close down in the Los Angeles area in California. This disease was described in detail in 1887 by N.B. Pierce (1856-1916) and was therefore named "Pierce's disease" (PD) of grapevine. A similar disease was recorded on peach (*Prunus persica*) in 1890 in Georgia (USA), with outbreaks in 1929, 1951 and 1976 and was named "phony peach disease" (PPD). The causal agent was described variously as a virus (Hutchins *et al*, 1953), a rickettsia-like bacterium (Goheen *et al*, 1973), until the organism was shown to be a thin, rod-shaped, Gram-negative bacterium and Koch's postulates were satisfied (Davis *et al*., 1978). In 1987, Wells *et al*. proposed to include the agent of these diseases in a new genus, *Xylella*, and named the organism *X. fastidiosa* based on its fastidious growth. Already in the 1940's sharpshooter leafhoppers and spittlebugs were identified as vectors of PD and PPD (Severin, 1949). Once the bacterium was described and more easily cultured, *X. fastidiosa* was found, with or without symptoms, in a large number of other hosts, among which the most important are *Prunus* spp. (almond leaf scorch, ALS in *Prunus amygdalus* and plum leaf scald, PLS in *P. domestica), Acer* spp., *Carya illinoinensis* (pecan), *Coffea arabica* (coffee leaf scorch, CLS, also pathogenic to *Citrus*), *Hedera helix, Morus rubra, Nerium oleander* (oleander leaf scorch, OLS), *Platanus occidentalis*, *Quercus* spp., *Ulmus americana*. Furthermore the bacterium was found in *Medicago sativa* (alfalfa dwarf), *Vinca major* and in *Persea americana* (avocado) (Montero-Astúa *et al*., 2008).

Many wild plants were found to carry the pathogen (often latently), such as grasses, sedges and trees (Freitag, 1951; Raju *et al*., 1982).

In 1987 in Brazil, a rapidly spreading disorder, called citrus variegated chlorosis (CVC) or X-disease of *Citrus*, was observed and *X. fastidiosa* was isolated from diseased trees. Characterization of *X. fastidiosa* culminated in whole genome sequencing, and the citrus strain of *X. fastidiosa* actually was the first plant pathogenic bacterium for which the whole genome was sequenced (Chang *et al.,* 1993).

I.1.2. Biology

X. fastidiosa (Wells *et al*., 1987) is a gram negative fastidious xylem limited bacterium with single (occasionally filamentous), non-motile, aflagellate straight rods (0.25 to 0.35 by 0.9 to 3.5 pm) cells. Colonies are gram negative bacteria, catalase positive, and oxidase negative, utilize hippurate, and produce gelatinase and often beta-lactamase but not beta-galactosidase, coagulase, lipase, amylase, phosphatase, indole, or H_2S. The bacterium is strictly aerobes with optimum growth at 26-28°C and pH 6.5-6.9 (Wells *et al*., 1987). It colonizes the water-conducting xylem vessels of plants (Figure 1) and develops biofilms that contribute to the blockage of sap flow, resulting in plant stress and disease. It moves downstream, but also is able to migrate upstream, via twitching motility, using type IV pili (Meng *et al*., 2005). Twitching motility is a mean of flagellar-independent bacterial movement over moist surfaces. Biofilm formation and cell-cell aggregation of *X. fastidiosa* requires the presence of type I pili while type IV pili are essential for motility (Li *et al*., 2007).

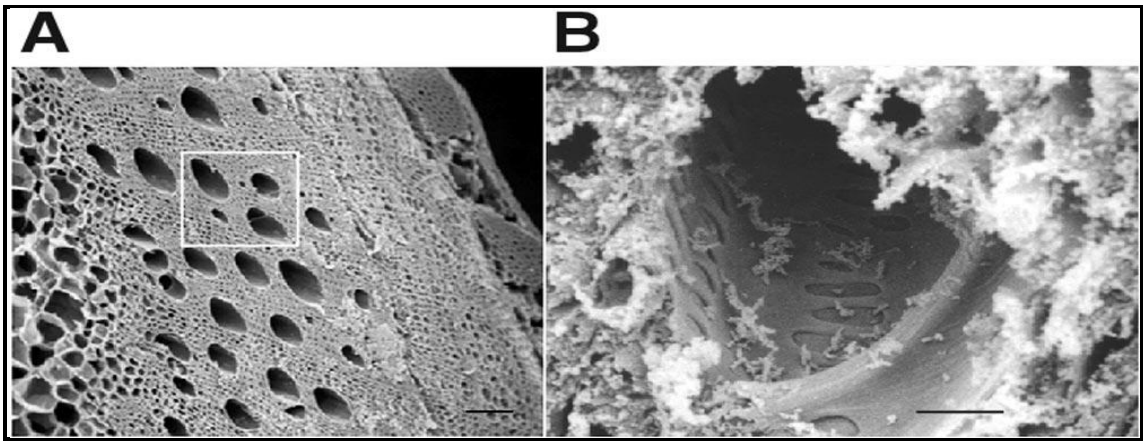

Figure 1: Scanning electron micrographs of tangential sections through grapevine and associated xylem vessels. (A) Water-conducting xylem vessels of a grapevine cross section serve as the habitat for *X. fastidiosa* cells. (B) View into a sectioned xylem vessel walls (Meng *et al*., 2005).

In advanced stages of infection, sap blocking biofilms are formed both in the host plant and in the foregut of the vectors (Figure 2). *X. fastidiosa* proliferates only in xylem vessels, in roots, stems and leaves. The vessels are ultimately blocked by bacterial aggregates and by tyloses and gums formed by the plant. In the vector biofilm the bacterial cells are pearly attached (Newman *et al*., 2004).

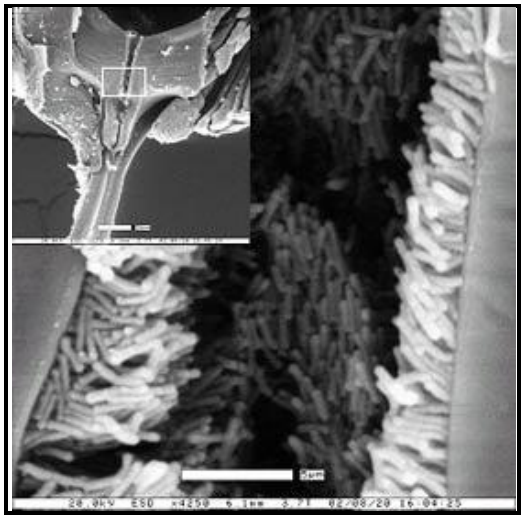

Figure 2: Cells of *X. fastidiosa* in biofilms on cuticle of insect foregut (Source: Almeida)

The bacterium is efficiently acquired by insect vectors, with no latent period, and persists in infective adults indefinitely (Severin, 1949). Bacterial colonization of plants is based on xylem vessels and movement through the xylem network via pit membranes. The bacterium can enter neighboring vessels through pits, after degradation of the pit membranes, which is apparently also triggered by a diffusible signal from the bacterium (Newman *et al*., 2004). Several bacterial components; such as type I pili, hemagglutinin, adhesins and finbriae; are implicated in *X. fastidiosa* biofilm formation (Chatterjee *et al*., 2008). Various vascular pathogens, such as *Xanthomonas* and *Clavibacter* spp. produce polysaccharides while colonizing plants. Interestingly, *X. fastidiosa* secretes exopolysaccharides (EPS) in both, plant vessels and insect vector foregut, which allow the formation of biofilms confirming that they are essential for plant virulence and vector transmission of *X. fastidiosa* (Killiny *et al*., 2013). EPS of *X. fastidiosa* contain a back-bone of β-1,4-glycosidic linkages which can be cleaved by endoglucanases (Roper *et al*., 2016).

I.1.3. Taxonomy and classification

Based on 16S ribosomal ribonucleic acid, Wells *et al*. (1987) found that different strains of the bacterium were related to xanthomonads. These bacteria form a distinct group, for which the name *Xylella fastidiosa* was proposed and a new genus was established with one species in the gamma subgroup of the eubacteria. The taxonomic relationship among 26 strains of *X. fastidiosa* from 10 hosts, conducted on sequenced 16S–23S intergenic spacer (ITS) region, showed a DNA relatedness ≥70% (Schaad *et al*., 2004). However, at high stringency (Tm –8 °C), three distinct genotypes (A, B, and C) were revealed. Taxon A included strains from cultivated grape, alfalfa, almond (two), and maple, interrelated by 85% (mean); taxon B included strains from peach, elm, plum, pigeon grape, sycamore, and almond (one), interrelated by 84%; and taxon C included only strains from citrus, interrelated by 87%. The mean reciprocal relatedness between taxons A and

B, A and C, and B and C, were 58, 41, and 45%, respectively. ITS results also confirmed the same grouping: taxons A and B, A and C, and B and C had identities of 98.7, 97.9, and 99.2%, respectively. Previous and present phenotypic data support the molecular data. Taxon A strains grow faster on Pierce's disease agar medium whereas B and C strains grow more slowly. Taxon B and C strains are susceptible to penicillin and resistant to carbenicillin whereas A strains are opposite. Each taxon can be differentiated serologically as well as by structural proteins and the names *X. fastidiosa* subsp. *piercei*, subsp. *multiplex* and subsp. *pauca* were respectively proposed for taxons A, B, and C. So far, 5 subspecies of *X. fastidiosa* are registered (Table 1).

Table 1. Host plants of different *X. fastidiosa* subspecies

Subspecies	**Host species**	**Family**	**References**
multiplex	*Prunus dulcis* (almond), *P. armeniaca* (apricot), *P. persica* (peach)	*Rosaceae*	Nunney *et al.*, 2013
	Olea europaea (Olive)	*Oleaceae*	
fastidiosa	*Vitis vinifera*	*Vitaceae*	Janse and Obradovic, 2010
sandyi	*Nerium oleander*	Apocynaceae	
tashke	*Chitalpa tashkentensis*	*Bignoniaceae*	
pauca	*Citrus* spp.	*Rutaceae*	Schaad *et al*., 2004

I.1.4. Geographical distribution

The distribution below is given for all host plants (Figure 3). For many years, *X. fastidiosa* remained confined to the Americas. In 1994, it was first noticed in Asia, in Taiwan causing leaf scorch on Asian pear (*Pyrus pyrifolia*). In the 2000s, it was also reported causing Pierce's disease in Taiwanese vineyards. In the EPPO region, the finding in Apulia (Southern Italy) represented the first confirmed detection in Europe. The introduction pathways of *X. fastidiosa* into Asia or Europe are unknown. However, it can be noted that EPPO member countries have intercepted several times *X. fastidiosa* on imported coffee plants from south America.

Asia: Iran, Taiwan (introduced, first found in Asian pears and then in grapevine). **North America**: Canada (Ontario), Mexico, USA (Alabama, Arizona, Arkansas, California, Delaware, District of Columbia, Florida, Georgia, Indiana, Kentucky, Louisiana, Maryland, Mississippi, Missouri, Montana, Nebraska, New Jersey, New Mexico, New York, North Carolina, Oklahoma, Oregon, Pennsylvania, South Carolina, Tennessee, Texas, Virginia, Washington).

South America: Argentina, Brazil (Bahia, Espirito Santo, Goias, Minas Gerais, Parana, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, Sao Paulo, Sergipe), Costa Rica, Paraguay, Venezuela.

Europe: *X. fastidiosa* is an EPPO A1 quarantine pathogen. The occurrence of Pierce's disease of grapevine was reported in Kosovo, although this report remained dubious because of the lack of further recoveries (EPPO RS N°98/157, 1998). In France, *X. fastidiosa* was detected in a single blemished apricot plant by serological assays based on immunofluorescence. All further serological and molecular tests failed to detect *X. fastidiosa* (Anses, 2012). In 2012, *X. fastidiosa* was isolated from coffee plants (*Coffea arabica* and *C. canephora*) growing in a confined glasshouse near Tours, France.

In October 2013, the occurrence of *X. fastidiosa* was reported in Southern Italy (Apulia region), associated to quick decline symptoms on olive trees (*Olea europea*) and leaf scorch on oleander and almond (Saponari *et al*., 2013).

In October 2015, the bacterium (*X. fastidiosa* subsp. *multiplex*) was discovered in France on the island of Corsica on *Polygala myrtifolia* plants (ornamentals). It was then found on the mainland, first in the municipalities of Nice and Mandelieu-la-Napoule (Alpes-Maritimes), and then other foci were found in Alpes-Maritimes and Var departments.

In April 2016, The Institute for National and International Plant Health of the Julius Kühn-Institut (JKI) has notified the first identification of an oleander sample found positive to *X. fastidiosa* in Saxony (Germany).

Finally, in November 2016, the Government of the Balearic Island (GOIB) has officially announced the first detection of the quarantine pathogen *Xylella fastidiosa* in Spain, in the town of Manacor on the island of Mallorca. The MLST analysis identified the three isolates as belonging to the X. fastidiosa subsp. fastidiosa ST-1 strain.

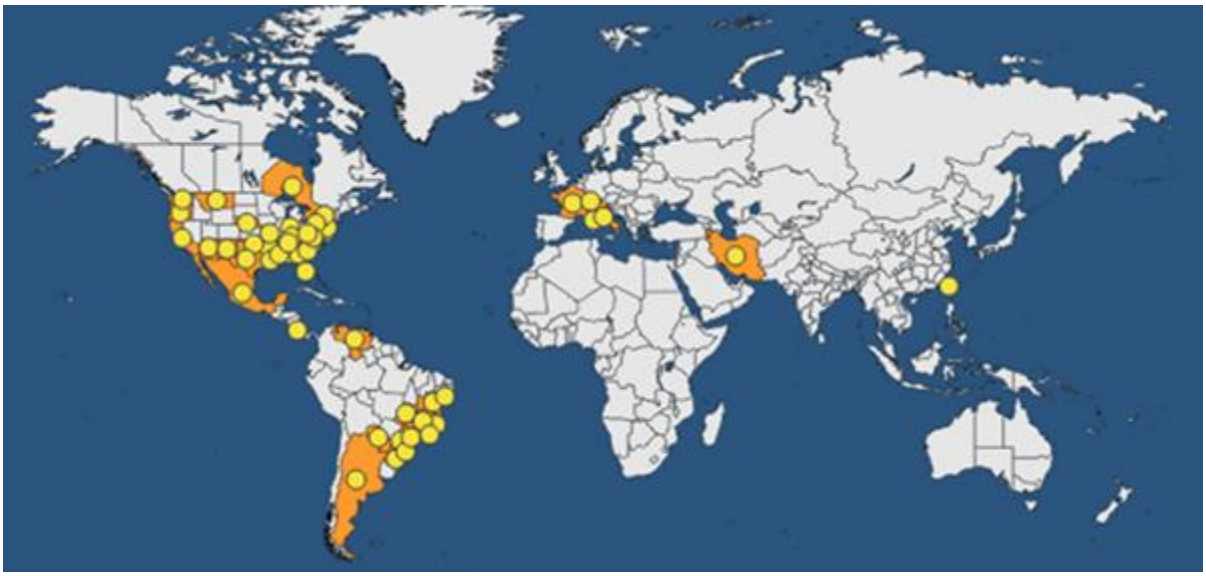

Figure 3: Geographical distribution of *Xylella fastidiosa* (EPPO/PQR 2016).

I.1.5. Symptoms

Most disease symptoms are associated with bacterial blockage of xylem fluid transport through the plant (water and nutrients). In susceptible host plants symptoms consist of marginal leaf scorching, wilting of foliage and withering of branches, dieback and stunting with eventual plant death.

Early symptoms appear as slight chlorosis or bronzing along the leaf margin or tip that intensify and that may become water-soaked before browning and drying.

These symptoms are first found on a few branches, later on almost all foliage. The affected area is delineated by a narrow chlorotic band that becomes clear in autumn. A premature defoliation takes place with new malformed leaves formed. Abnormally shaped fruit may also be formed and stems may show internal and external discoloration, dieback and abnormal growth, leading to eventual death of the host.

Grapevines. The most characteristic symptom of primary infection is leaf scorch. An early sign is sudden drying of part of a green leaf, which then turns brown while adjacent tissues turn yellow or red (Figure 4, A). The desiccation spreads and the whole leaf may shrivel and drop, leaving only the petiole attached. Diseased stems often mature irregularly, with patches of brown and green tissue. In later years, infected plants develop late and produce stunted chlorotic shoots. They rarely survive more than one or two years, despite any signs of recovery.

Peaches. Young shoots are stunted and bear greener and denser foliage than healthy trees. Lateral branches grow horizontally or droop, so that the tree seems uniform, compact and rounded (Figure 4, D). Leaves and flowers appear early, and leaves remain on the tree longer than on

healthy trees. Affected trees yield increasingly fewer and smaller fruits until, after 3-5 years, they become economically worthless.

Citrus. Trees can start showing the symptoms of variegated chlorosis from nursery size up to 7-10 years of age. These younger trees become systemically affected by *X. fastidiosa*. Usually, trees more than 15 years old are not totally affected, but rather have one or two major scaffold branches showing symptoms. Affected trees show foliar chlorosis resembling zinc deficiency with interveinal chlorosis. The chlorosis appears on young leaves as they mature and may also occur on older leaves. Newly affected trees show sectoring of symptoms, whereas trees which have been affected for a period of time show the variegated chlorosis throughout the canopy. As the leaves mature, small, light-brown, slightly raised gummy lesions (becoming dark-brown or even necrotic) appear on the underside, corresponding to the yellow chlorotic areas on the upper side (Figure 4, B). Fruit size is greatly reduced. The sugar content of affected fruit is higher than in non-affected fruit, and the fruit has a hard rind, causing damage to juicing machines. Blossom and fruit set occur at the same time on healthy and affected trees, but normal fruit thinning does not occur on affected trees and the fruits remain small but open earlier. Since more fruits remain, total production is not greatly reduced. On affected trees of cv. Pera and other orange cultivars, fruits often occur in clusters of 4-10, resembling grape clusters. Affected trees show stunting and slow growth rate. Twigs and branches die back and the canopy thins, but affected trees do not die.

Olive. The outbreak in Italy is characterized by extensive leaf scorch and dieback of olive trees (*Olea europaea*), some of which are over 100 years old, over a large area estimated in ca. 8 000 hectares. A disease with similar symptoms was reported on olive trees from South California in 2010 and *X. fastidiosa* was associated with it. Later on, it was found that *X. fastidiosa* did not cause olive leaf scorch/branch dieback, but olive may serve as an alternative, albeit suboptimal,

host that may contribute to the epidemiology of *X. fastidiosa*-elicited diseases in California as a source of inoculum (Krugner *et al*., 2014).

Figure 4: **A.** Typical leaf scorch symptoms, caused by *Xylella fastidiosa* subsp. *fastidiosa* on grapevine leaves; **B.** Typical symptoms on leaves (variegated spots) and fruits (dwarf growth) of Citrus caused by *Xylella fastidiosa* subsp. *pauca*; **C.** Severe symptoms of leaf scorch on a *Platanus occidentalis* (sycamore) caused by *X. fastidiosa* subsp. *fastidiosa*; **D.** Reduced growth of peach (on the left) affected by phony peach disease (Janse and Obradovic, 2010).

I.1.6. Vectors

X. fastidiosa is a xylem-limited bacterium that in nature is exclusively transmitted by xylem-fluid feeding insects. These insects belong to the order Hemiptera, sub-order Cicadomorpha (de Jong, 2013). They have sucking mouthparts (mandibular and maxillary stylets) that allow them to reach

the xylem of plants, from which they ingest sap. Due to the very poor nutritional value of xylem fluid, xylem-fluid feeders ingest large amounts of crude sap and produce big amounts of liquid excretions. They are generally not direct pests unless present at very high population levels. Within Cicadomorpha, the three superfamilies, *Cercopoidea*, *Cicadoidea* and *Membracoidea* include xylem-fluid feeding groups, but, while all *Cercopoidea* (known as spittlebugs or froghoppers) and *Cicadoidea* (cicadas) are regarded as xylem-fluid feeders, only the subfamily *Cicadellinae* (known as sharpshooters) within the family *Cicadellidae* includes actually xylem-fluid feeders. Even though other sap-sucking insects also feed on xylem, only these three groups of 'specialists' in xylem-fluid feeding have been shown to be vectors of *X. fastidiosa.* Spittlebugs, cicadas and sharpshooters are heterometabolous insects that develop through egg, five nymphal and one adult (winged) stages. Nymphs of cicadas and of spittlebugs of the family *Cercopidae* are subterranean root feeders, while nymphs of spittlebugs of the family *Aphrophoridae* and of sharpshooters develop on the parts of host plants above the ground. All adults feed and live on the aerial parts of host plants. The transmission of *X. fastidiosa* by insects is peculiar in that it does not require a latent period and is of persistent type (Almeida *et al*., 2005). Bacteria are restricted to the alimentary canal and do not infect systemically the insect body. They adhere to and multiply in the cibarium and precibarium (part of the foregut). This implies that vectors lose the infectivity with moulting, as the foregut is of ectodermal origin and is renewed with it. Therefore, newly emerged adults must feed on an infected plant to become infectious. Once infected, adult vectors can transmit during their whole lifetime, because the bacterium multiplies and persists in the vector foregut (Almeida *et al*., 2005). The bacterium is not transovarially transmitted to the progeny of the vector (Freitag, 1951). For all these reasons and also because of their high mobility, winged adults are mostly responsible of *X. fastidiosa* spread. It is important to remember that, since the bacterium is restricted to the foregut (Purcell and Finlay, 1979), the number of bacterial cells per insect is low and therefore a very sensitive diagnostic tool, like

PCR, is needed to detect the presence of *X. fastidiosa* in the vectors. Although restricted to xylem-fluid feeding insects, transmission of *X. fastidiosa* is known to be poorly specific and therefore all xylem-fluid feeding insects are considered potential vectors, until proven otherwise (Frazier, 1944; Purcell, 1989; Almeida *et al*., 2005). However, transmission efficiency varies substantially depending on insect species, host plant, and *X. fastidiosa* genotype (Redak *et al*. 2004).

Non-European vectors

Since *X. fastidiosa* has been found and studied primarily in the Americas and causes disease in different crops in the Nearctic and Neotropics regions, its vectors have been identified and studied in these biogeographical areas only. Known vectors of *X. fastidiosa* are listed by Redak *et al.* (2004).

Potential European vectors

With the exception of *Philaenus spumarius* (Aphrophoridae), an old world species introduced in North America, all the known American vectors are absent in Europe (de Jong, 2013). According to Janse and Obradovic (2010) only two species, *Cicadella viridis* and *Philaenus spumarius*, are possible vectors for Europe. Other xylem-fluid feeders, should indeed be considered as potential vectors according to Frazier (1944) and Purcell (1989).

A list of potential vectors of *X. fastidiosa* in Europe was drawn from the Fauna Europaea database (de Jong, 2013) using the following criteria: all the xylem-fluid feeding insects were included, provided that their presence was certain. As stated before, cicadas are xylem-fluid feeders and are thus expected to be potential vectors, although their role in *X. fastidiosa* transmission is poorly understood. In Italy, about 16 species of cicadas of families *Cicadidae* and *Tibicinidae* are known, out of 60 species reported in Europe, most having a very restricted area of distribution (de Jong, 2013). Sharpshooters (Cicadellidae subfamily Cicadellinae) are by far the

most important vectors of *X. fastidiosa* in the Americas and only few species are present in Europe. *C. viridis* is widespread in Europe but only in humid areas. On the contrary, a relatively high number of spittlebug species, which are less important as vectors in America, occur in Europe and some, like *P. spumarius*, are very common. It has to be noted that, while the sharpshooters in America overwinter as adult and can maintain *X. fastidiosa* during winter, the European sharpshooters and most of the European spittlebugs (Aphrophoridae, but not Cercopidae) overwinter as egg (Nickel and Remane, 2002) and therefore, if infected, cannot sustain overwintering of *X. fastidiosa*, since transovarial transmission does not occur (Freitag, 1951).

I.2. The Olive Quick decline syndrome

I.2.1. Current situation in Apulia

In 2013, *X. fastidiosa* was reported in southern Italy associated with quick decline symptoms on olive trees, oleander and almond (Saponari *et al*., 2013). *X. fastidiosa* was found initially in the area of Gallipoli (around 8 000 ha of olive trees, with a significant part severely affected) and it was subsequently found in many other sites, first to the north and later also to the east of the initially reported outbreak areas. *X. fastidiosa* has been identified from olive plants based on PCR detection, ELISA, indirect immunofluorescence, electron microscopy and immunogold labelling, as well as by laboratory culture. The genotype of the strain of *X. fastidiosa* present in Italy is considered to be a new genetic variant within the subspecies *pauca* (Cariddi *et al*., 2014). It has been shown that the strain present in Italy is very homogeneous, and identical to a variant infecting oleander in Costa Rica. This also represents the first report of subspecies *pauca* in Costa Rica (Nunney *et al*., 2014). It was assigned a new sequence type (ST) profile, ST 53, and named CoDiRO for "Complesso del Disseccamento Rapido dell' Olivo". Concatenated sequences of the

seven MLST genes (Figure 5) showed that the CoDiRO strain is a "divergent" variant within the subspecies *pauca*.

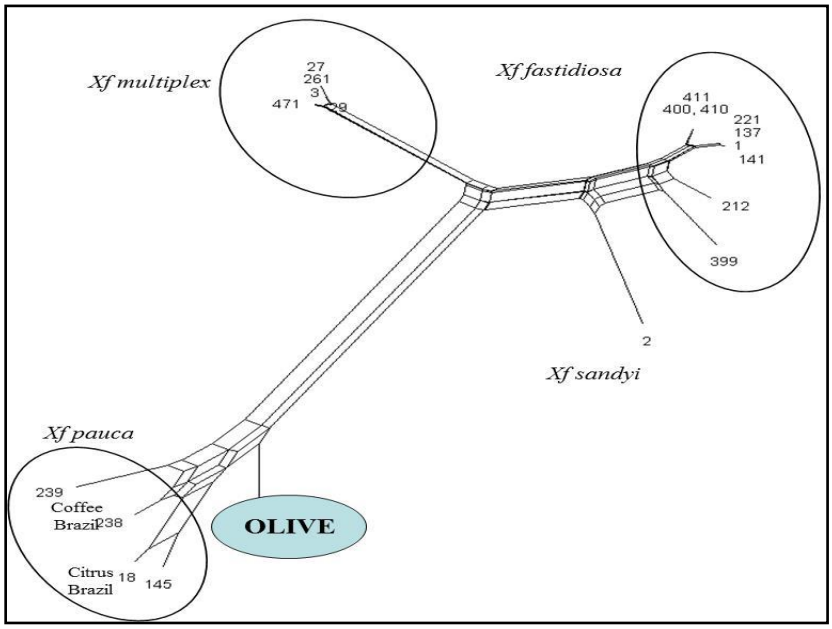

Figure 5: Phylogenetic tree of the Apulian isolate of *X. fastidiosa* derived from multilocus sequence typing (MLST) based on the concatenated sequences of seven genes. The Italian CoDiRO strain is indicated by the green circle (olive) (Maria Saponari, CNR, Bari, Italy)

I.2.2. Host plants

The bacterium was isolated on periwinkle wilt gelrite and buffered cysteine–yeast extract media, from symptomatic natural infected oleander and periwinkle infected by *X. fastidiosa*-positive spittlebugs. Later on, it was isolated from *Olea europea*, *Olea oleaster*, *Prunus dulcis*, *Prunus avium*, *Polygala myrtifolia*, *Westringia fruticosa* (Maria Saponari and Donato Boscia, CNR, Institute for Sustainable Plant Protection, personal communication, October 2014). The current list of *X. fastidiosa* host plant species consists of 359 plant species (including hybrids) from 204 genera and 75 different botanical families. Compared to the previous database, 44 new species

and 2 new hybrids, 15 new genera and 5 new families were found. The majority of the additional species (70%) were reported in Apulia, Corsica and southern France (EFSA, 2016).

In olive trees, symptoms are found on all known varieties. Old varieties, such as Ogliarola Salentina, Cellina di Nardò and the common varieties Frantoio and Coratina, appear quite susceptible while the variety Leccino seems less susceptible, although there is much uncertainty about such indications because such records are based on field observations and still have to be fully demonstrated. Although the disease was more frequently found in old trees, presumably because of the severity of symptoms, it has also been observed on young plants (Cariddi *et al*., 2014). First leaf scorch or, more often, desiccation symptoms generally appear on one or two branches, and then appear randomly on the rest of the canopy. It is thought that the dieback symptoms take several years to extend to the whole plant.

I.2.3. Vectors

Since the discovery of the *X. fastidiosa* -associated epidemics in olive groves in 2013, field surveys and transmission experiments have been carried out throughout the year, mainly by sweep nets, in the infested areas, both on olive trees and on grasses. Collected insects were further identified and tested in the laboratory for the presence of *X. fastidiosa* by PCR. These investigations were part of the work performed in this thesis. Thereby, further information on vector situation in Apulia are reported in the results of objectives II and III of this dissertation.

Chapter II: Identification of potential insect vectors of *Xylella fastidiosa* in Apulia region and study of their seasonal dynamics in relation with disease diffusion.

II.1. Background

The widespread of the bacterium in Apulia region prompted an investigation for the identification of the local insects, mainly leafhoppers (*Cicadellidae*) and spittlebugs (*Aphrophoridae*), which could be responsible of its transmission in Apulia. No species of sharpshooters, which are by far the most important vectors in the Americas, was previously reported in Europe. Putative vectors for Europe are considered *Cicadella viridis* L. (Cicadellidae) and *Philaenus spumarius* L. (Aphrophoridae) (Janse and Obradovic, 2010), even though the list of potential vectors should be extended to all the xylem-fluid feeders according to Purcell (1989) but also phloem feeders since they can feed occasionally on xylem (Pompon *et al*., 2011). To this aim, all Auchenorrhyncha species present in the contaminated area were considered. Captured Auchenorrhyncha individuals were identified and quantified in order to study their seasonal dynamics then tested for the presence of *X. fastidiosa*.

II.2. Material and Methods

II.2.1. 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* (Figure 6). 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 Holzingher *et al*. (2003). For species identification, male genitalia was dissected and kept in KOH (10%) for 24 hrs; 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.

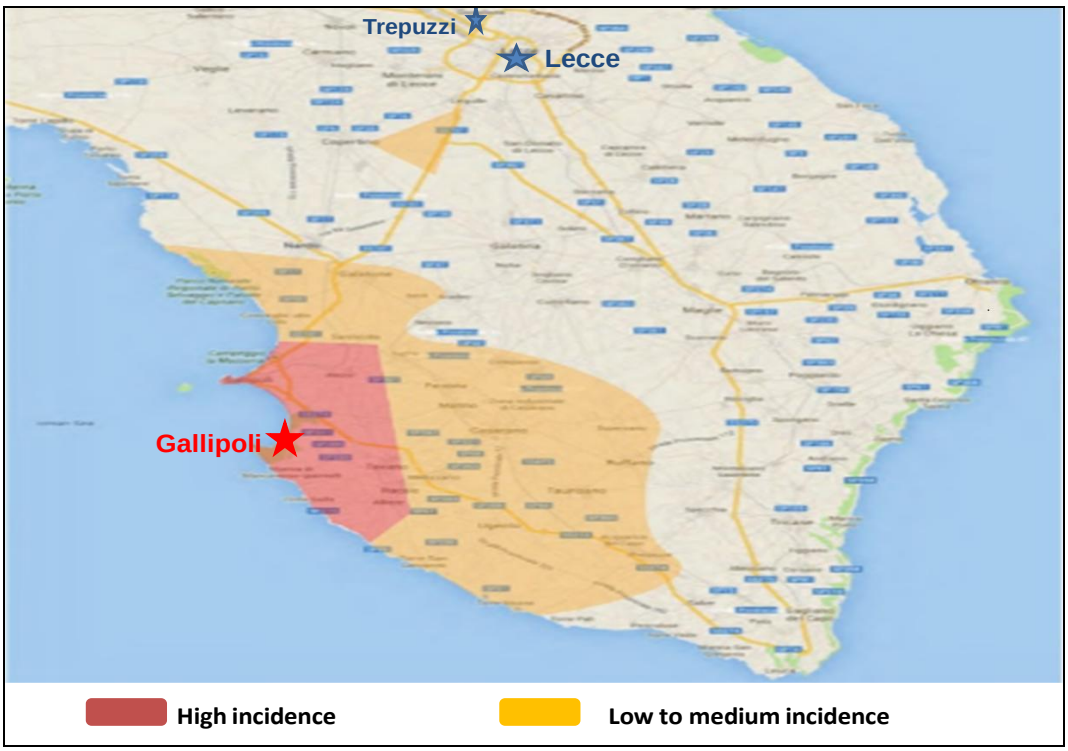

Figure 6: Distribution of olive quick decline in Apulia region (situation in October 2013).

II.2.2. Extraction and amplification of X. fastidiosa DNA

Insect heads were macerated individually using a sterile pestle in a tube containing 200 µl of extraction buffer (Triton X 1%, Tris-HCl 20 mM, EDTA 2 mM). After the homogenization, 180 µl of suspension were transferred to a new tube and left to incubate at 60° C for 30 min to allow complete lysis of cells. Two hundred microliters of chloroform-isoamyl alcohol (24:1) were added to the suspension and centrifuged at 12,000 g for 10 min. DNA was precipitated by adding 1 vol of cold isopropanol and incubated overnight at -20° C. After a new centrifugation at 20,000 g for 20 min, the pellet was washed with 70% ethanol, dried and finally resuspended in 20 µl of sterile water. DNA extracts from insect samples were tested by PCR with RST31 (GCGTTAATTTTCGAAGTGATTCGATTGC) and RST33 (CACCATTCGTATCCCGGTG) specific primers targeting the RNA polymerase sigma-70 factor genomic area and generating an amplicon of 733 bp (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 10 ng/µl, 1.25 U of Go Taq polymerase (Promega, Madison, WI, USA), 1× Go Taq Flexi DNA Buffer, 0.2mM dNTPs, 0.3 µM forward and reverse primers, all in a final reaction volume of 25 µl. Samples were put in the thermocycler (IQ5 Thermocycler, BioRad, USA) with the following program: initial denaturation at 94° C for 4 min, 35 cycles at 94° C for 30 s, 55° C for 30 s and 72° C for 40 s, and a final extension at 72° C for 7 min. Products were visualized by ethidium bromide staining after electrophoresis in 1.2 % agarose gel.

II.2.3. Cloning and sequence analysis

PCR amplicons were transformed in StrataClone^{TM} PCR Cloning vector pSC-A (Stratagene, CA, USA), subcloned into *Escherichia coli* DH5α or SoloPACK cells and custom sequenced (Primm, Milan, Italy). Four copies of each DNA clone were sequenced bi-directionally to eliminate any

sequence ambiguity. The sequences obtained were cleaned from vector with the assistance of the DNA Strider 1.1 program (Marck, 1988). Sequence similarities were analyzed with BLASTN at the National Center for Biotechnology Information website (Altschul *et al*., 1990). Multiple sequence alignments were generated by using the default options of CLUSTALX 1.8 (Thompson *et al*., 1997). Sequences obtained from this study were deposited in GenBank.

II.2.3.1. Ligation

PCR amplicons corresponding to different insect species were considered. Two µl of each PCR product were mixed with 3 µl of cloning buffer and 1 µl of vectors and the mix was incubated at room temperature for 15 min.

II.2.3.2. Transformation of competent cells

Fifty microliters of bacterial suspension were added to the mix and immerged in ice for 20 min. After a heat shock at 42°C for 45 sec, the mix was replaced in ice. Then the samples were added to 800 µl of LB without ampicilline and shaked at 37°C for 90 min. Samples were centrifuged at 5 000 rpm for 3 min, and the pellet (100 µl) was plated using sterile glass rod onto solid LB plate previously prepared adding 35 µl of Xβ galactosidose. Plates were incubated overnight at 37°C.

II.2.3.3. Extraction of DNA plasmid

From each plate, single white colonies, likely containing the recombinant plasmids, were collected and diluted in bacterial tubes containing 3 ml of LB with ampicilline and incubated overnight at 37°C with agitation. An aliquot (1.5 ml) from each tube was transferred to another eppendorf tube and centrifuged at 13 000 rpm for 30 sec. The supernatant was discarded and the pellet resuspended in the remaining 1.5 ml in the bacterial tube. After a new centrifugation at 13 000 rpm for 30 sec, the supernatant was discarded.

The isolation of the plasmid DNA was done using Quick lyse Qiagen Mini prep kit. Thereby, 400 µl of lysis buffer (QLL buffer) and the mix were homogenized on vortex. This mixture was transferred onto collection tubes with filters and centrifuged at 13 000 rpm for 1 min; then 400 µl of washing buffer (QLW buffer) and the mix were added and again centrifuged at 13 000 rpm for 1 min. The liquid was discarded keeping the covering filters, and again centrifuged to eliminate the remaining ethanol. Then, the covering filters containing the DNA were transferred on new eppendorf tubes where 50 µl of elution buffer (QLE buffer) were added and centrifuged at 13 000 rpm for 1 min. Covering filters were then discarded and the DNA kept into the eppendorf tubes.

II.2.3.4. Enzymatic digestion

Three microliters of DNA were mixed with 1 µl of restriction enzyme buffer H (10pb), 0.2 µl of EcoRI and 5.8 µl of sterile water. The mix was incubated at 37°C for 1h until used for electrophoretic analyses.

II.2.4. 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%; sub-rare, 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.

The mean of each monthly collection (M_c) and *X. fastidiosa*-infection (M_i) were calculated to compare abundance of captured species and correlation with the rate of infection along a single vegetative season (12 months), applying the following formulas:

$$M_c = (N_1+N_2)/2$$

$$M_i = (X_1+X_2)/2$$

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 harbour 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).

II.3. Results

II.3.1. Identification and abundance of insects

Overall, 2398 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 2). 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* (Stäl) (Hemiptera: Cicadellidae) (1.6%), *Allygus modestus* Scott (Hemiptera: Cicadellidae) (1.2%) and *Fieberiella florii* (Stäl) (Hemiptera: Cicadellidae) (1.1%), while the remainder of the captured species were classified as sub-rare with less than 1% abundance (Table 2). *Philaenus spumarius* was clearly the most captured species in the olive groves (Figure 7).

Table 2. Classification and total number of captured species during the two-year survey.

Infra order	Superfamily	Family	Species	N[a]	P
Cicadomorpha	Cercopoidea	Aphrophoridae	*Philaenus spumarius* (L.)	955	39.8
			Neophilaemus campestris	207	8.6
		Cercopidae	*Cercopis sanguinolenta*	12	0.5
	Cicadoidea	Cicadellidae	*Allygus modestus* Scott	28	1.2
			Anoplotettix putoni Ribaut	172	7.2
			Fieberiella florii (Stäl)	26	1.1
			Synophropsis lauri (Horvart)	86	3.6
			Euscelis lineolatus Brullé	195	8.1
			Conosanus obsoletus	13	0.5
			Exitianus capicola (Stäl)	39	1.6
			Thamnotettix zelleri	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		2398	

[a] N: number of individuals captured, [b] P (%): Percentage of individuals of each species

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_cT= 256), whereas May was the month with the highest species richness (10 out of 15 species collected) (Table 3).

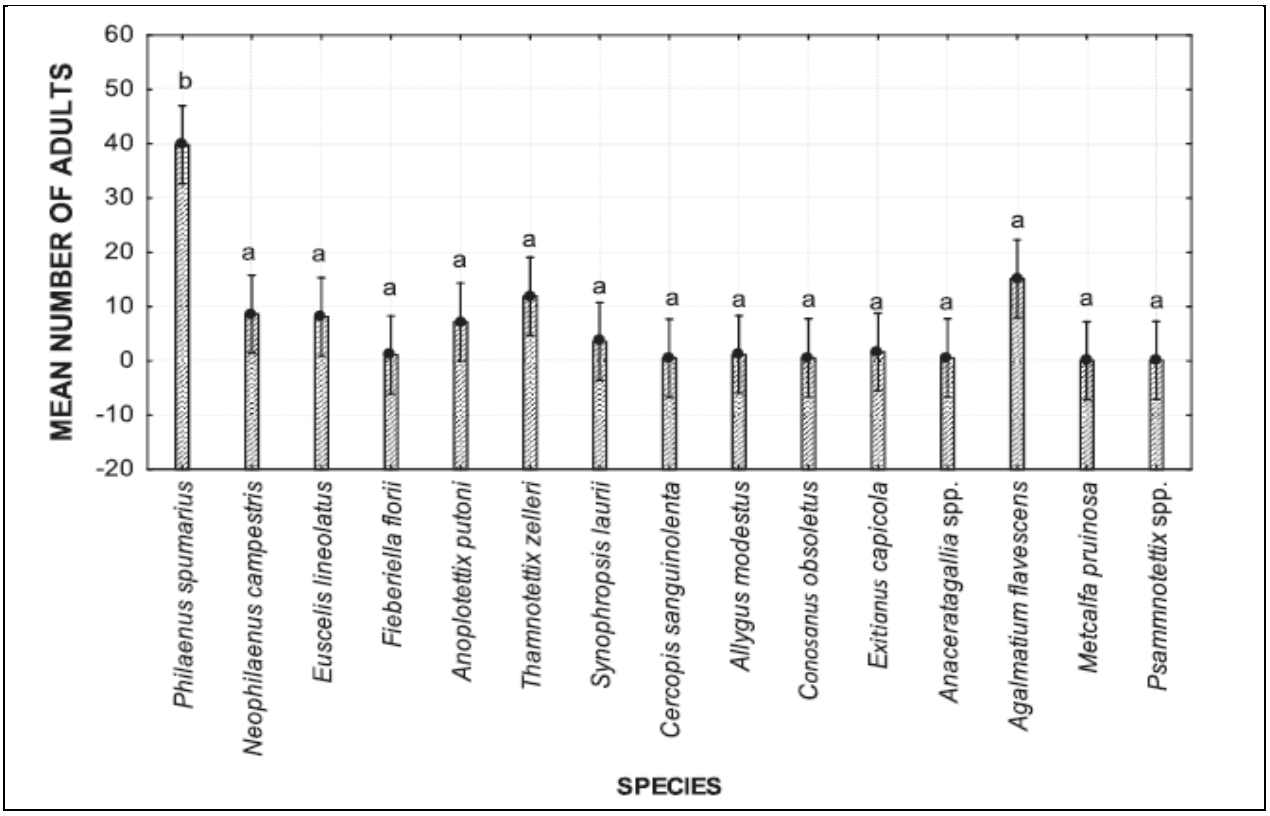

Figure 7: LS Mean of total number of individuals captured for each species during two years of study. Letters above each bar indicate significant differences using one-way ANOVA with Tukey's HSD test (F= 8.05, dF= 14, $P < 0.001$). Vertical bars denote 0.95 confidence intervals.

II.3.2. Detection and seasonal fluctuations of X. fastidiosa infection in captured insects

Only three out 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 2398 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.

Table 3. Average monthly abundance of Auchenorrhyncha species captured during a two-years survey.

Species	M_c [a]											
	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	1010	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	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	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

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* (Table 4).

Results of PCR assays are reported in Table 4, sub-divided 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%).

Table 4. Average monthly incidence of *Xylella fastidiosa*-infected Auchenorrhyncha specimens collected during a two-year survey.

Month	*P. spumarius*		*N. campestris*		*E. lineolatus*	
	M_c [a]	M_i [b]	M_c	M_i	M_c	M_i
October	25.5	8.0	16.5	6.0	4.5	0
November	5.0	0.5	6.5	2.0	13.0	0.5
December	3.5	0	5.0	1.5	24.0	5.5
January	4.0	1.0	3.0	2.0	25.5	11.0
February	0	0	0	0	13.0	0
March	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
August	101.0	20.0	4.5	0	0	0
September	148.5	21.0	13.0	1.0	0	0

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

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) (Figure 8 A, B).

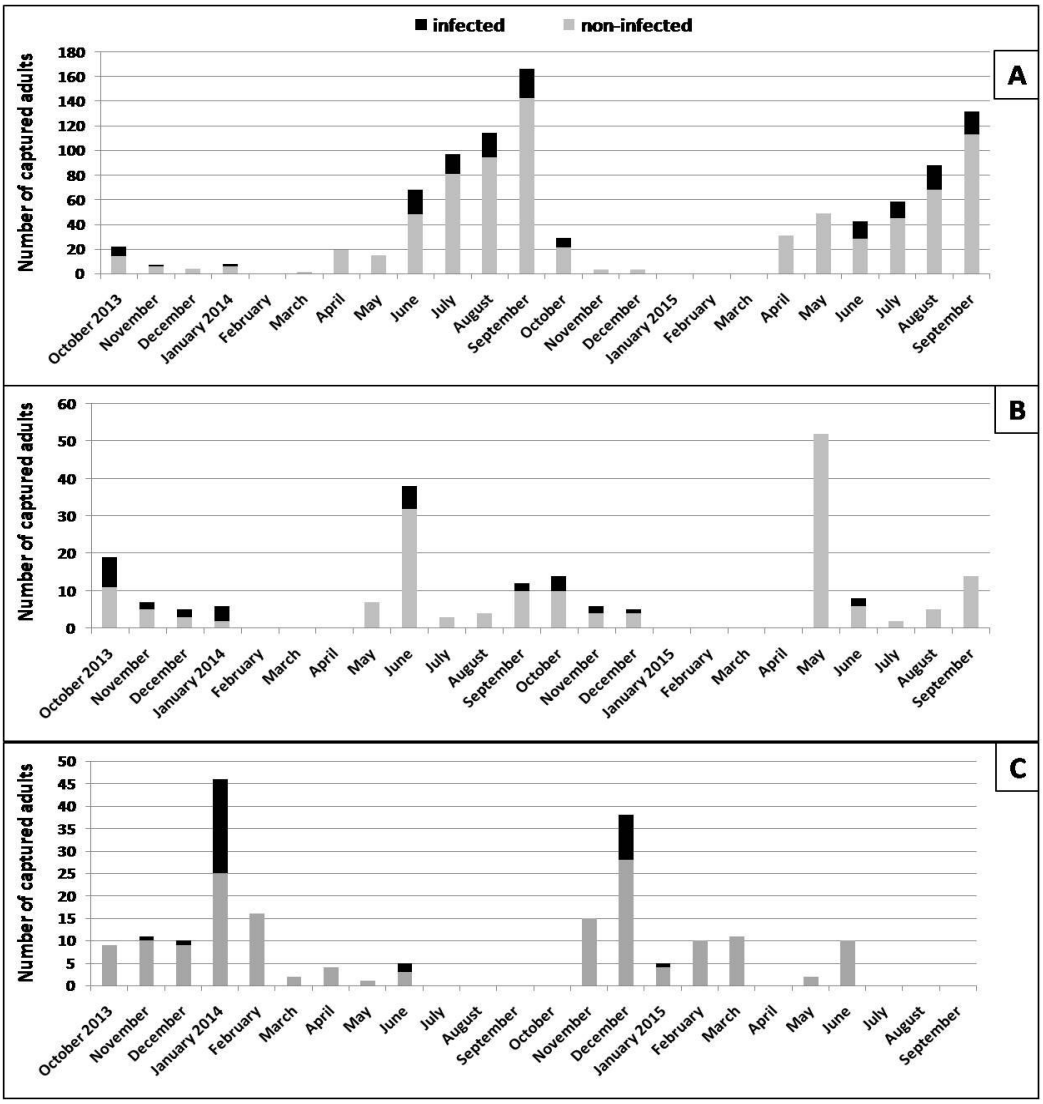

Figure 8: Seasonal population dynamics of *Xylella fastidiosa*-infected and non-infected adults of *Philaenus spumarius* (A), *Neophilaenus campestris* (B), and *Euscelis lineolatus* (C) in olive orchards.

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*, n = 115, *N. campestris*, n = 59 and *E. lineolatus*, n = 46) were found positive for *X. fastidiosa* by PCR (Figure 8). 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* (Figure 8, A).

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 $M_c = 25.5$ and $M_i = 11$ (Table 4), which was supported by the high coefficient of correlation (r = 0.90) (Figure 9, C). Similarly, the highest number of infected *P. spumarius* individuals coincided with greatest abundance in September with $M_c = 148.5$ and $M_i = 21$ (Table 4), resulting in a high coefficient of correlation (r = 0.92) (Figure 9, A).

Unlike *P. spumarius*, the peak of *N. campestris* population was in May with $M_c = 29.5$, when incidence of infected adults was null, while highest incidence of infected adults occurred in October ($M_i = 6$). 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 (r= 0.42) (Figure 9, B). Population density was high in May ($M_c = 29.5$) and June ($M_c = 23$), decreased steadily from July to August and then increased again in September ($M_c = 13$) and October ($M_c = 16.5$) (Table 4).

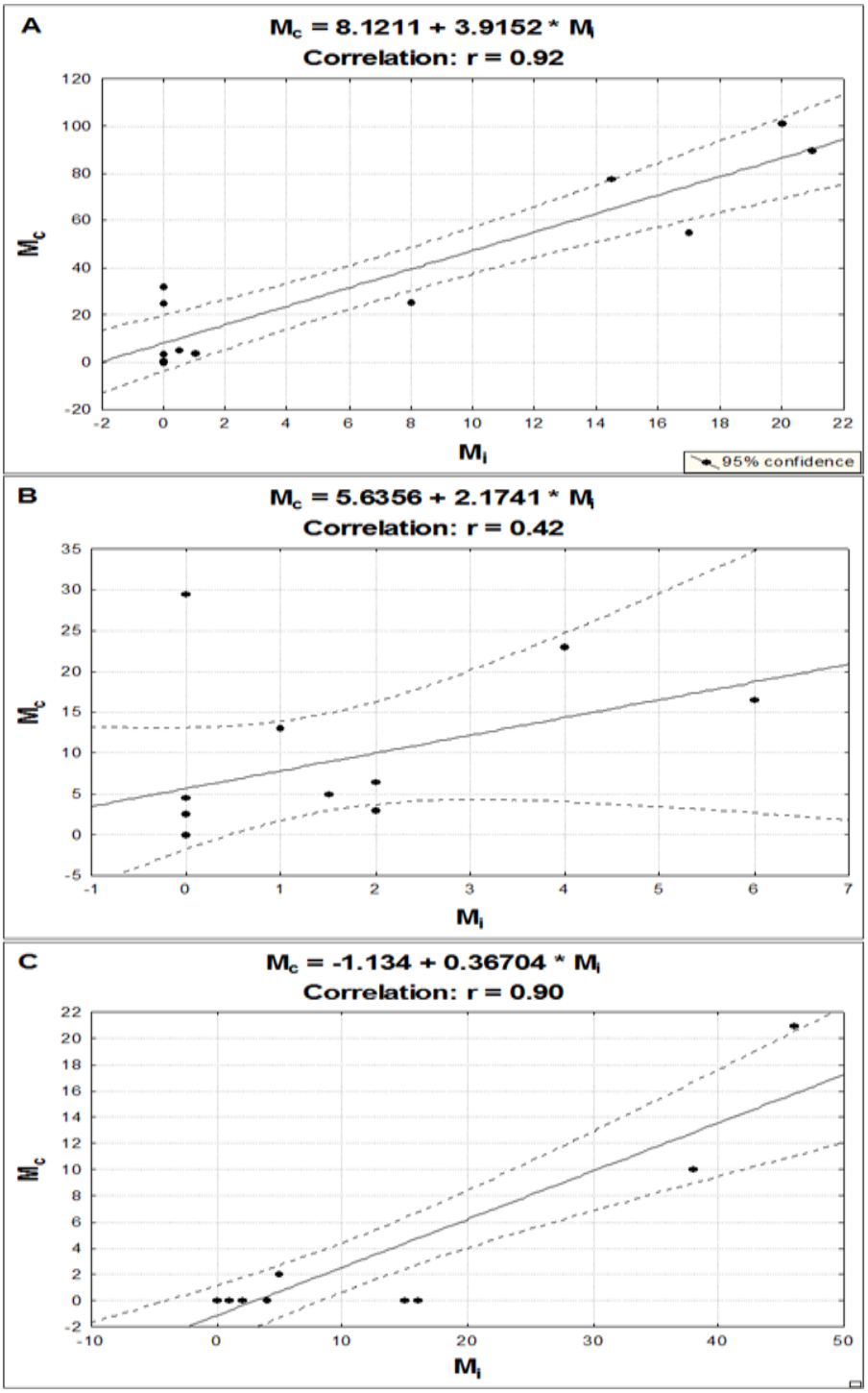

Figure 9: Relationship between seasonal abundance (Mc) of *Xylella fastidiosa*-infected insects (overall abundance) and the relative incidence of infected specimens (Mi) along a single season. (A) *Philaenus spumarius.* (B) *Neophilaenus campestris*. (C) *Euscelis lineolatus*.

II.3.3. Sequences analysis

Six PCR amplicons, two from each one of the 3 Xf-positive insect species (*P. spumarius, N. campestris* and *E. lineolatus*), were chosen for cloning and sequencing. The 6 sequences obtained were 99.3-100% identical among them at nucleotide level, and 3 of them (one sequence from each insect) were deposited in GenBank (accession no. HG939491, HG939505 and HG939506). BlastN analysis showed that all these sequences had the highest nucleotide identity (99.6-99.7%) with *X. fastidiosa* DNA fragments OL-X4-5 (accession no. HG532021) and OL-G (accession no. HG532022) from Apulian olive trees. However, they shared 96-98% identity with homologous fragments of isolates from other countries, and in particular 98% with the strains of subspecies fastidiosa GB514 (CP002165 from Texas-USA) and M23 (CP001011 from California-USA), and 97% with the strain of subspecies pauca 9a5c (AE003849).

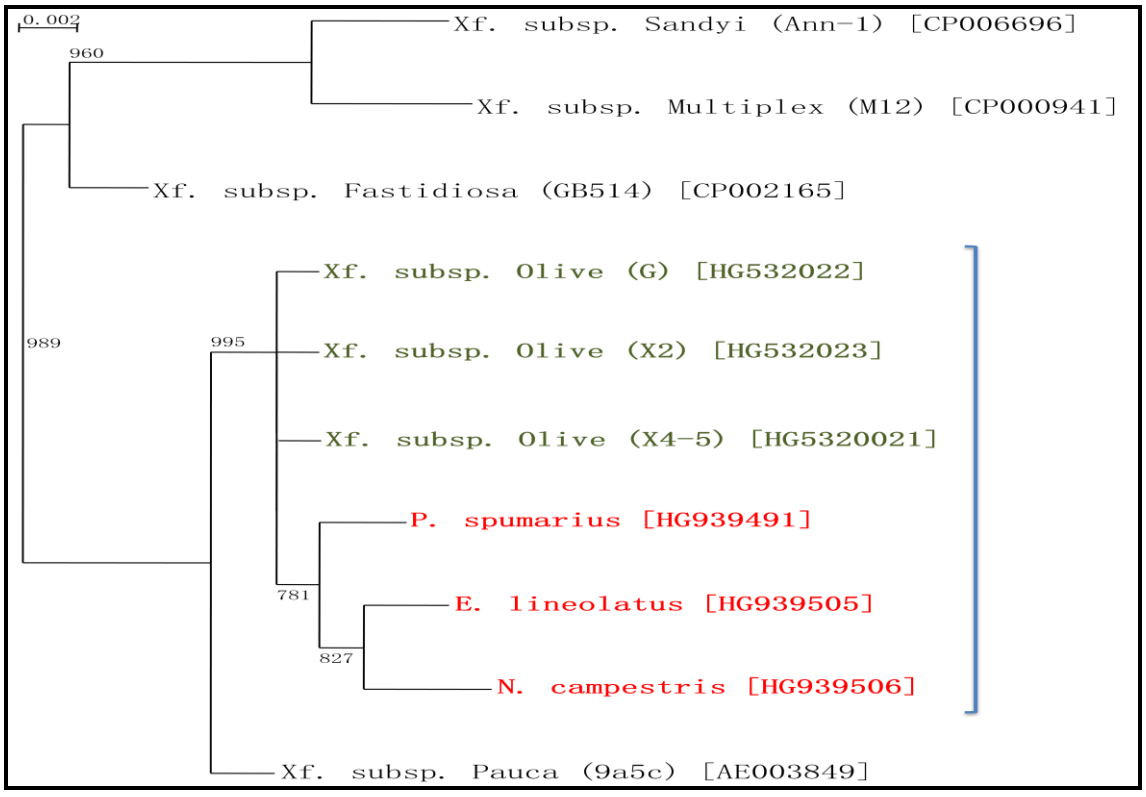

Figure 10: Position of the three *X. fastidiosa* strains from 3 insect species (*P. spumarius*, *N. campestris* and *E. lineolatus*) and 3 strains obtained from olive plant tissue (Xf. subsp. olive) in the phylogenetic tree.

Based on this sequence analysis, a phylogenetic tree (Figure 10) was deduced, which confirmed that the 3 sequences from insects were in the same clade with the strains of *X. fastidiosa* from Apulian olive trees, thus supporting that a single *X. fastidiosa* strain is present in that area. Whether the insects acquired the bacterium directly from olive plants or by alternative hosts, still remains to be established.

II.4. 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 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 two-year study, *X. fastidiosa* was detected by PCR in *P. spumarius*, *N. campestris* and *E. lineolatus*. 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: (1) adults were collected prior to coming in contact with *X. fastidiosa*-infected olive trees and/or (2) acquisition efficiency from olive trees during winter months is low for this species.

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 epidemiology. 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 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 non-cultivated 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.

Chapter III: Evaluation of the possibility to utilize potential vectors as indicators of the presence of the bacterium in apparently uncontaminated areas so called "Spy Insect approach"

III.1. Background

The quick dissemination of *X. fastidiosa* prompted an effective strategy for quickly monitoring this bacterium in a certain area in order to limit its spread. Since *X. fastidiosa* is a vector-borne bacterium, a survey on potential insect vectors present in the areas surrounding the infection sites (buffer zone) was carried out in order to evaluate the possibility to detect *Xf*-positive insects (so called "spy insects"), which can be used as indicators of the bacterium presence in apparently uncontaminated areas.

III.2. Material and methods

III.2.1. Insects capture and identification

From May to June 2014, adult insects were collected from 9 olive orchards from Trepuzzi area, where an isolated *Xf*-outbreak was present (Figures 6). All around the severely infected olive orchard (C), eight other orchards, apparently *Xf*-free, were identified symmetrically distributed from 4 sides [North (N1, N2), South (S1, S2), East (E1, E2) and West (W1 and W2)], at a distance of 500mt and 1000mt, respectively (Figure 11). A sweeping net was used to manually trap the insects from both olive canopy and ground vegetation. The insects captured were carefully collected by aspiration directly *in loco*, put in small tubes containing 70% ethanol and brought to the laboratory for identification. The classification and nomenclature of captured leafhoppers and froghoppers were based on Ribaut (1952), Della Giustina (1989) and Holzingher *et al*. (2003) keys. To accomplish their identification, male genitalia were dissected and kept in

KOH (10%) for 24 hrs. Then, they were mounted on glass slide in Berlese's liquid and observed under stereoscopic microscope.

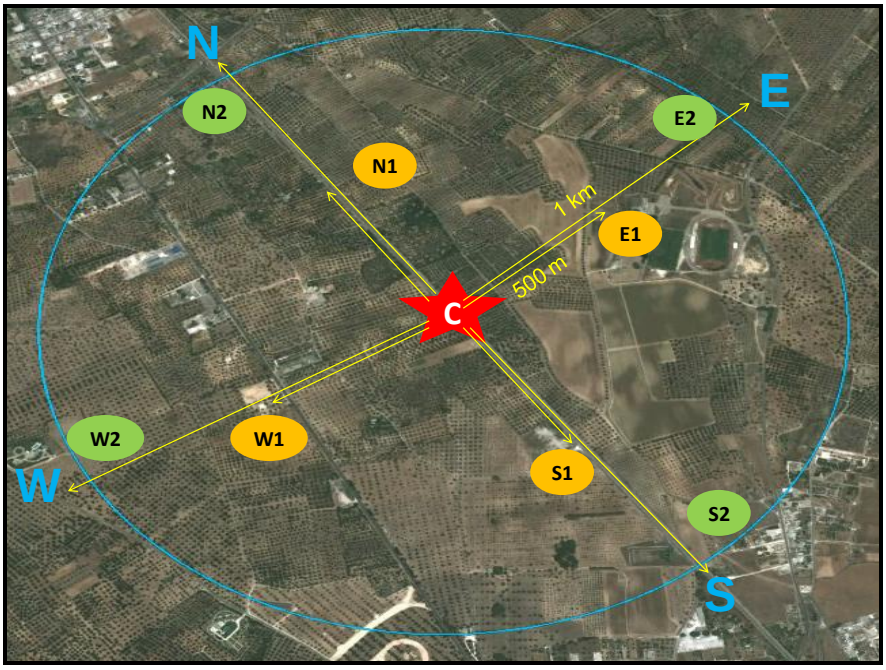

Figure 11: Design of the area of study in Trepuzzi.

III.2.2. Extraction of X. fastidiosa DNA from insects and PCR assays

Only adults were considered and the total of captured specimens were tested. Due to the multiplication and accumulation of bacteria particles in the foregut (Janse and Obradovic, 2010), the heads of single adult specimens were detached from the body as described by Bextine *et al*. (2004) and used for DNA extraction as follows. Each head was grinded using sterile pestles in a tube containing 200 µl of extraction buffer (Triton-X 1%, Tris-HCl 20 mM, EDTA 2 mM). After homogenization, 180 µl of suspension were transferred to a new tube and left to incubate at 60° for 30 min to allow full cell lysing to take place. Two hundred microliters of chloroform-isoamyl alcohol (24:1) were added to the suspension and centrifuged at 10,000 rpm for 10 min. DNA was

precipitated by adding 1 vol of cold isopropanol and incubated overnight at -20 °C. After a new centrifugation at 13,000 rpm for 20 min, the pellet was washed with 70% ethanol, dried and finally resuspended in 20 µl of sterile water.

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

III.3. Results

III.3.1. Insects capture and identification

Six insect species belonging to the taxon *Auchenorrhyncha* were captured from the 9 orchards located in the infected outbreak and the surrounding buffer zone at Trepuzzi. The captures were carried out in two different periods, i.e. on May and June, respectively. Only adults were taken into consideration and identified. The captured species were: the Aphrophoridae *Philaenus spumarius* (L.) and *Neophilaenus campestris* (Fallén), and the Cicadellidae *Thamnotettix zelleri* (Kirschbaum), *Anoplotettix putoni* (Ribaut), *Fieberiella florii* (Stål) and *Euscelis lineolatus* (Brullé). In the two catches, no substantial difference was observed in the species distribution according to the sampled orchards. The most frequent species were *P. spumarius* (40.7% in May

and 39.7% in June) and *N. campestris* (34.8% in May and 31.3% in June) followed by *A. putoni* and *F. florii*. Adults of these four species were captured both in May and June, whereas the adults of *T. zelleri* were collected only in May and those of *E. lineolatus* only in June, even though with a low prevalence (2%).

III.3.2. PCR assays

As shown in Table 5, in the first capture (May) 17 out of 76 (22.4%) adults were found positive to *X. fastidiosa* in the infected focus (C), whereas they were all negative in the olive orchards of the surrounding buffer zone, except for a single site 500 mt far from the central outbreak, where 4 out of 44 (9%) adults were found positive. All these 21 (17+4) *Xf*-positive adults belonged to the species *P. spumarius*. The complete absence of positive insects in the remaining seven orchards in the buffer zone could be likely justified by the low frequency of infected insects even in the infected outbreak (C). The low rate of infected insects even in the infected area may be explained by the fact that the adults captured in May were likely members of the new generation that still had not chance to come in contact with *Xf*-infected sources and for the absence of a trans-stadial or transovarial transmission of the bacterium in the insects (Hill and Purcell, 1995).

Furthermore, PCR results of the second capture of insects in June showed that the *Xf*-positive samples were more numerous not only in the infected outbreak (34.1% of total), but also in olive orchards in the buffer zone at 500 mt and 1 km far from it, where *Xf*-positive insects ranged from 0 to 35 % (Table 5).

Table 5. Incidence of Xf-positive Hemiptera insects captured from the buffer zone in May and June 2014.

Orchards	Insects captured *	Number of adults / species						% of infection
		P. spumarius	*N. campestris*	*E. lineolatus*	*F. florii*	*T. zelleri*	*A. Putoni*	

May	*C*	tot	34	4	0	11	8	19	22,4%
		inf	17	-	-	-	-	-	
	N1	tot	13	7	0	0	12	5	0%
		inf	-	-	-	-	-	-	
	N2	tot	15	11	0	1	9	0	0%
		inf	-	-	-	-	-	-	
	S1	tot	21	14	0	1	6	2	9%
		inf	4	-	-	-	-	-	
	S2	tot	24	32	0	0	4	2	0%
		inf	-	-	-	-	-	-	
	E1	tot	20	19	0	0	0	0	0%
		inf	-	-	-	-	-	-	
	E2	tot	17	20	0	0	11	1	0%
		inf	-	-	-	-	-	-	
	W1	tot	15	17	0	0	3	7	0%
		inf	-	-	-	-	-	-	
	W2	tot	21	30	0	0	2	4	0%
		inf	-	-	-	-	-	-	
June	*C*	tot	17	7	0	2	0	15	34,1%
		inf	14	-	-	-	-	-	
	N1	tot	15	4	3	0	0	2	12,5%
		inf	-	2	1	-	-	-	
	N2	tot	12	17	0	2	0	2	6,0%
		inf	2	-	-	-	-	-	
	S1	tot	12	6	2	0	0	0	35,0%
		inf	6	-	1	-	-	-	
	S2	tot	4	13	0	0	0	15	12,5%
		inf	-	4	-	-	-	-	
	E1	tot	11	5	0	4	0	3	21,7%
		inf	5	-	-	-	-	-	
	E2	tot	9	16	0	0	0	6	0,0%
		inf	-	-	-	-	-	-	
	W1	tot	8	6	0	0	0	0	14,3%
		inf	-	2	-	-	-	-	
	W2	tot	12	5	0	4	0	13	0,0%
		inf	-	-	-	-	-	-	

*tot= total; inf= infected

More precisely, in the infected orchard, the 14 out 41 (34.1%) positive adults were all of the species *P. spumarius*. In the same month, *X. fastidiosa* was detected also in insects collected from 6 out of 8 orchards in the buffer zone, all 4 orchards at a distance of 500mt and 2 at 1km far from the outbreak. The highest rate of infected specimens in the buffer zone was noticed in an orchard 500mt far from the centre, where 7 out of 20 (35%) adults were positive, 6 of which were *P. spumarius* and 1 *E. lineolatus*. At a distance of 1km far from the infected site, the highest infection rate was 12.5%. Six out of 130 specimens captured from the four orchards 1 km far from the outbreak were positive to *X. fastidiosa*, i.e. 2 *P. spumarius* and 4 *N. campestris*. Accordingly, considering all over the two captures (May and June) and the 6 tested insect species, 3 species resulted *Xf*-positive, i.e. *P. spumarius*, *N. campestris* and *E. lineolatus* (Table 6).

Table 6. Incidence of infected specimens of the three Xf-positive species during the two months of survey throughout the area of study.

Number of infected/captured adults (- = no captures)										
Species	Period of captures	Orchards								
		C	N1	N2	S1	S2	E1	E2	W1	W2
P. spumarius	May	17/34	0/13	0/15	4/21	0/24	0/20	0/17	0/15	0/21
	June	14/17	0/15	2/12	6/12	0/4	5/11	0/9	0/8	0/12
N. campestris	May	0/4	0/7	0/11	0/14	0/32	0/19	0/20	0/17	0/30
	June	0/7	2/4	0/17	0/6	4/13	0/16	0/5	2/6	0/5
E. lineolatus	May	-	-	-	-	-	-	-	-	-
	June	-	1/3	-	1/2	-	-	-	-	-

In general, *P. spumarius* was the most abundant species and showed the highest rate of infection throughout the area of study, especially in the central infected site, where it was the only infected species with rate of infection that reached 50% and 82% in May and June, respectively. Adults of *N. campestris* were as well abundant in both months but none of the tested specimens was positive in May and only 6 out 69 adults were positive in June throughout the area

of study. Conversely, adults of *E. lineolatus* were totally absent in May and only 5 adults were captured in June from two orchards in the buffer zone at a distance of 500mt far from the outbreak. Although with low prevalence, *E. lineoaltus* showed a high rate of infection since 2 out 5 (40%) adults captured were positive to *X. fastidiosa*.

III.4. Discussion

The results of the present study, although preliminary, provide new information on the potential role of some Auchenorrhyncha species in the epidemiology of *X. fastidiosa*, associated with Olive quick decline syndrome (OQDS) in Apulia (south Italy). *X. fastidiosa* was detected by PCR in three out of six species captured, i.e. *P. spumarius*, *N. campestris* and *E. lineolatus*, thus confirming the preliminary results provided in chapter 2. Moreover, the presence of infected adults of these three species in the buffer zone up to a distance of 1 Km far from the infection site confirmed the importance of the use of the "spy insects" for early revealing the presence of the pathogen in apparently free areas before symptoms become visible on the plants. Nevertheless, the presence of infected insects 1 km far from the *Xf* outbreak has two possible explanations: the first one is that the insects have acquired the bacterium from still symptomless infected hosts (olive trees or other plant species) already present in the "*Xf*-free" area; the second one is that the insects acquired the bacterium directly from the infected site and moved up to 1 Km far from it. If this second hypothesis is true, a larger radium for the buffer zone should be adopted, whose the measure needs to be established by further surveys.

Chapter IV: Effect of *X. fastidiosa* endoglucanases on the acquisition, retention of the bacteria by an efficient insect vector

IV.1. Background

X. fastidiosa maintains several endoglucanases in its genome that facilitate systemic colonization of host plants via enzymatic degradation of the plant cell wall. Specifically, these enzymes cleave the β-1,4 glycosidic linkages of cellulose, which is one of the main polysaccharides composing the plant cell wall. In addition to their roles *in planta*, we have speculated that these enzymes may also contribute to the dissolution of *X. fastidiosa* biofilm (both *in planta* and in the insect vector) through degradation of the protective exopolysaccharide (EPS) matrix, which contains a backbone of β-1,4 glycosidic linkages (Backus *et al.*, 2012). The mechanism of biofilm dissolution in *X. fastidiosa* has remained largely unexplored. To explore this process, the mutants constructed at Roper's lab (Department of plant pathology and Microbiology, University of California Riverside) present a unique opportunity. Thereby, utilizing *X. fastidiosa* endoglucanase mutants and blue-green sharpshooter insects (*Graphocephala atropunctata*), laboratory-based transmission assays with artificial feeding sachets were conducted to explore the effect of these enzymes on the acquisition and retention of *X. fastidiosa*.

IV.2. Material and methods

IV.2.1. Bacterial strains and growth conditions

Wild type *X. fastidiosa* Temecula1 (Guilhabert *et al*., 2001) and Δ*engXCA2* mutant strain (Roper *et al*., 2016), were used. *X. fastidiosa* Temecula1 wild type and Δ*engXCA2* mutant strains were grown for 7 days at 28°C on solid PD3 medium. For acquisition tests, strains were prepared as previously described (Rapicavoli *et al*., 2015). In brief, wild type *X. fastidiosa* and Δ*engXCA2*

mutant cells were harvested from PD3 plates and suspended in liquid XFM medium (without filter-sterilized supplement: l-asparagine, l-cysteine, l-glutamine, and bovine serum albumin). Bacterial cell suspensions were adjusted to OD600=0.25 (approximately 10^8 cells/mL); 20µL aliquots of cell suspensions were striped onto XFM-pectin (0.01%) medium. Cells were incubated at 28°C for an additional 7 days, suspended in artificial insect diet solution (L-glutamine, 0.7mM; L-asparagine, 0.1mM; sodium citrate, 1mM; pH 6.4) (Killiny and Almeida, 2009), and suspensions were adjusted to 10^8 cells/mL (OD600 0.25).

IV.2.2. Insect maintenance

Adult blue-green sharpshooters (BGSS), *Graphocephala atropunctata* (Signoret) (Hemiptera, Cicadellidae), were reared on sweet basil (*Ocimum basilicum* L.) under greenhouse conditions (22 ± 5°C). Only second generation adults were used in the acquisition tests.

IV.2.3. Insect acquisition experiments

Acquisition experiments using artificial diets were performed as previously described (Killiny and Almeida, 2009). Retention and acquisition from three different treatments, i.e. wild type Xf (Temecula 1), Δ*engXCA2* mutant and diet only (negative control). In total, 75 BGSS adults were tested per treatment and they were divided into 3 time intervals (1 day, 4 days and 7 days) in order to compare colonization patterns and quantity of bacterial cells acquired over time. Therefore, groups of 25 individuals per treatment were tested at each time interval.

Insects were caged individually in artificial sachets constructed from the base of a 5mL pipette tip, sealed with a moist cotton ball. Each replicate consisted of 35µL of bacterial cell suspensions loaded onto a layer of stretched parafilm, with the drop later being covered with another layer of parafilm. Insects were given a 6-hr acquisition access period (AAP) at 20°C. To remove any unbound cells present in the ingested solutions, insects were transferred to healthy basil plants for

a clearing and multiplication period. After 1, 4 and 7 days of clearing and multiplication period at room temperature, 10 insect heads per treatment were placed into 4% glutaraldehyde fixative for scanning electron microscopy, and 15 whole insects were immediately stored at -20°C until DNA extraction and qPCR analysis.

IV.2.4. Quantification of X. fastidiosa

The Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.) was used for genomic DNA extractions from individual BGSS heads, with a liquid nitrogen pre-treatment. Using a disposable microtube pestle, individual heads were ground to a fine powder with liquid nitrogen. Samples were then processed according to the standard DNeasy protocol, and the resulting DNA was concentrated using a Speedvac, prior to use in qPCR. For use in quantification of *X. fastidiosa* in the insect samples, standard curves were created using a 2:1 concentration ratio of BGSS genomic DNA and *X. fastidiosa* genomic DNA. To prepare the DNA for the insect sample standard curve, cultured *X. fastidiosa* cells were adjusted to an OD600=0.25, corresponding to approximately 1×10^8 cells/mL (1×10^5 cells/µL), and genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.). The resulting DNA was mixed with insect DNA in a 2:1 concentration ratio. From this mixture, ten fold serial dilutions were made creating a range of standards from 30,000 – 3 *X. fastidiosa* cells/µL. Quantitative PCR was performed using a C1000 thermal cycler fitted with the CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). TaqMan primers and probe targeting the ITS region of the *X. fastidiosa* Temecula1 genome were *Xf*ITSF6 (5'-GAGTATGGTGAATATAATTGTC-3'), *Xf*ITSR6 (5'-CAACATAAA CCCAAACCTAT-3'), and *Xf*ITS6-probe1 (5'- (6-FAM) CCAGGCGTC CTCACAAGTTA (BHQ1a-6FAM) -3').BHQ1a is Black Hole Quencher 1 (Eurofins MWG Operon, Huntsville, AL, U.S.A.). PCRs were performed in a volume of 25µL and contained 2.5µL of 10x Buffer Mix (Qiagen, Valencia, CA, U.S.A.), 2.5µL of $MgCl_2$ Buffer

(Qiagen, Valencia, CA, U.S.A.), 200μM of each dNTP (Qiagen, Valencia, CA, U.S.A.), 400nM of each primer, 250nM of the probe, 1.25 units of Taq DNA polymerase, and 1μL of sample or standard template. PCRs were performed using the following conditions: an initial denaturation step at 94°C for 5 min, followed by 38 cycles of denaturation at 94°C for 20 sec, primer annealing at 63°C for 30 sec, and primer extension at 72°C for 30 sec, with a final extension step at 72°C for 2 min.

IV.2.5. Scanning electron microscopy

Using a series of ethanol dilutions, BGSS heads were dehydrated and critical point dried using the Tousimis Autosamdri-815B critical point dryer (Tousimis, Rockville, MD, U.S.A.). Foreguts were gently dissected from insect heads and mounted onto copper tape attached to aluminum stubs. Samples were coated in a platinum/palladium (Pt/Pd) mixture using the Cressington 108 Auto Sputter Coater (Cressington Scientific Instruments Ltd., Watford, England, UK) and imaged using the Philips XL30 FEG scanning electron microscope (FEI, Hillsboro, OR, U.S.A.).

IV.2.6.Data analysis

Mean separation for the comparison number of *X. fastidiosa* cells per insect's foregut between wild type *Xf* and the mutant at each time interval was made with one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. Data analysis described and graphs were treated using the statistical software STATISTICA (version 7.0.61.0).

IV.3. Results

IV.3.1. Quantitative comparison (qPCR)

Overall, 14 and 16 out of 45 BGSS adults that were fed respectively on artificial diet containing wild type bacteria and Δ*engXCA2* mutant, were *xf*-positive. However, none of the 45 BGSS adults that were fed on artificial diet only, was positive by qPCR. Over time; 6, 5 and 3 sharpshooters were able to acquire the wild type bacteria at 1, 4 and 7 days after acquisition, respectively. Similarly, 7, 4 and 5 sharpshooters fed on the Δ*engXCA2* mutant were able to acquire the bacteria respectively at the same time intervals. Therefore, no significant difference was noticed between both treatments in term of acquisition rate at any time interval.

From another hand, insects tested after one day of multiplication showed that there is no difference in term of number of cells acquired between both treatments. However, when tested after 4 and 7 days of multiplication period, sharpshooters that were fed suspensions of the Δ*engXCA2* mutant harbored significantly greater (F= 6.7, df= 7, P= 0.03) cells than those that were fed wild type bacteria (Figure 12).

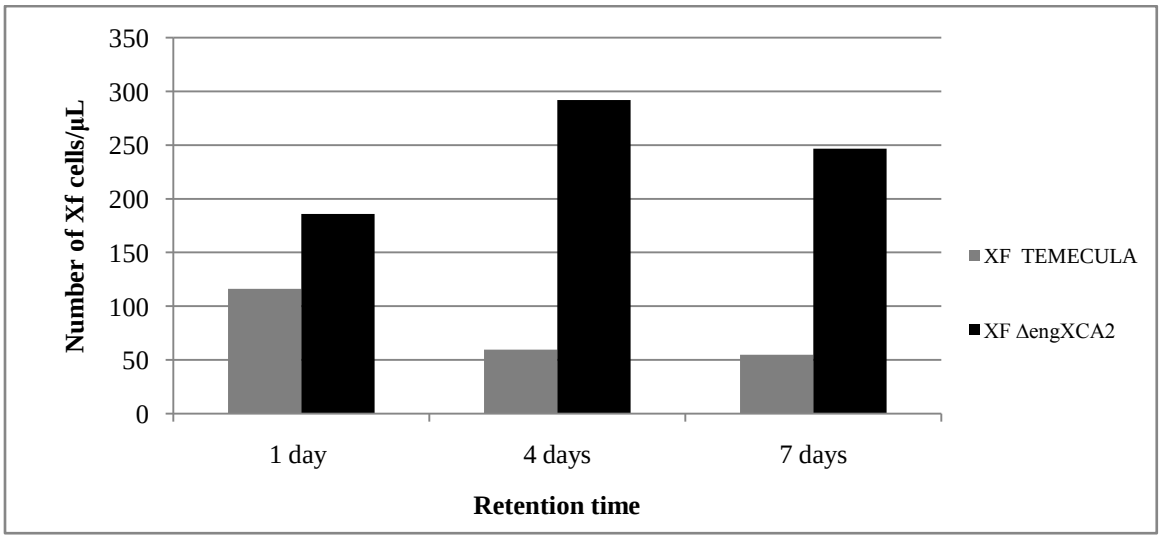

Figure 12: Mean of *X. fastidiosa* cells number detected per 1µL of DNA extracted from BGSS head along 3 retention time intervals after acquisition from artificial diets containing suspensions of wild type bacteria (Temecula 1) compared to those fed on suspensions of Δ*engXCA2* mutant.

IV.3.2. Qualitative comparison (SEM)

After feeding insects on artificial diets containing wild type bacteria and Δ*engXCA2* mutant, BGSS foreguts contained large, flocculent microcolonies of bacteria (Figure 13).

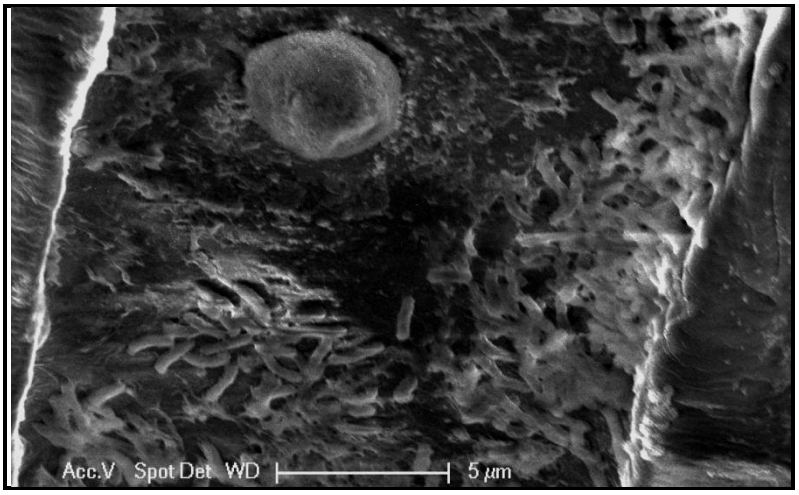

Figure 13: Scanning electron micrograph illustrating *X. fastidiosa* biofilms within the precibarial trough of BGSS fed on artificial diet containing bacterial suspensions. Bar = 5 μm.

One day after acquisition, no difference was observed between both treatments in term of foregut colonization patterns. However, Δ*engXCA2*-fed sharpshooters foreguts observed after 4 and 7 days of multiplication period showed that bacterial aggregates were more abundant and accumulated into dense colonies while those observed in wild type-fed insects were clearly fewer and sparse in the foregut. Insects fed on artificial diet only, showed no visible bacterial colonization in both regions, i.e. cibarium and precibarium (Figures 14 and 15).

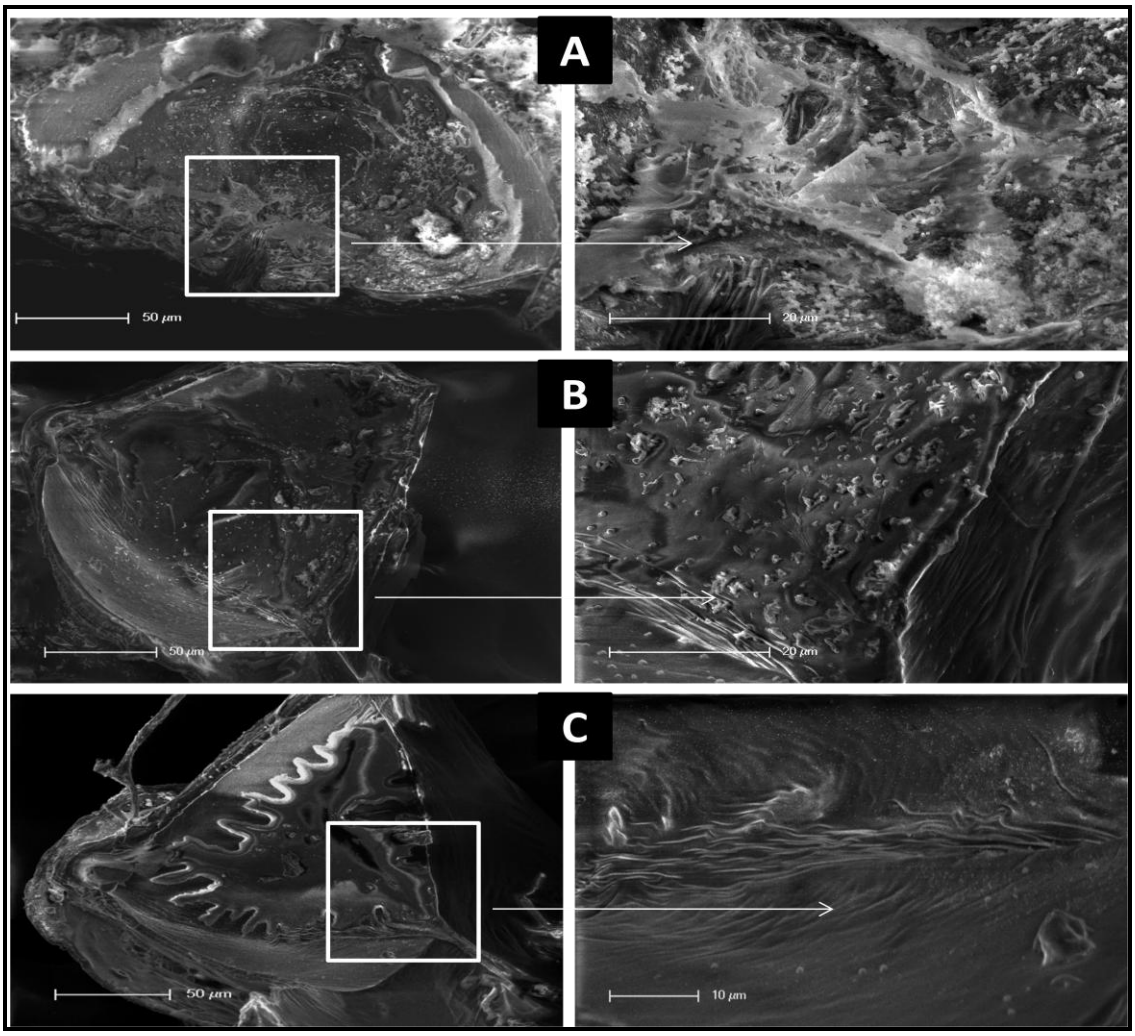

Figure 14: Scanning electron micrographs of the epipharyngeal surface of the foregut of BGSS fed on artificial diets containing Δ*engXCA2* mutant (A), wild type *X. fastidiosa* (B) and artificial diet only (C) for 6 hours. Images are obtained after 7 days of multiplication period and are representative of 20 total replicates per treatment (10 replicates per time interval, i.e. 4 and 7 days of multiplication period). Diet-fed insects represent negative controls. Micrographs illustrate bacterial colonization along the insect's cibarium and precibarium, Bar = 50 µm (left) and details of bacterial biofilms in the precibarium channel and cibarium's ventral surface, Bar = 20 µm (right).

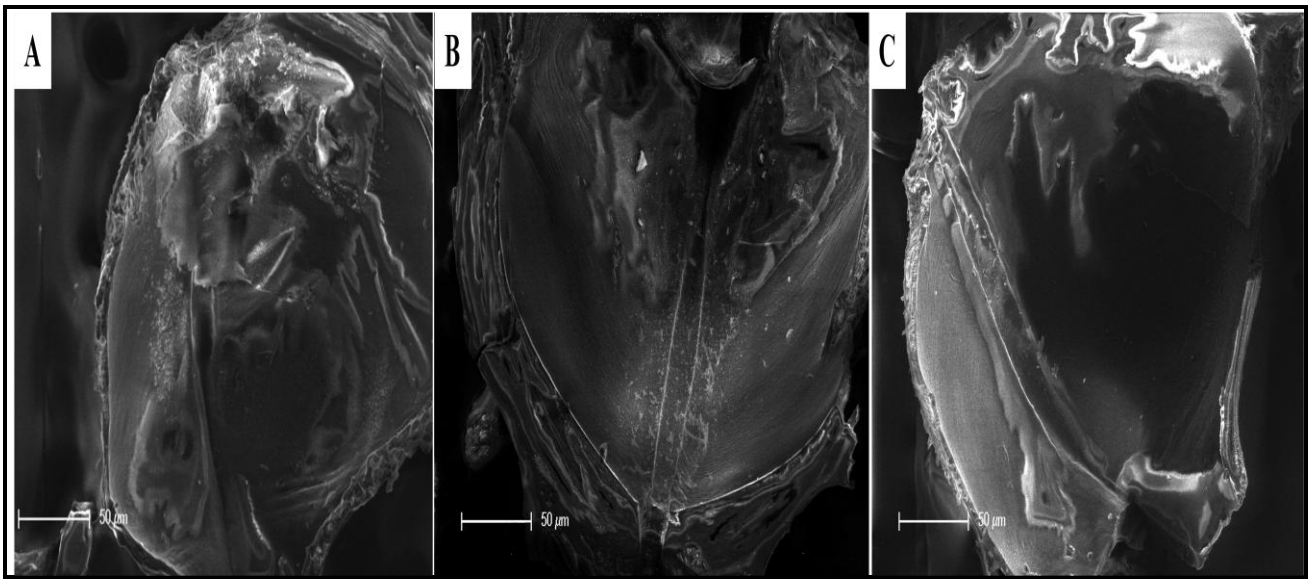

Figure 15: Scanning electron micrographs of the hypopharyngeal surface of the cibarium of BGSS fed on artificial diets containing Δ*engXCA2* mutant (A), wild type *X. fastidiosa* (B) and artificial diet only (C) for 6 hours. Images are obtained after 7 days of multiplication period on basil and are representative of 20 total replicates per treatment (10 replicates per time interval, i.e. 4 and 7 days of multiplication period). Diet-fed insects represent negative controls. Bar = 50 µm.

IV.4. Discussion

Insect vectors are the only means for plant-to-plant dissemination of *X. fastidiosa*. Therefore, the bacterium depends equally on successful plant and insect colonization for its survivorship and spread. Pathogen acquisition , retention and inoculation are combined to generate an estimate of overall transmission efficiency. This process depends heavily on the properties of the bacterial cell surface, because it is the first surface to interact with the surrounding environment, thus playing an important role in cell- cell and host-microbe interactions (Walker et.,2004; Clements *et al*., 2008). *X. fastidiosa* have evolved surface-associated polysaccharides, namely LPSs and EPSs, that aid in attachment, protection and survival under pressures exerted by their specific host environment. In the insect and plant hosts, *X. fastidiosa* seemingly follows the canonical steps involved in biofilm formation, which begins with initial attachment to the surface, followed

by aggregation/microcolony formation, macrocolony formation and biofilm maturation. Particularly, EPS is important for the development of biofilms through forming a matrix responsible for biofilm stability (Flemming and Wingender, 2010). This matrix contains a backbone of β-1,4 glycosidic linkages which can be degraded by endoglucanases. *X. fastidiosa* contains these enzymes in its genome which facilitate systemic colonization of host plants. However, there are no studies that explored the role of endoglucanases in degrading EPS and consequently their effect on biofilm formation and maturation in insect vectors.

For this aim, we used a Δ*engXCA2* mutant of *X. fastidiosa* constructed at Caroline Roper's lab (Department of plant pathology and microbiology, UC Riverside). Δ*engXCA2* is an endoglucanases minus mutant which present a unique opportunity to demonstrate if the presence or absence of these enzymes may interfere with the transmission process of *X. fastidiosa*.

Our study showed that in vitro, endoglucanases do not affect the acquisition rate of the bacterium by BGSS. However, it was shown that these enzymes modulate the over time retention of *X. fastidiosa*. Thus, it was found that at 4 and 7 days of multiplication period after acquisition, number of *Xf* cells and bacterial colonization in BGSS foreguts were greater when insects were fed on artificial diets containing Δ*engXCA2* suspensions compared to those fed on the wild type bacteria. These findings confirm the fact that sparse amounts of EPS are associated with the cells during the initial attachment, with the bulk of the EPS being produced during the late phase of biofilm maturation where substantial amounts can be seen intercalating the biofilm matrix of a mature biofilm (Roper *et al*., 2007). Similarly, previous study showed that EPS does not impair initial attachment to the insect foregut cuticule but may impair subsequent steps in the insect colonization process (Killiny and Almeida., 2009; Killiny *et al*., 2013).

These results lead us to bear out the hypothesis that endoglucanases may contribute to the dissolution of *X. fastidiosa* biofilms in insect foregut through the degradation of the EPS matrix. Thus, *X. fastidiosa* produces more biofilms in the absence of endoglucanases and bacteria adheres

more to the insect foregut cuticule so will be less egested into basil during the multiplication period. Although promoting, these results are still preliminary and need to be confirmed through performing in vitro inoculation assays then plant-to-plant transmission experiments.

Conclusions

The present thesis provide new information on the potential role of some insects of Auchenorrhyncha in the epidemiology of *X. fastidiosa*, associated with olive quick decline in Apulia region (Italy) as well as a novel biocontrol approach for vector transmission of the bacterium. Our findings presented the first identification of three potential vectors of *X. fastidiosa* in Apulia region, i.e. *Philaenus spumarius* L. (Aphrophoridae), *Neophilaenus campestris* Fallén (Aphrophoridae) and *Euscelis lineolatus* Brullé (Cicadellidae). *P. spumarius*, which is the most abundant species, was later confirmed as a vector of the bacterium while further transmission trials are still required for the other two species. Moreover, we provided the first qualitative and quantitative data about main Auchenorrhyncha species occurrence and abundance over the year in the Xf-infected area. The study of the seasonal fluctuations of three identified potential vectors allowed us to confirm that transstadial or transovarial transmission of *X. fastidiosa* does not occur since all the individuals of the new generation were Xf-negative. Thereby, we 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.

From another hand, the potential role of these insects as " spy insects" useful to reveal the presence of the pathogen in apparently free areas (buffer zones, uncontaminated areas), before symptoms become visible on the plants. In a preliminary application of this technical approach in the buffer zone surrounding a well circumscribed focus of infection at Parabita (Lecce province), the bacterium was detected in the insects also at a distance of 1 Km far from the infection site in symptomless olive orchards which were supposed to be *Xf*-free. Thus, a larger radium for the buffer zone should be adopted, whose the measure needs to be established by further surveys.

Finally, we explored the role of endoglucanases in degrading EPS and consequently their effect on biofilm formation and maturation in insect vectors. Even though preliminary, our results showed that these enzymes may contribute to the dissolution of *X. fastidiosa* biofilms in insect foregut through the degradation of the EPS matrix and consequently modulate the over time retention of *X. fastidiosa* by the blue green sharpshooter. These results are still to be confirmed through performing in vitro inoculation assays then plant-to-plant transmission experiments.

References

Almeida, R.P.P., Blua, M.J., Lopes, J.R.S. and Purcell, A.H. 2005. Vector transmission of *Xylella fastidiosa*: applying fundamental knowledge to generate disease management strategies. Ann. Entomol. Soc. America 98: 775-786.

Anses (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), 2012. Evaluation du Risque Simplifiée de *Xylella fastidiosa*. Available online: http://www.anses.fr/Documents/SVEG2012sa0121Ra.pdf

Altschul, S.F., Stephen, F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Backus, E.A., Andrews, K.B., Shugart, H.J., Greve, L.C., Labavitch, J.M., and Alhaddad, H. 2012. Salivary enzymes are injected into xylem by the glassy-winged sharpshooter, a vector of *Xylella fastidiosa*. J. Insect Physiol. 58: 949–959.

Baugnée, J.Y. 2011. La Cicadelle du laurier *Synophropsis lauri* (Hemiptera: Cicadellidae) en Belgique. Phegea 39: 34-38.

Bextine B., Tuan, S.J., Shaikh, H., Blua, M., and Miller, T.A. 2004. Evaluation of methods for extracting *Xylella fastidiosa* DNA from the glassy-winged sharpshooter. J. Econ. Entomol. 97: 757-763.

Cariddi, C., Saponari, M., Boscia, D., De Stradis, A., Loconsole, G., Nigro, F., Porcelli, F., Potere, O., and Martelli, G.P. 2014. Isolation of a *Xylella fastidiosa* strain infecting olive and oleander in Apulia, Italy. J. Plant Pathol. 96: 1-5.

Chatterjee, S., Almeida, R.P.P. and Lindow, S. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. Annu. Rev. Phytopathology 46: 243-271.

Chang, C.J., Garnier, M., Zreik, L., Rossetti, V., and Bovè, J.M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. Curr. Microbiol. 27: 137-142.

Clements, A., Gaboriaud, F., Duval, J.F.L., Farn, J.L., Jenney, A.W., Lithgow, T., Wijburg, O.L.C., Hartland, E.L. and Strugnell, R.A. 2008. The major surface-associated saccharides of *Klebsiella pneumoniae* contribute to host cell association. PLoS One 3: e3817.

Davis, M.J., Purcell, A.H., and Thomson, S.V. 1978. Pierce's disease of grapevines isolation of the casual bacterium. Science. 199: 75-77.

Della Giustina, W. 1989. Homoptères Cicadellidae, pp. 1-350. *In Faune de France*' 73 (3), Fédération Française des sociétés de sciences naturelles and INRA (ed.), Paris, France.

de Jong Y.S.D.M. (2013). Fauna Europaea version 2.6. Ed de Jong YSDM. Web Service. Available online: http://www.faunaeur.org

EFSA - European Food Safety Authority. 2015. Scientific Opinion on the risk to plant health posed by *Xylella fastidiosa* in the EU territory, with the identification and evaluation of risk reduction options. EFSA Journal 13 (1): 3989-4251.

EFSA - European Food Safety Authority. 2016. Update of a database of host plants of *Xylella fastidiosa*. EFSA Journal 14 (2): 4378-4422.

Elbeaino, T., Yaseen, T., Valentini, F., Ben Moussa, I.E., Mazzoni, V., and D'Onghia, A.M. 2014. Identification of three potential insect vectors of *Xylella fastidiosa* in southern Italy. Phytopathol. Mediterr. 53: 328-332.

Flemming, H.C. and Wingender, J. 2010. The biofilm matrix. Nat. Rev. Microbiol. 8: 623-633.

Frazier, N.W. 1944. Phylogenetic relationship of the nine known leafhopper vectors of Pierce's disease of grape. Phytopathology 34: 1000– 1001

Freitag, J.H. 1951. Host range of the Pierce's disease virus of grapes as determined by insect transmission. Phytopathology 41: 920-932.

Goheen, A.C., Nyland, G. and Lowe, S.K. 1973. Association of rickettsia-like organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63: 341-345.

Guilhabert, M.R., Hoffman, L.M., Mills, D.A. and Kirkpatrick, B.C. 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant Microbe Interact. 14:701-706.

Hail, D., Mitchell, F., Lauzière, I., Marshall, P., Brady, J., and Bextine, B. 2010. Detection and analysis of the bacterium, *Xylella fastidiosa*, in glassy-winged sharpshooter, *Homalodisca vitripennis*, populations in Texas. J. Insect Sci. 10: 168.

Hill, B.L., and Purcell, A.H. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathology 85: 209-212.

Holzinger, W.E., Kammerlander, I., and Nickel, H. 2003. The Auchenorrhyncha of Central Europe (Fulgoromorpha, Cicadomorpha excl. Cicadellidae). *In* H. Nickel (ed.), Brill Publishers, Leiden, The Netherlands, p. 673.

Hutchins, L.M., Cochran, L.C., Turner, W.F. and Weinberger, J.H. 1953. Transmission of phony disease virus from tops of certain affected peach and plum trees. Phytopathology 43: 691-696.

Janse, J.D., and Obradovic, A. 2010. *Xylella fastidiosa*: Its biology, diagnosis, control and risks. J. Plant Pathol. 92: 35-48.

Krczal, G., Krczal, H., and Kunze, L. 1988. *Fieberiella florii* (Stål), a vector of apple proliferation agent. Acta Hortic. 235: 99-106.

Killiny, N. and Almeida, R.P.P. 2009. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. Appl. Environ. Microbiol. 75:521-528.

Killiny, N., Martinez, R.H., Dumenyo, C.K., Cooksey, D.A. and Almeida, R.P.P. 2013. The exopolysaccharide of *Xylella fastidiosa* is essential for biofilm formation, plant virulence, and vector transmission. Mol. Plant Microbe Interact. 26:1044-1053.

Krugner, R., Sisterson, M.S., Chen, J.C., Stenger, D.C., and Johnson, M.W. 2014. Evaluation of olive as a host of *Xylella fastidiosa* and associated sharpshooter vectors. Plant Dis. 98: 1186-1193.

Lepsï, J. 1993. Taylor's power law and measuring variation in the size of populations in space and time. Oikos 68: 349-356.

Li, Y., Hao, G., Galvani, C.D., Meng, Y., De La Fuente, L., Hoch, H.C. and Burr, T.J. 2007. Type I and type II pili of *Xylella fastidiosa* affect twitching motility, biofilm formation, and cell-wall aggregation. Microbiology 153: 719-726

Li, W.B., Pria, W.D., Teixeira, C., Miranda, V.S., Ayres, A.J., Franco, C.F., Costa, M.G., He, C.X., Costa, P.I., and Hartung, J.S. 2001. Coffee leaf scorch caused by a strain of *Xylella fastidiosa* from citrus. Plant Dis. 85: 501-505.

Lopes, J.R.S, and Krugner, R. 2016. Transmission ecology and epidemiology of the citrus variegated chlorosis strain of *Xylella fastidiosa*. In: Brown, J.(ed.) Vector-mediated transmission of plant pathogens. American Phytopathological Society Press. (In press).

Marck, C. 1988. DNA Strider: a "C" programme for the fast analysis of DNA and protein sequences on the Apple Macintosh family computers. Nucleic Acids Research 16: 1829-1836.

McArdle, B.H., and Gaston, K.J. 1995. The temporal variability of densities: back to basics. Oikos 74: 165-171.

Meng, Y., Li, Y., Galvani, C.D., Hao, G., Turner, J.N., Burr, T.J. and Hoch, H.C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. J. Bacteriol. 187: 5560-5567.

Montero-Astúa M. 2008. Isolation and molecular characterization of *Xylella fastidiosa* from coffee plants in Costa Rica. J. Microbiol. 46: 482-490.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, M.V.B.C., and Stall, R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84: 456-461.

Mircetich, S.M., Lowe, S.K., Moller, W.J., and Nyland, G. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. Phytopathology 66: 17-24.

Newman, K.L., Almeida, R.P., Purcell, A.H. and Lindow, S.E. 2004. Cell-to-cell signalling controls *Xylella fastidiosa* interactions with both insects. Proceedings of the National Academy of Sciences, USA 101: 1737-1742.

Nunney, L., Ortiz, B., Russell, S.A., Sánchez, R.R. and Stouthamer, R. 2014. The complex biogeography of the plant pathogen *Xylella fastidiosa*: genetic evidence of introductions and subspecific introgression in Central America. PLoS ONE 9: e112463.

Nunney, L., Vickerman, D.B., Bromley, R.E., Russell, S.A. and Hartman, J.R. 2013. Recent evolutionary radiation and host plant specialization in the *Xylella fastidiosa* subspecies native to the United States. Appl. Environ. Microbiol. 79: 2189–2200.

Pompon, J., Quiring, D., Goyer, C., Giordanengo, P., and Pelletier, Y. 2011. A phloem-sap feeder mixes phloem and xylem sap to regulate osmotic potential. J.Insect Physiol. 57: 1317-1322.

Purcell, A.H. 1989. Homopteran transmission of xylem-inhabiting bacteria, pp. 243-266. In K.F. Harris (ed.), Advances in Disease Vector Research, vol. 6 Springer, New York, NY.

Purcell, A.H., and Finlay, A. 1979. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. Phytopathology 69: 393–395.

Raju, B.C., Wells, J.M., Nyland, G., Brlansky, R.H., and Lowe, S.K. 1982. Plum leaf scald isolation and culture and pathogenicity of the causal agent. Phytopathology 72: 1460-1466.

Rapicavoli, J.N., Kinsinger, N., Perring, T.M., Backus, E.A., Shugart, H.J., Walker, S. and Roper, M.C. 2015. O-antigen modulates insect vector acquisition of the bacterial plant pathogen, *Xylella fastidiosa*. Appl. Environm. Microbiol. 81: 8145-8154.

Redak, R.A., Purcell, A.H., Lopes, J.R.S., Blua, M.J., Mizell, R.F., and Andersen, P.C. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. 49: 243-270.

Ribaut, H. 1952. Homoptères Auchénorhynches II. (Jassidae), pp. 1-474. *In* P. Lechevalier (ed.), Faune de France, vol. 57, Paris, France.

Roper, M.C., Kirkpatrick, B., Labavitch, J. and Cosgrove, D. 2016. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the type II secretion system and their secretion machinery. CDFA Final Report 073116. https://static.cdfa.ca.gov/PiercesDisease/reports%2F2016%2FRoper%20%20CDFA%20Final%20Report%20073116.pdf

Roper, M.C., Greve, L.C., Warren, J.G., Labavitch, J.M. and Kirkpatrick, B.C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Mol. Plant. Microbe. Interac. 20, 411-9.

Sanderlin, R.S., and Heyderich-Alger, K.I. 2000. Evidence that *Xylella fastidiosa* can cause leaf scorch disease of pecan. Plant Dis. 84: 1282-1286.

Saponari, M., Boscia, D., Nigro, F., and Martelli, G.P. 2013. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). J. Plant Pathol. 95: 659-668.

Saponari, M., Loconsole, G., Cornara, D., Yokomi, R.K., De Stradis, A., Boscia, D., Bosco, D., Martelli, G.P., Krugner, R., and Porcelli, F. 2014. Infectivity and transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. J. Econ. Entomol. 107: 1316-1319.

Schaad N.W., Postnikova E., George L., Fatmi M. and Chang C. (2004). *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp. piercei, subsp. nov., *X. fastidiosa* subsp. multiplex, subsp. nov., and *X. fastidiosa* subsp. pauca, subsp. nov. Sys. App. Microbiol. 27: 290-300.

Severin, H.H.P. 1949. Transmission of the virus of Pierce's disease of grapevines by leafhoppers. Hilgardia 19: 190-206.

Severin, H.H.P. 1950. Spittle-insect vectors of Pierce's disease virus. Hilgardia 19: 357-382.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

Walker, S.L., Redman, J.A., Elimelech, M. 2004. Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport. Langmuir 20:7736-7746.

Wells, J.M., Raju, B.C., and Nyland, G. 1983. Isolation culture and pathogenicity of the bacterium causing phony disease of peach *Prunus persica*. Phytopathology 73: 859-862.

Wells, J.M., Raju, B.C., Hung, H.Y., Weisburg, W.G., Mandelco-Paul, L. and Brenner, D.J. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem-limited fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Sys. Bacteriol. 37:136-143.