Università degli studi di Milano

Sustainable integrated management of grapevine Bois noir and its associated insect vectors

By

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Matriculation No.: R12053

Ph.D. School of "Agriculture, Environment, and Bioenergy"

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Department of Agricultural and Environmental Sciences.

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Matriculation No.: R12053

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy in
Agriculture, Environment and Bio-energy

Candidate: Abdelhameed Moussa

Supervisor: Prof. Fabio Quaglino

Supervisor: Prof. Nicola Mori

The Coordinator: Prof. Daniele Bassi

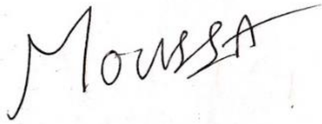
Date: November 30, 2020

Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Signature *Date. November 30, 2020*



Preface

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Milan. The research described herein was conducted under the supervision of Prof. Fabio Quaglino and Prof. Nicola Mori at the Department of Agriculture and Environmental Sciences, between October 2017 and November 2021.

Part of this work has been presented in papers published or submitted in peer-reviewed journals and proceedings, as follows:

Chapter 2

Moussa A, Mori N, Faccincani M, Pavan F, Bianco PA, Quaglino F (2019). *Vitex agnus-castus* cannot be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by '*Candidatus* Phytoplasma solani' in northern Italian vineyards: Experimental evidence. Annals of Applied Biology 175 (3), 302-312 (doi: 10.1111/aab.12542).

Chapter 3

Moussa A, Maixner M, Stephan D, Santoiemma G, Passera A, Mori N, Quaglino F (2020). Entomopathogenic nematodes and fungi acting for the control of *Hyalesthes obsoletus* (Hemiptera: Auchenorrhyncha: Cixiidae. BioControl (doi: 10.1007/s10526-020-10076-1)

Chapter 4

Quaglino F, Sanna F, **Moussa A**, Faccincani M, Passera A, Casati P, Bianco PA, Mori N (2019). Identification and ecology of alternative insect vectors of '*Candidatus* Phytoplasma solani' to grapevine. Scientific Reports 9 (1), 1-11 (doi: 10.1038/s41598-019-56076-9).

Chapter 5

Moussa A, Passera A, Sanna F, Faccincani M, Casati P, Bianco PA, Mori N, Quaglino F (2020). Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection. FEMS Microbiology Ecology 96 (11), fiaa203 (doi: 10.1093/femsec/fiaa203).

Chapter 6

Moussa A, Quaglino F, Faccincani M, Bianco PA, Mori N (2019). "Bois noir" incidence reduction by grafting recovered grapevine shoots. Phytopathogenic Mollicutes 9 (1), 181-182. [Extended abstracts of the 4th International Phytoplasmologist Group (IPWG) Meeting, Sept 08-12, Valencia, Spain].

During the PhD thesis, I was involved in other research activities concerning grapevine Bois noir and its causal agent. Results of such studies, not included in this thesis, have been presented in papers published or submitted in peer-reviewed journals, as follows:

Passera A, Zhao Y, Murolo S, Pierro R, Arsov E, Mori N, **Moussa A**, Silletti MR, Casati P, Panattoni A, Wei W, Mitrev S, Materazzi A, Luvisi A, Romanazzi G, Bianco PA, Davis RE, Quaglino F (2020) Multilocus genotyping reveals new molecular markers for differentiating distinct genetic lineages among "*Candidatus* Phytoplasma solani" strains associated with grapevine Bois noir. Pathogens 9, 970 (doi: 10.3390/pathogens9110970).

Quaglino F, Passera A, Faccincani M, **Moussa A**, Pozzebon A, Sanna F, Casati P, Bianco PA, Mori N (2020). Molecular and spatial analyses reveal new insights on Bois noir epidemiology in Franciacorta vineyards. Annals of Applied Biology (under revision).

Abstract

Bois Noir (BN) is a disease of the grapevine yellows (GY) complex associated with '*Candidatus* Phytoplasma solani' (CaPsol) strains, which causes economic crop losses in viticulture worldwide. The epidemiology of BN is very complex due to the involvement of different herbaceous plants and several insect vectors that transmit CaPsol to grapevine. Therefore, the BN containment is very difficult and require massive efforts for possible spread reduction. The heavy application of chemical insecticides was not successful to control the insect vector presence within the vineyard. The thesis work was framed considering the directives provided by the European council 2009/128/EC regarding the promotion of low use of pesticides in sustainable management approaches. In the present thesis dissertation, CaPsol insect vectors and diseased grapevines were the main targets prioritized for successful containment of BN in organically cultivated vineyards in northern Italy. Since *H. obsoletus* is the widely distributed insect vector in Europe, the management of the leafhopper population was carefully considered. The use of *Vitex agnus-castus* as trap plant for *H. obsoletus* as an indirect control strategy was evaluated. *Vitex agnus-castus* tended to be a preferred host plant for *H. obsoletus*, but transmission trials demonstrated its ability to harbor CaPsol and indicated the impossibility of using this plant to avoid BN spread. In addition, the efficacy of different entomopathogenic nematodes and fungi as direct control strategy were evaluated against *H. obsoletus* nymphs and adults. Their application in a laboratory and semi-field conditions showed a promising killing effect that can be implemented for insect vector control in open field. Due to the very low density of *H. obsoletus* population in heavily BN infected vineyards questions were raised to figure out the other possible presence of alternative insect vectors. Surveys on Auchenorrhyncha coupled with molecular analyses revealed the presence of numerous putative vectors. Some of them, selected on the basis of their abundance, CaPsol-infection rate and CaPsol strains harbored,

went through transmission trials. Eight insects were found able to transmit CaPsol to grapevines. Characterization of the bacterial microbiota associated with *H. obsoletus* and the alternative insect vectors indicated an interesting perspective regarding the microbial signatures associated with xylem- and phloem-feeding insects, and determinants that could be relevant to establish whether an insect species can be a vector or not, opening up new avenues for developing microbial resource management-based approaches. Moreover, grafting of materials collected from recovered grapevines was conducted in field trials with the aim to evaluate its preventive and curative potentials against BN. Results of symptom observation and CaPsol molecular detection on grafted and non-grafted grapevines showed that grafting of recovered shoots can have a curative effect, increasing the natural recovery. Results obtained in this PhD thesis opened new perspectives to develop integrated sustainable strategies for BN management.

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I wish to extend my profound gratitude to my supervisor Prof. Fabio QUAGLINO for providing me with the opportunity to complete my PhD under his supervision. I am highly grateful for his great tolerance, advice, help and daily presence throughout my PhD. He helped me and kept me active throughout my PhD. I really appreciate his untiring efforts in reading my manuscripts to attain a professional standard. I am so proud of the technical and scientific aspects I have learned from him that will promote my future career in academia.

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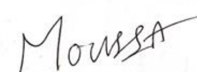
Abdelhameed Moussa



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Chapter. 1: General Introduction

1. Grapevine

The European grapevine (*Vitis vinifera* L.) is considered one of the ancient and most important cultivated crops worldwide. Grapes total area harvested worldwide in 2018 was estimated to be around 7.16 million hectares with a total production estimated to be around 79.12 million tones. Europe is the leading global grape producer, with about 50% of the world's grapevine growing area, with 3.3 million hectares (FAOSTAT, 2020). Viticulture is facing many threats worldwide that would jeopardize the total yield and area cultivated, mainly due to anthropogenic disturbances (Fraga *et al.*, 2020). Commercial cultivars are greatly affected by a large number of pests that cause serious damages pre- and/or post-harvest periods, affecting production, processing and export, along with fruit quality (Armijo *et al.*, 2016).

Unlike other living organisms, plants are restricted in their ability to travel long distances, it is critical that plant pathogens develop their own strategies that allow them to move from plant to plant. In contrast to plants, plant-feeding arthropods often fly long distances. Insects are abundant and have evolved a diverse range of plant feeding and colonization strategies that enable pathogens to adapt in various ways to utilize these herbivores as vectors for their transmission (Szklarzewicz and Michalik, 2017). The relationship of insects with plant-parasitizing bacteria seems to have arisen independently many times. Insects are the main way of transmission and spread for several infections' bacterial strains. Many other bacteria utilize insects as primary vectors and form symbiotic relationships with them (Moussa *et al.*, 2020).

Presently, rising temperatures in the climate change context are predicted to impact grapevine cultivation through modulating the risks of pests outbreaks and emergence of insect-borne diseases (Jactel, Koricheva and Castagneyrol, 2019). Grapevine is a connecting hub for complex communities and interact in trophic networks with a range of organisms both cryptic and above-ground which have adverse effects on production. The unfavorable effects could be a direct through feeding on plant tissues or indirect through vectoring of plant diseases (Reineke and Thiéry, 2016).

2. Phytoplasmas

Phytoplasmas insect- transmitted pleomorphic phloem restricted intracellular obligate bacteria are associated with several economic diseases infecting vegetable and fruit crops

(Doi *et al.*, 1967; Bertaccini and Lee, 2018) (fig.1). It was formerly called mycoplasma-like organism (MLOs). Phytoplasmas are with variable sizes from 200 to 800 nm, possess a very small genome of about 680–1600 kb where UGA is used as a stop codon instead as a tryptophan codon as in several other mycoplasmas and multiply in isotonic niches of plant phloem tissues and insect hemolymph. It is sensitive to tetracycline antibiotics, but not to penicillin – Base composition of DNA: low content of guanine and cytosine (23 – 29 mol%) (IRPCM and Spiroplasma, 2004).

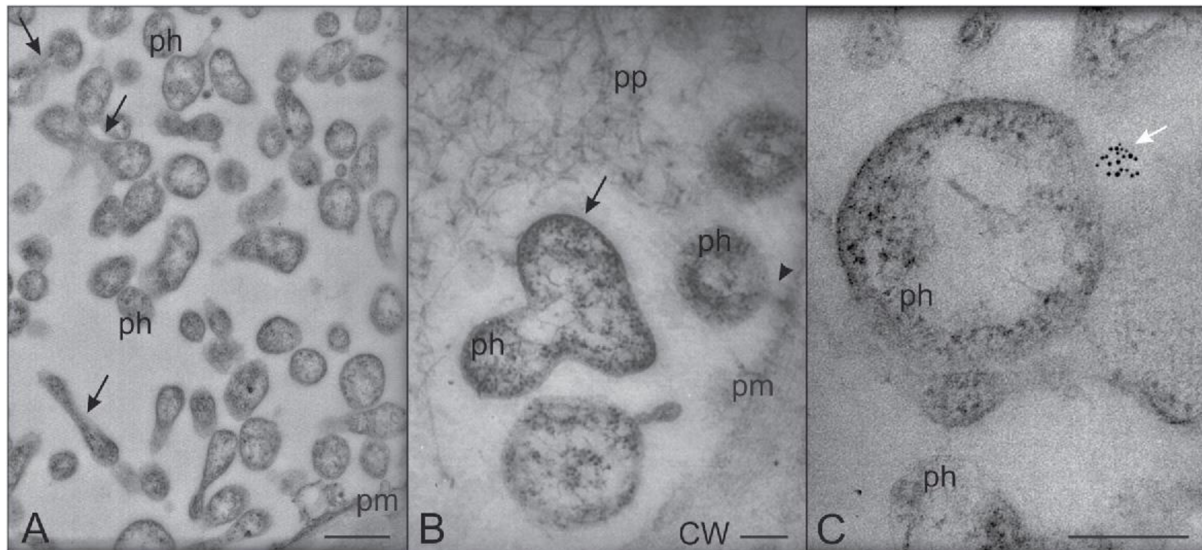

Figure 1: Transmission electron microscopy micrographs of phytoplasmas floating in the sieve element (SE) lumen. (A, B) Phytoplasmas are mostly roundish, sometimes elongated; a few are dividing (black arrows). (C) Aggregates of SE actin form unipolar fields on the phytoplasma surface in the SE lumen (white arrow). The arrowhead in (B) indicates the attachment of a phytoplasma to the SE plasma membrane. In (A), the bar corresponds to 500 nm; in (B) and (C) the bars correspond to 200 nm. CW: cell wall; ph: phytoplasma; pm: plasma membrane; pp: phloem protein.(Musetti et al., 2016)

Phytoplasmas lack several metabolic pathways for the synthesis of compounds that are very crucial for their survival and multiplication such as the genes responsible for the biosynthesis of amino acids, fatty acids, the tricarboxylic acid (TCA), and oxidative phosphorylation (ATP production) (Angelini *et al.*, 2018). Therefore, phytoplasma obtain the trophic substances from the host plants acting as obligate parasite, solely dependent on the host. The existence of phytoplasma in plant is localized exclusively in the sieve tubes where they multiply and systemically move within the host with a concentration that is often low particularly in woody hosts (Berges, Rott and Seemüller, 2000). Grapevine yellows (GY) are diseases associated to different phytoplasmas that occur in many grape growing areas all over around the world and have an increasing significance. Almost identical symptoms of the GY

syndrome are caused by different phytoplasmas and appear on leaves, shoots and clusters of grapevine.

During the last decade, massive progress has been made in detecting, identifying and classifying phytoplasmas. The use of hybridization, restriction fragment length polymorphism (RFLP) and sequence analyses of polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) resulted in the characterization and assigning of GY phytoplasmas to six different groups. These are the elm yellows (EY), stolbur (STOL), X-disease, aster yellows (AY), Australian grapevine yellows (AUSGY) and faba bean phyllody (FBP) phytoplasma groups, respectively (Bertaccini and Lee, 2018).

3. Phytoplasmas insect vectors

The Hemipterans are the main vectors of phytoplasma which is a large and diverse order of exopterygote sab sucking insects, that occur throughout the world. The order is now divided into 3 suborders: Heteroptera (true bugs), Sternorrhyncha (scale insects, aphids, whiteflies and psyllids) and Auchenorrhyncha (leafhoppers, planthoppers, cicadas, treehoppers and spittlebugs). The most successful suborders of phytoplasma vectors is the Auchenorrhyncha (Cicadellidae and Fulgoromorpha) and Sternorrhyncha (family: Psyllidae) (Fig.2) (Alma *et al.*, 2015).

Hoppers possess several characteristics that make them very efficient vectors of phytoplasmas:

A. They are hemimetabolous; thus, nymphs and adults feed similarly and are in the same physical location and often both immatures and adults can transmit phytoplasmas.
B. They feed specifically and selectively on certain plant tissues, which makes them efficient vectors of pathogens residing in those tissues.
C. They have a propagative and persistent relationship with phytoplasmas.
D. They have obligate-symbiotic prokaryotes that are passed to the offspring by transovarial transmission, the same mechanisms that allow the transovarial transmission of phytoplasmas.

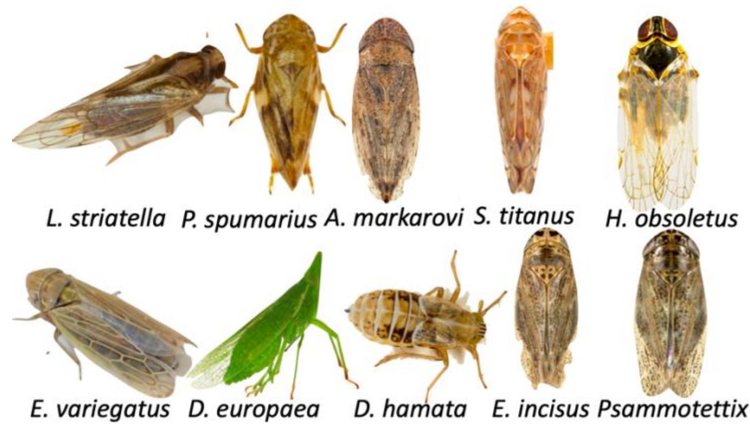

Figure 2: Hemipterans insect vectors of phytoplasma

4. Transmission patterns of Phytoplasmas

Transmission of phytoplasma include different patterns such as, transovarial transmission and seed transmission. They are efficiently spread via vegetative propagation such as cuttings, grafting, and micropropagation practices (Bertaccini and Lee, 2018). The host range of phytoplasmas is directly coupled to the feeding preference of their insect vector. The degree of specialization or plasticity of vectors with respect to their host plants drives their ability of spreading phytoplasma diseases.

4.1. Phytoplasmas Insect Transmission Process

Transmission process of phytoplasmas typically consists of three phases: Acquisition access period (AAP), Latency period (LP), and Inoculation access period (IAP) (Fig.3). Each insect species has a typical AAP, LP, IAP. Phytoplasmas are transmitted by insect vectors in a persistent-propagative manner, requiring short AAP (a few days), long LP (weeks) and medium-short IAP (Alma, Lessio and Nickel, 2019). Phytoplasmas overwinter in insect vectors or in perennial plant hosts and interact with insect hosts also reducing or enhancing their fitness (Sugio *et al.*, 2011) (Fig.4).

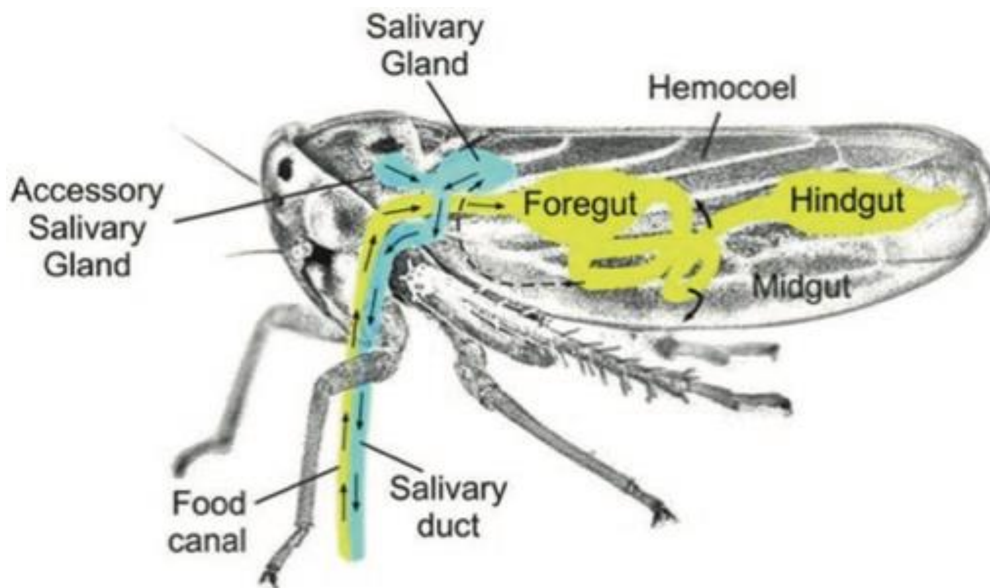

Figure 3: Pathways of phytoplasmas through the insect body (Alma, Lessio and Nickel, 2019)

a- Acquisition:

Acquisition access period (AAP) when phytoplasmas are sucked from the phloem sieve tubes by the vector's mouth parts (insects are infected). The acquisition is mainly performed by nymphs, which hatch from the egg on host plants which are already infected (Alma *et al.*, 2015). Nymphs of vectors (leafhoppers, planthoppers, and psyllids) are generally sedentary, moving from plant to plant only by walking or jumping. In some cases (e.g. Cixiidae) they are born and develop underground on the roots; therefore, acquisition may be successful only if adults are laying eggs directly on infected plants.

b- Latency:

Latency period (LP) is the period which is necessary for phytoplasma multiplication and circulation inside the insect body, including the salivary glands. The length of latency period (LP) depends on the multiplication kinetics of phytoplasmas in the vector's body. Factors influencing the LP include temperature and carbon dioxide (Galetto *et al.*, 2011) and this may result in shorter/longer LP depending on the season, with consequences on the diseases' outbreak.

c- Inoculation:

Inoculation access period (IAP), when phytoplasmas are injected into the host plant (insects are infective). IAP is generally made by adults. Nymphs cannot fly and are unable to

move from an infected to a healthy host plant; moreover, usually they become adults during LP. Inoculation may occur in different moments of the season, depending on the biology of vectors and their infective status. Early-season inoculation happens when adult vectors arriving into crop fields are already infective. Transovarial transmission allows the vector to maintain a source of inoculum throughout generations, without relying on host plants as sources. This is particularly important for phytoplasmas affecting annual crops (Rashidi *et al.*, 2014).

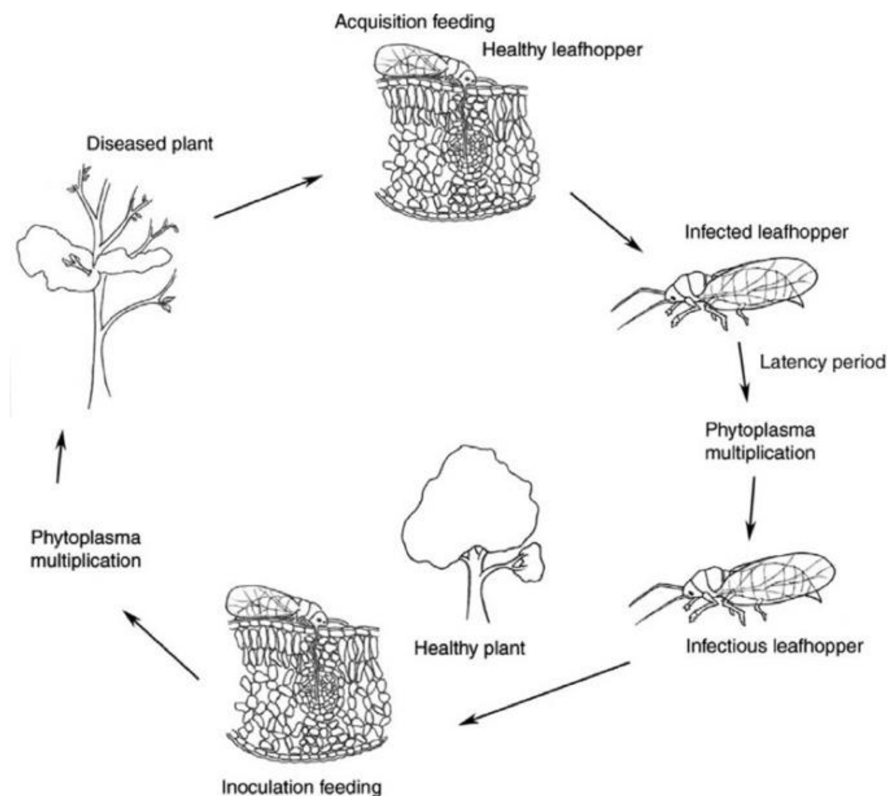

Figure 4: Pathway for phytoplasma acquisition and transmission by hemipteran vectors (Christensen et al., 2005).

5. Grapevine Yellows Complex Impact and Symptoms:

Grapevine yellows (GY) complex is an important threat to viticulture that snitching the attention worldwide and became a top priority when considering the cultivation of grapevine (Moussa *et al.*, 2019). Grapevine yellows (GY) are diseases of *Vitis vinifera*, characterized by similar symptoms, but different aetiology and epidemiology. The most economically important grapevine diseases associated with phytoplasma are Bois noir (BN), Flavescence doreé (FD) and general Grapevine yellows (GY). Symptoms of GY diseases are very similar in *Vitis vinifera,* regardless the phytoplasma present, though the agents of GY

are often different. The main symptoms associated with GY diseases are present on the leaves, bunches, and canes. The symptoms on the leaves and canes are the first ones to appear in the heavily infected plants as soon as the sprouting has started: internodes are short, and canes appear rubbery and not lignified or can show dark pustules, depending on the variety; necrosis of terminal buds may occur (Angelini *et al.*, 2018). *V. vinifera* varieties vary among each other in symptoms expression, phytoplasma concentration, and occurrence of infected plants under field and laboratory conditions. Variation in susceptibility could depend on genetic or epigenetic features of the grapevine variety/species or by the feeding behavior of the insect vectors (Eveillard *et al.*, 2016).

The manifested symptoms of phytoplasma on the infected plants. could be related to the following possible physiological changes including (Ermacora and Osler, 2019):

a- Interruption to the hormonal system with subsequent disturbance in the balance of the growth regulators since it is considered a typical "auxonic diseases.". This interruption will lead do general malformation in different plant organs (Arashida *et al.*, 2008).

b- The accumulation of organic solutes (e.g., amino acids and sugars) due to the partial or total blockage of the phloem flux (Pagliari *et al.*, 2017).

c- Phloem flux blockage eventually lead to a noticed reduction in the primary storage compounds in sink organs, such roots (Guerriero *et al.*, 2012).

6. Recovery phenomenon:

Interestingly, the natural recovery phenomena from GY disease that results in the disappearance of symptoms was reported in most of infected vineyards. In the recovered grapevines, phytoplasmas were not present when molecularly detected with the advancened molecular techniques. Therfore, the insect vectors will not be able to acquire phytoplasma for further infections. (Galetto *et al.*, 2016). The rate of recovery, however, depends on infective vector reinoculation and grapevine variety (Maggi *et al.*, 2017) and it does not appear to be contingent on the strain of phytoplasma (Constable, 2010). Although the underlying physiological and molecular mechanisms are not yet completely known, H_2O_2 accumulation, resulting in lower phytoplasma titer, has been correlated with the recovery phenomenon (Gambino *et al.*, 2013).

7. Bois Noir

7.1. History, Importance, Distribution, and associated agent

Bois Noir (BN) disease was first reported in a vineyard located in northeastern France in 1961 (Caudwell *et al.*, 1997). In the course of disease spread Germany was the first station, exactly in the vineyards of the Mosel and Rhein Valley where similar symptoms had been observed. Based on the experimental evidences, proved that BN was originally named "Vergilbungskrankheit" (VK) ; now BN and VK are considered to be the same disease (Belli, Bianco and Conti, 2010).

Bois noir (BN) is the widespread GY complex disease in the Euro-Mediterranean region, where complete yield loss and even grapevine death can be exacerbated (Belli, Bianco and Conti, 2010; Moussa *et al.*, 2019), South America (Chile) (Gajardo et al., 2009), North America (Canada) (Rott *et al.,* 2007), Asia (China, Middle East) and South Africa (Choueiri *et al.,* 2002; Duduk *et al.,* 2010; Salem *et al.,* 2013; Mirchenari *et al.,* 2015) (Fig. 5). BN is associated with Stolbur group (16SrXII) of the species '*Candidatus* Phytoplasma (*Ca*. P.) solani' (subgroup 16SrXII-A) by phytoplasma strains (Bois noir phytoplasma strains, BNp) (Quaglino *et al.*, 2013).

Figure 5: Bois Noir reports (in red) in Vitis vinifera all over the world.

7.2. Symptoms and transmission:

Generally, BN symptoms in grapevine are clearly visible in September. Symptoms are not distinguishable from other GYs, but between the different *V. vinifera* cultivars. The main symptoms manifested on grapevine include leaf and veins discoloration with leaf blade down curling, improper lignification of shoots that eventually turn black, fruit malformation due to

abortion of clusters or berry shriveling, and significant vigor reduction in the infected branches (Fig.6).

Symptoms of BN remains limited to parts of the infected veins for several years (Maixner, 2011; Quaglino *et al.*, 2016; Murolo *et al.*, 2020). There are several insect vectors transmitting '*Ca*. P. solani' in the Euro-Mediterranean area. The main insect vector that is responsible of the spread in the European vineyards is *Hyalesthes obsoletus* (Maixner, 1994). *Reptalus panzeri* Low (Homoptera: Cixiidae) and *R. quinquecostatus* have been reported as vectors of '*Ca*. P. solani' in Serbian and French vineyards (Cvrković *et al.*, 2014; Chuche *et al.*, 2016), whereas *Macrosteles quadripunctulatus* (Kirschbaum) (Homoptera: Cicadellidae) was verified to have the ability to transmit CaPsol to potted grapevine plants (Batlle *et al.*, 2008). In addition, *Anaceratagallia ribauti* (Ossiannilsson) (Homoptera: Cicadellidae) and *Reptalus quinquecostatus* (Dufour) (Homoptera: Cixiidae) were confirmed as vectors but not to grapevine (Riedle-Bauer, Sára and Regner, 2008; Chuche *et al.*, 2016).

Figure 6: General symptoms of Bois Noir manifested on two different V. vinifera varieties

Other studies reported that different Cixiidae and Cicadellidae species have been captured within or near BN-diseased vineyards and found to contain and transmit CaPsol including at least eight species (*Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Philaenus spumarius*, and *Psammotettix alienus/confinis*) (Quaglino *et al.*, 2019).

7.3. *Hyalesthes obsoletus* Signoret:

Hyalesthes obsoletus Signoret (Homoptera: Cixiidae) is considered the main insect vector in the Europe and the Mediterranean area (Maixner, 1994; Johannesen *et al.*, 2008). It is a polyphagous vector that acquires '*Ca*. P. solani' during feeding on the roots of the herbaceous plants. *H. obsoletus* is a palearctic monovoltine xerothermic insect species that completes its life cycle mainly on nettle (*Urtica dioica* L.) and bindweed (*Convolvulus arvensis* L.) (Langer and Maixner, 2004) but also on other host plants (Kosovac *et al.*, 2019; Moussa *et al.*, 2019). The herbaceous host plants significantly influence distribution and density of *H. obsoletus* as well as the levels of infestation of vector populations (Maixner, 2007). In summertime (fig.7), females lay eggs on the root collar of host plants. upon egg hatching, the five nymphal instars migrate into the soil to the roots of infected plants from which they can acquire '*Ca*. P. solani'. After a latency period, *H. obsoletus* becomes able to transmit '*Ca*. P. solani' to plants for the duration of its life. Overwintering occurs as second-third instar nymphs in the soil. In early spring, fourth and fifth instar nymphs migrate to the soil surface.

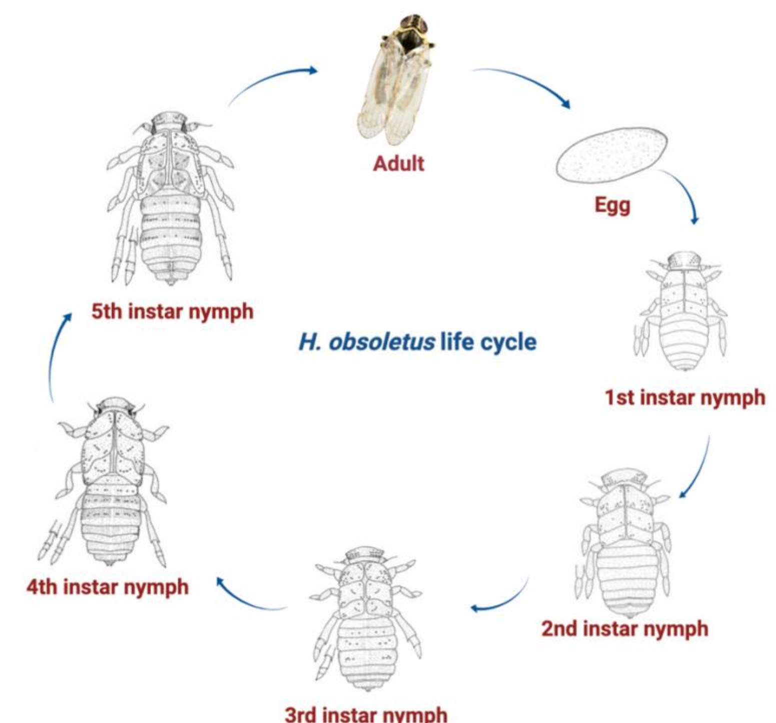

Figure 7: H. obsoletus life cycle (Cargnus et al., 2012)

Adults emerge from end of May till end of June and they fly from beginning of July to end of August, based on climate, host plant, and region (Alma *et al.*, 2015). During their flights, *H. obsoletus* adults can occasionally feed on grapevine and, if infected, transmit '*Ca*.

P. solani’. However, due to their limited feeding activity on grapevine and the short lifespan of the adult stage, they cannot transmit ‘*Ca*. P. solani’ from vine to vine (Fig.3). Grapevine is therefore a dead-end host for the pathogen (Bressan *et al.*, 2007) .

7.4. Bois Noir Molecular Epidemiology:

Based on the unique biological properties and unshared molecular markers within multiple genes (*tufB, rplV rpsC, secY*), the phytoplasma associated with BN disease has been identified as ‘*Ca.* P. solani’ (Quaglino *et al.,* 2013). The ‘*Ca*. P. solani’ strains associated with BN in Europe were assigned to the subgroup 16SrXII-A (Bertaccini *et al.,* 1995), more diversity was found lately, and subgroups 16SrXII-F, 16SrXII-G, 16SrXII-J, and 16SrXII-K were also differentiated (Quaglino *et al.,* 2009, 2017).

Moreover, sequence analysis of the *tufB* gene revealed that three BN tuf-types are present in grapevines and alternative plant hosts, according to ecological diverse pathosystems: (i) bindweed, *H. obsoletus*, grapevine *tuf*-type b, (ii) nettle – *H. obsoletus* – grapevine tuf-type a, and (iii) *Calystegia sepium* – *H. obsoletus* – grapevine *tuf*-type c *tuf*-type c (Langer and Maixner 2004). *C. arvensis* and *U. dioica* have been reported as being the main host plants of *H. obsoletus* in Germany, northern Italy, Spain, and Austria (Mori *et al.,* 2013). Recently, in Austria, Aryan *et al.,* (2014) detected a high incidence of a *tuf*-type b with a distinguished HpaII-restriction profile designed as *tuf* type b2 that appears to have different ecological features.

The biological complexity of BN disease has stimulated research on molecular markers for verification of possible genetic diversity related to pathogenicity of the strains. MLST on variable genes, such as *secY, vmp1*, and *stamp*, provided evidence of high variability among BN strains within the *tuf*-types (Foissac *et al.,* 2013; Murolo and Romanazzi 2015). For example, based on RsaI-RFLP analyses of vmp1 gene amplicons, ‘*Ca.* P. solani’ populations show 23 profiles (Pacifico *et al.,* 2009; Murolo *et al.,* 2014) (Fig. 5.4). Based on phylogenetic analysis of concatenated nucleotide sequences of the genes vmp1 and stamp for 76 ‘*Ca*. P. solani’ strains, 49 *vmp1/stamp* sequence variants were grouped into five vmp1/stamp clusters. The cluster *vmp1/ stamp-4* included BN strains (*tuf*-type a) associated with nettle-related biological cycle, while the other four clusters included BN strains (*tuf*-type b) associated with bindweed-related biological cycle (Quaglino *et al.,* 2016). Several weeds, such as *Chenopodium album* and *Malva sylvestris*, host the ‘*Ca*. P. solani’ in or around infected vineyards and can therefore play a role in BN diffusion (Marchi *et al.,* 2015; Mori *et*

al., 2015; Oliveri *et al.,* 2015). In the Euro-Mediterranean regions, the main BN insect vector is *H. obsoletus*, a polyphagous cixiid living preferentially on nettle (*Urtica dioica*), bindweed (*Convolvulus arvensis*), mugwort (*Artemisia vulgaris*), and chaste tree (*Vitex agnus-castus*) inside and/or around vineyards (Alma *et al.,* 1988; Sforza *et al.,* 1998; Langer and Maixner 2004; Sharon *et al.,* 2005). Recently, *Reptalus panzeri* and *R. quinquecostatus* have been reported as vectors of BN in Serbian and France vineyards, respectively (Cvrković *et al.,* 2014; Chuche *et al.,* 2016), and *Anaceratagallia ribauti* was reported as vector of "stolbur" phytoplasmas even if not to grapevine (Riedle-Bauer *et al.,* 2008). Molecular epidemiology approaches, using *vmp1*- and *stamp*-based markers, increased the knowledge of the population variability of BN throughout vineyards and their surroundings in the Mediterranean area (Fialová *et al.,* 2009; Fabre *et al.,* 2011; Foissac *et al.,* 2013; Murolo *et al.,* 2014; Landi *et al.,* 2015; Murolo and Romanazzi 2015). Moreover, recent studies reported the direct epidemiological role of *V. agnus-castus* in the *H. obsoletus*-mediated BN transmission to grapevine (Kosovac *et al.,* 2016) and the ability of *R. panzeri* to transmit '*Ca.* P. solani' from corn with reddening disease to grapevine (Cvrković *et al.,* 2014). The complexity of BN disease epidemiology renders it difficult to design efficient control strategies. Insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus* (Maixner 2007; Mori *et al.,* 2008).

7.5. Containment strategies:

The containment of phytoplasma diseasesd is mainly based on the control of the insect vectors, the use of phytoplasma resistant varieties, elimination of infected plant, application of tetracycline antibiotics on infected plants, cultivation of healthy rootstocks. Phytoplasmas insect vectors control is currently prioritized for limiting outbreaks of phytoplasma diseases which relies heavily on the use of insecticides (Firrao, Conci and Locci, 2007). The complexity of BN disease epidemiology renders it difficult to design efficient control strategies particularly after the confirmation of other insect vectors including *Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Philaenus spumarius*, and *Psammotettix alienus/confini* (Quaglino *et al.*, 2019).

Application of insecticides on the grapevine canopy have no influence neither on the disease nor on the insect vectors, as demonstrated for *H. obsoletus* (Mori *et al.*, 2008; Maixner, Weintraub and Jones, 2010). The management of insect vectors host plants in the

vineyards and surrounding areas should be considered with utmost attention for BN control (Quaglino *et al.*, 2019). Thus, preventive measures, such as the use of phytoplasma-free propagation materials (i.e., mother plants and grafted cuttings) and treating of cuttings through thermotherapy, are applied to limit long-distance dissemination and in-field spread of the disease (Mannini, 2007). Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed (Bertin *et al.*, 2010) (ii) trunk cutting above the graft union on symptomatic grapevines (Kast, Stark-Urnau and Bleyer, 2007; Riedle-Bauer *et al.*, 2010); and (iii) treatments with resistance inducers (Romanazzi, D'Ascenzo and Murolo, 2009; Romanazzi, Murolo and Feliziani, 2013). Moreover, volatiles from host plants and resistance inducers can be used for reducing vineyard colonisation by *H. obsoletus* (Riolo *et al.*, 2017; Minuz *et al.*, 2020).

8. Aim of this thesis

The major aim of this PhD thesis was to investigate the possible containment strategies of Bois Noir disease in organically cultivated vineyards. The investigations targeted the two main pillars of Bois noir disease, *Candidatus* Phytoplasma solani and the associated insect vector(s). The direct action on the phytoplasma was done through the use of grafting of Bois noir recovered grapevine shoots as a possible preventive and curative measures against disease onset. Whereas the actions that were taken against insect vectors were as the following:

1- Checking the possibility of using *Vitex agnus-castus* as a trap plant for *Hyalesthes obsoletus* and its ability to harbour CaPsol.
2- Study the efficacy of different entomopathogenic nematodes and entomopathogenic fungi against nymphs and adults of *H. obsoletus* develop effective and innovative approaches to control the main vector of CaPsol.
3- Studies on the Auchenorrhyncha community present in the vineyard for possible identification and detection of putative insect vectors of '*Ca*.P. solani'.
4- Description of the bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection for possible development of microbial resources management (MRM).

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CHAPTER. 2: *Vitex agnus-castus* cannot be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by '*Candidatus* Phytoplasma solani' in northern Italian vineyards: experimental evidence

1- Summary

Bois noir (BN), the most prevalent disease of the grapevine yellows complex, causes considerable yield loss in vineyards. BN is associated with phytoplasma strains of the species '*Candidatus* Phytoplasma solani' (taxonomic subgroup 16SrXII-A). In Europe, the BN phytoplasma is transmitted to grapevine mainly by *Hyalesthes obsoletus*, a polyphagous cixiid completing its life cycle on stinging nettle and field bindweed. As a result of the complexity of BN epidemiology, no effective control strategies have been developed. In previous studies conducted in the eastern Mediterranean coast of Israel, chaste tree (*Vitex agnus-castus*) was found to be the preferred host plant of *H. obsoletus* but did not harbor BN phytoplasma. Thus, a 'push and pull' strategy was suggested based on the fact that chaste tree plants located at vineyard borders was an effective trap plant for *H. obsoletus* adults. However, in other studies carried out in the eastern Adriatic coast of Montenegro, chaste tree was found to be a key source plant for BN phytoplasma transmission to grapevine. This study aimed to investigate (i) the interaction between chaste tree and *H. obsoletus* through survival, attractiveness and oviposition experiments conducted comparing the behavior of *H. obsoletus* in chaste tree versus stinging nettle and grapevine and (ii) the capability of chaste tree to harbor '*Ca*. P. solani' in northern Italy through transmission trials. *H. obsoletus* adults were found to survive on chaste tree and grapevine over a 1week period and prefer chaste tree to grapevine. Moreover, *H. obsoletus* produced eggs and overwintered as nymphs on chaste tree, even if at a lesser extent than on stinging nettle. *H. obsoletus* originating from nettle was found able to transmit '*Ca.* P. solani' to chaste tree (2 plants of 16 were found infected by the BN phytoplasma strain St5 identified in *H. obsoletus* specimens). These results increased our knowledge about the role of *Vitex agnus-castus* as host plant of *H. obsoletus* and BN phytoplasma in northern Italy and do not recommend considering chaste tree as trap plant at vineyard borders.

2- Introduction

Europe is the world leader in grape production with almost half of the global vine production area. Italy is the second top producer of grapes after China with about 8.2 million tons (FAO, 2016). Quality and quantity of grape production are damaged by a broad range of pathogens associated with diseases affecting the main cultivated grapevine varieties (Bellée, Cluzet, Dufour, Mérrillon, & Corio-Costet, 2018). Among these diseases, the grapevine yellows (GY) complex is one of the most important threats to viticulture in many countries

(Magarey, 2017). The GY putative causal agents are phytoplasmas (‘*Candidatus* Phytoplasma’), obligate parasitic phloem-limited bacteria lacking cell walls that are transmitted by insect vectors to plants (Angelini et al., 2018). Interestingly, even if undistinguishable based on symptoms, the main diseases within the GY complex are associated with genetically distinct phytoplasmas, belonging to at least six ‘*Ca*. Phytoplasma’ species, characterized by different biological features that reflect on disease epidemiological patterns (Angelini et al., 2018; Belli, Bianco, & Conti, 2010). Bois noir (BN) is the most widespread disease of the GY complex in the Euro-Mediterranean area, where it may lead to a total yield loss and even grapevine death (Belli et al., 2010; Pavan, Mori, Bressan, & Mutton, 2012). BN is associated with grapevine infection by phytoplasma strains (Bois noir phytoplasma strains, BNp) of the species ‘*Candidatus* Phytoplasma (*Ca*. P.) solani’ (subgroup 16SrXII-A) (Quaglino et al., 2013). In the Euro-Mediterranean regions, the main ‘*Ca.* P. solani’ insect vector is *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) (Johannesen et al., 2008; Bressan et al., 2007; Maixner, 1994; Sforza, Clair, Daire, Larrue, & Boudon-Padieu, 1988), a polyphagous planthopper living preferentially on stinging nettle (*Urtica dioica* L.), field bindweed (*Convolvulus arvensis* L.), stinking hawk's-beard (Crepis foetida L.) and *Artemisia spp*. in and/or around vineyards (Alma, Arnò, Arzone, & Vidano, 1988; Cargnus, Pavan, Mori, & Martini, 2012; Kosovac et al., 2013; Langer & Maixner, 2004; Mori et al., 2008b, 2013; Sforza et al., 1988; Weber & Maixner, 1998). Recently, *Reptalus panzeri* Low (Homoptera: Cixiidae) has been reported as vector of ‘*Ca*. P. solani’ (CaPsol) in Serbian vineyards (Cvrkovic, Jovic, Mitrovic, Krstic, & Toševski, 2014), whereas *Macrosteles quadripunctulatus* (Kirschbaum) (Homoptera: Cicadellidae) was found able to transmit CaPsol to potted grapevine plants (Batlle, Altabella, Sabaté, & Laviña, 2008). In addition, *Anaceratagallia ribauti* (Ossiannilsson) (Homoptera: Cicadellidae) and *Reptalus quinquecostatus* (Dufour) (Homoptera: Cixiidae) were reported as vectors but not to grapevine (Chuche, Danet, Salar, Foissac, & Thiéry, 2016; Riedle-Bauer, Sára, & Regner, 2008). Other studies reported that different Cixiidae and Cicadellidae species have been captured within or near BN-diseased vineyards and found to contain CaPsol (Oliveri et al., 2015; Šafářová, Lauterer, Star ý, Válová, & Navrátil, 2018) but are not currently considered involved in CaPsol transmission to grapevine. Sequence analysis of *tufB* gene revealed that two main ‘*Ca*. P. solani’ tuf-types are present on grapevines and alternative plant hosts, according to diverse ecological patho-systems: (i) field bindweed—*H. obsoletus*—grapevine *tuf*-type b and (ii) stinging nettle—*H. obsoletus* grapevine tuf-type a (Langer & Maixner, 2004). Recently, in Austria, Aryan, Brader, Mörtel, Pastar, and Riedle-Bauer (2014) detected

a dominant presence of a *tuf*-type b with a distinguished HpaII-restriction profile designed as *tuf*-type b2 that appears to have different ecological features. Interestingly, recent evidence highlighted the existence of new BN epidemiological cycles of '*Ca*. P. solani' *tuf*-type b. Such strain, sourced by *C. foetida* in the Balkan region and *Vitex agnus-castus* L. in the eastern Adriatic coast of Montenegro, is transmitted to grapevine by *H. obsoletus* population associated with these plants (Kosovac et al., 2016, 2019). Moreover, several weeds, such as *Chenopodium album* L. and *Malva sylvestris* L., host the '*Ca*. P. solani' in or around infected vineyards and can therefore play a role in BN spread (Marchi et al., 2015; Mori et al., 2015; Oliveri et al., 2015). Molecular epidemiology approaches, using *vmp*1- and *stamp*-based markers, have enhanced our knowledge to be increased of the populations of BN throughout vineyards and their surroundings in the Mediterranean area (Fabre, Danet, & Foissac, 2011; Fialová et al., 2009; Foissac et al., 2013; Landi et al., 2015; Murolo, Mancini, & Romanazzi, 2014; Murolo & Romanazzi, 2015; Pierro et al., 2018b; Pierro et al., 2018a). The complexity of BN disease epidemiology renders it difficult to design efficient control strategies. Insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus* (Maixner, 2010; Mori, Pavan, Bondavalli, et al., 2008b). The management of *H. obsoletus* host plants in the vineyards and surrounding areas is therefore considered crucial for BN control (Maixner, 2010; Mori et al., 2012; Panassiti, Hartig, Fahrentrapp, Breuer, & Biedermann, 2017). Thus, preventive measures, such as checking the health status of propagation materials (i.e., mother plants and grafted cuttings) and treating of cuttings through thermotherapy, are applied to limit long-distance dissemination and in-field spread of the disease (Mannini, 2007). Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed (Picciau et al., 2010); (ii) trunk cutting above the graft union on symptomatic grapevines (Kast, Stark-Urnau, & Bleyer, 2008; Riedle-Bauer, Hanak, Regner, & Tiefenbrunner, 2010); and (iii) treatments by resistance inducers (Romanazzi, D'Ascenzo, & Murolo, 2009; Romanazzi, Murolo, & Feliziani, 2013). Volatiles from host plants can be used for reducing vineyard colonization by H. obsoletus (Riolo, Minuz, Peri, & Isidoro, 2017). In Israel, chaste tree (*Vitex agnus-castus* L.) is a plant where *H. obsoletus* can complete its life cycle (Sharon et al., 2005). In both olfactometric and field studies, chaste tree was more attractive than grapevine for *H. obsoletus* adults (Riolo et al., 2012; Sharon et al., 2005; Zahavi, Peles, Harari, Soroker, & Sharon, 2007). Therefore, a 'push and pull' strategy based on the use of chaste tree as a trap plant at vineyard borders to reduce the vector population living inside the vineyards was suggested (Zahavi et al., 2007). The rationale for this strategy was reinforced

by the fact that in Israel, chaste tree was never found infected by '*Ca*. P. solani' and thus cannot serve as an inoculation source for grapevine (Sharon et al., 2015). This study aimed to investigate the role of *V. agnus-castus* as a host plant of *H. obsoletus* and CaPsol in northern Italy to evaluate its possible use as trap plant at vineyard borders. The interaction between chaste tree and *H. obsoletus* was examined through survival, attractiveness and oviposition trials, whereas the capability of chaste tree to harbor CaPsol was studied through transmission trials in controlled conditions.

3- MATERIALS AND METHODS

3.1. Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine.

Hyalesthes obsoletus adults were collected by using a sweep net and pooter in the Veneto region on July 4, 2016, and June 27, 2017, from stinging nettle plants, growing along a ditch bordering a BN infected vineyard (45°23´32.42´´N; 11°09´45.62´´E) and were maintained for 10 days under controlled conditions [25 ± 3° C, 70 ± 5% RH, 16:8 (L: D) daily light cycle] in insect-proof cages on potted plants of chaste tree, stinging nettle and grapevine. These potted plants were prepared employing chaste tree plants generated by tissue culture in Guagno nursery (Padova, Italy), stinging nettle plants transplanted from field, and 1-year grapevine (Chardonnay) plantlets grafted on SO4 rootstock in Vivai Cooperativi Rauscedo (Pordenone, Italy). The plants, grown in 5 L pots, were in good vegetative conditions and did not show symptoms of biotic and abiotic stresses. The three plant species had similar volume and leaf density (diameter about 0.3 m and height approximately 0.8 m). Both years, the *H. obsoletus* individuals, collected from stinging nettle, were randomly confined on eight singularly caged potted plants for each of the three host species. In 2016, an average number of 28.5, 14.1 and 25.1 adults were placed on chaste tree, stinging nettle and grapevine respectively; in 2017, an average number of 14.6, 15.3 and 17.6 adults were placed on chaste tree, stinging nettle and grapevine respectively. Species identity was confirmed based on the taxonomic keys by Bertin, Picciau, Acs, Alma, and Bosco (2010). During the 10-day confinement, the number of dead individuals was daily counted. On the last sampling day, the number of alive individuals was also counted, to calculate the total number in each cage. Kaplan–Meier analysis was used to estimate the survival curve on the three plants, and the comparison between two survival curves was made by the log-rank test (XLSTAT statistical software).

3.2. Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

The attractiveness of chaste tree for *H. obsoletus* collected on stinging nettle was evaluated under laboratory, semi-field and field conditions. In the laboratory, the experiment was conducted in 2017 using *H. obsoletus* adults captured on stinging nettle. Before their use in the experiment, the adults were left on Petri dishes with water for 12 hr. The planthoppers then underwent a choice test using a custom made two-choice olfactometer [following Dicke, Sabelis, & De Jong, 1988] between shoots of chaste tree and grapevine (cv. Chardonnay), chaste tree and stinging nettle, stinging nettle and grapevine. The shoots were in good condition and did not show any symptoms of biotic and abiotic stresses. Forty individuals (20 females and 20 males) were tested for each comparison. If 10 min after positioning the insect was still at the start on the olfactometer, the test was considered as 'No choice'.

Data analysis was performed on the individuals that chose one of the two plants under comparison. To establish if the proportion of males and females that were attracted by one of the two plants was different, a Fisher's exact test was used (GraphPad Instat 3.0 program). To test if one plant was preferred by adults more than the other in comparison, a G-test of goodness of fit (IBM SPSS Statistics 20) was used. Because the percentages of males and females that chose one of the two plants under comparison always differed by no more than 7%, this last analysis was conducted pooling together the adults of the two sexes. In 2016 and 2017, nine cages (0.5 m × 0.5 m × 1.0 m) containing potted plants of two species, namely chaste tree and grapevine (n. three cages) or chaste tree and nettle (n. three cages) or nettle and grapevine (n. three) were prepared. The origin and the vegetative status of the plants were the same of those used in the survival experiment. The plants of the two species under comparison inside each cage were pruned to similar volume and leaf density (diameter of about 0.3 m and height of about 0.5 m). In each cage, 20 *H. obsoletus* adults (10 females and 10 males) (captured on stinging nettle) were confined. Cages were maintained under controlled conditions [25 ± 3°C, 70 ± 5% RH, 16:8 (L:D) daily light cycle]. Observation of adult insect's position was performed 1, 4 and 8 hr. after caging. If the insect was on the net or on the bottom of the cage, the position was considered 'No choice'. Data analysis was performed on the individuals that chose one of the two plants under comparison using a paired-sample t test (GraphPad Instat 3.0 program). The field trial was conducted in 2017 in a ~3.7 ha ploughed field (45°23´ 34.92´´N; 11°09´ 39.10´´ E) with one side (103 m long)

bordered by a ditch covered with stinging nettle harboring large *H. obsoletus* populations. At the time of the adults' flight period, the stinging nettle along the ditch was mowed. Potted chaste tree, grapevine and nettle plants were placed in the field at 5, 10 and 20 m from the border on the day of mowing. For each distance, six groups of the three plants were considered, one for each of the three species. The distance between each plant group was 15 m and 1.0 m between each plant within the group. All potted plants of the three species under comparison were pruned to similar volume and leaf density (diameter of about 0.4 m and height of about 0.9 m) and irrigated twice a week. The plants were in good vegetative condition and did not show any symptoms of biotic and abiotic stress. The presence of *H. obsoletus* adults on the three potted-plant species was monitored after nettle mowing by handmade transparent sticky traps (148 mm × 210 mm) positioned within their canopy. The number of individuals captured during the first and second week was counted. To compare field-trial data (number of *H. obsoletus* adults captured), a three-way ANOVA was used, considering sampling time (first and second week from stinging nettle mowing), host plant (stinging nettle, grapevine and chaste tree) and distance from *H. obsoletus* source (5 m, 10 m and 20 m). Before analysis, data normality was tested with the Shapiro–Wilk test, homogeneity was tested with Levene's variance test, the presence of outliers was assessed, and the data were log(x+1) transformed. For post hoc comparisons of means, LSD 5% (least significant difference between two means at the 5% level) was used (XLSTAT statistical software).

3.3. Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Insects proof cages (0.5 m × 0.5 m × 1.0 m) were arranged on potted plants of chaste tree (grown from tissue culture Guagno nurseries Padova) and transplanted stinging nettle. Four and eight potted plants for each species were considered in 2016 and 2017 respectively. The pots had 50 L of capacity, and the holes at the bottom were closed with insect-proof net to allow water flow but prevent the hatched nymphs escaping. Plants were trimmed to approximately 0.4 m in diameter and about 0.9 m in height. In each cage, 100 H. obsoletus adults (50 females and 50 males), collected on stinging nettle on July 21, 2016, and July 14, 2017, were confined with the plants. The cages were maintained in an open field during winter. In February 2017 and 2018, *H. obsoletus* nymphs were extracted from the soil by Berlese funnel and identified and counted under stereomicroscope. Nymphs were identified

using the dichotomous keys of Cargnus et al. (2012). Data collected in the 2 years were analyzed together using a paired-sample t test (GraphPad Instat 3.0 program).

3.4. Transmission trials of BN phytoplasmas to chaste tree

In 2017, adults of *H. obsoletus* were collected on stinging nettle in a ditch bordering two BN-affected vineyards in Lombardy (Brescia province: 45°35´37.72´´ N; 10° 09´33.36´´E) and Veneto (Verona province: 45° 23´32.42´´N; 11°09´45.62´´E) regions. Capturing of adults was carried out by using a sweep net and pooter. The captured insects were kept in jars for transport to the laboratory. The transmission trials were conducted with 24 chaste tree plants, that tested PCR negative for '*Ca*. P. solani' in a greenhouse under controlled conditions (25 ± 3° C, 70 ± 5% RH) located in Verona province (45° 20´ 13.72´´ N; 11°13´ 03.28´´ E). The plants were singularly caged and divided into three groups: (i) plants TBS1-TBS8, with confined *H. obsoletus* individuals collected in Brescia (30 adults per plant), (ii) plants TVR1-TVR8, with confined *H. obsoletus* individuals collected in Verona (30 adults per plant) and (iii) plants T1–T8, without insects (control plants). Transmission trials were conducted at the end of adult survival. The plants were kept in an insect-free greenhouse during and after the transmission period.

Dead insects (136 in plants TBS1-TBS8; 146 in plants TVR1-TVR8), collected from the end of June till mid-July 2017, were stored in absolute ethanol at 4° C. The presence of '*Ca*. P. solani' was tested by nested PCR-based amplification of the stamp gene (Fabre et al., 2011) using the total nucleic acids (TNAs) extracted from both the individual insect specimens (Marzachì et al., 2008) and the leaves of chaste tree plants (Angelini et al., 2018), collected in October 2017 and 2018, placed in plastic bags (Bioreba, France) and stored at −30° C.

Briefly, TNAs were extracted from ethanol preserved insects (dried by filter paper) through homogenization in a CTAB-based buffer [2% w/v cetyltrimethyl-ammonium-bromide (CTAB); 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HCl pH 8.0; 0.5% ascorbic acid]. After incubation at 60° C for 20 min, TNAs were separated with one volume of chloroform: isoamyl alcohol 24:1 v/v solution and precipitated with the addition of one volume of cold isopropanol. The TNAs pellet was then washed with 70% ethanol, air dried, dissolved in 30 µ L TE pH 8.0 and maintained at −20° C until use. TNAs were extracted from 1 g of each plant sample through homogenization by a mechanical pestle in a pre-warmed CTAB-based buffer (2.5% w/v CTAB; 100 mM Tris pH 8.0; 1.4 M NaCl; 50 mM EDTA pH

8; 1% PVP-40; 0.5% ascorbic acid). After incubation at 60° C for 30 min, TNAs were extracted with one volume of chloroform: isoamyl alcohol (24:1) and precipitated with one volume of isopropanol. Pellets were washed with 70% ethanol, air-dried, suspended in 400 μ L of TE pH 8.0 buffer. TNAs were re-precipitated with two volumes of absolute ethanol and 10% volume of sodium acetate 3 M pH 5.2. Pellets were washed with ethanol 70% and 80%, air-dried, suspended in 100 μ L of TE pH 8.0 buffer and maintained at −30° C until use.

Nested PCR-based amplification of the stamp gene was conducted using the primer pair *Stamp-F* (5´-GTAGGTTTTGGATGTTTTAAG-3´)/ *Stamp-R0* (5´-AAATAAAAGAACAAGTA-TAGACGA-3´) followed by the primer pair *Stamp-F1* (5´-TTCTTTAAACACACCAAGAC-3´)/ *Stamp-R1* (5´-AAGCCAGAATTTAATCTAGC-3´) (Fabre et al., 2011). PCR reactions were conducted in the thermocycler Applied Biosystems 2720 (Applied Biosystems, Monza, Milan) with the following conditions: 4 min at 94° C; 35 cycles consisting in 30 s at 94° C, 30 s at 56° C (direct PCR) or 52° C (nested PCR) and 1 min 30 s at 72° C; 7 min at 72° C. Reactions were performed in 25 μ L volume containing 50 μ M of each dNTP, 0.4 μ M of each primer, 1.5 mM MgCl2 , 1× polymerase buffer, 1 unit GoTaq polymerase enzyme (Promega, Milan, Italy).

PCR products were analyzed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator. PCR products (Stamp-F1/Stamp-R1), amplified from insect adults and chaste tree samples, were sequenced in both strands (Sanger method, 5× coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pair in the software BioEdit, version 7.2.6 (Hall, 1999). Obtained stamp gene nucleotide sequences were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analysed by Sequence Identity Matrix to estimate genetic diversity. Stamp sequence variants, identified in the study, were aligned and compared with representative sequences of previously defined sequence variants (Pierro, Passera, Panattoni, Casati, et al., 2018a; Pierro, Passera, Panattoni, Rizzo, et al., 2018b); a nucleotide sequence identity of 100% was necessary for the attribution to such sequence variants.

4. Results

4.1. Survival of *Hyalesthes obsoletus* from stinging nettle on chaste tree and grapevine

Adults of *H. obsoletus* collected from stinging nettle can survive on chaste tree and grapevine for some days, but the survival curves were lower than those on stinging nettle with statistical differences in both years (2016: grapevine vs stinging nettle, $X^2 = 251.4$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 104.6$, $p < 0.0001$; 2017: grapevine vs stinging nettle, $X^2 = 151.2$, $p > 0.0001$; chaste tree vs stinging nettle, $X^2 = 66.6$, $p < 0.0001$) (Figure 8). Survival on chaste tree and grapevine fell below 50% after 3 days in 2016 and after 5 days in 2017. In 2016, the survival curve on chaste tree was significantly better than on grapevine (X^2= 4.87, p = 0.016), but in 2017, there was no significant difference (X^2 =1.47, p = 0.16).

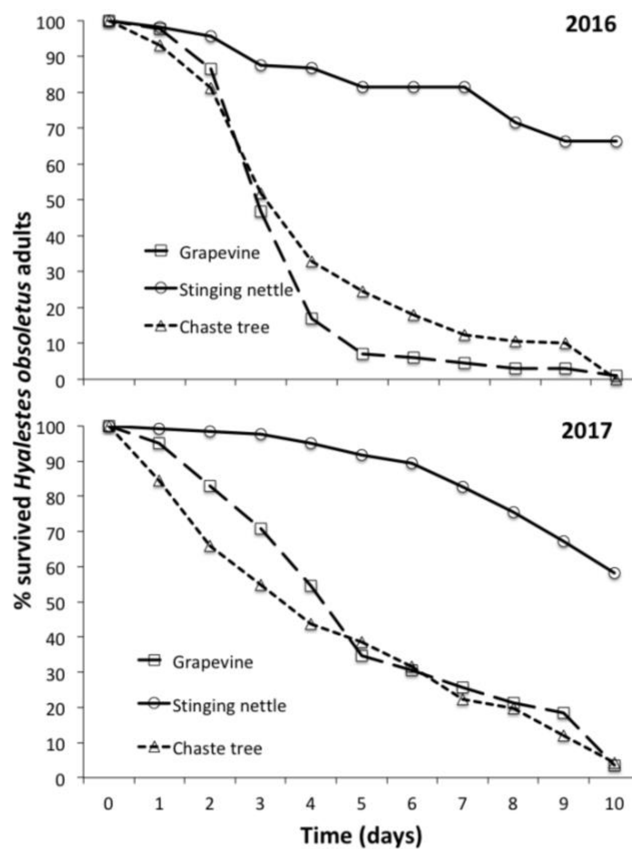

Figure 8: Survival of H. obsoletus from stinging nettle recorded in 2016 and 2017 on three different plant species

4.2. Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle

In the laboratory experiment, the proportion of males and females that chose one of the two plants under comparison with 'no-choice' individuals were not significantly different ($p = 0.10$ for grapevine vs chaste tree, $p = 1$ for chaste tree vs stinging nettle, $p = 1$ for grapevine vs stinging nettle, Fisher's Exact Test). *Hyalesthes obsoletus* adults did not show any significant preference for grapevine vs chaste tree ($G = 0.081$, $p = 0.78$), chaste tree vs stinging nettle ($G = 0.081$, $p = 0.78$) or grapevine vs stinging nettle ($G = 2.19$, $p = 0.14$) (Figure 9).

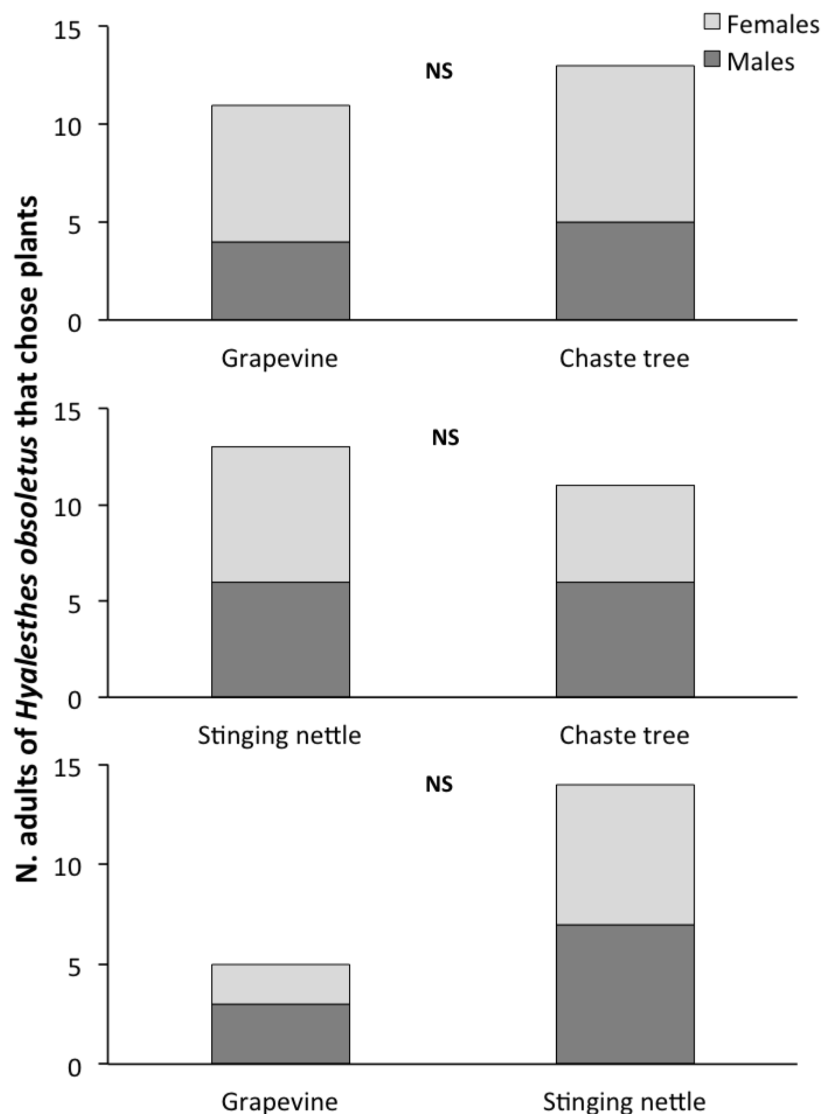

Figure 9: Number of H. obsoletus individuals (males and females) out of 40 that moved towards the two plants in comparison in two-choice olfactometer tests. NS indicates not significant differences (α = 0.05) at G-test of goodness of fit

In the semi-field experiment, there were significant differences in the choice of plant species by *H. obsoletus* adults collected on stinging nettle (Figure 10). In particular, chaste tree was significantly preferred to grapevine in both 2016 (t = 2.80, df = 8, p = 0.02) and 2017 (t = 2.80, df = 8, p = 0.02); stinging nettle was significantly preferred to grapevine in both 2016 (t = 3.39, df = 8, p = 0.0095) and 2017 (t = 5.58, df = 8, p = 0.0005); and stinging nettle was significantly preferred to chaste tree in 2017 (t = 2.44, df = 8, p = 0.04), but not in 2016 (t = 1.42, df = 8, p = 0.19).

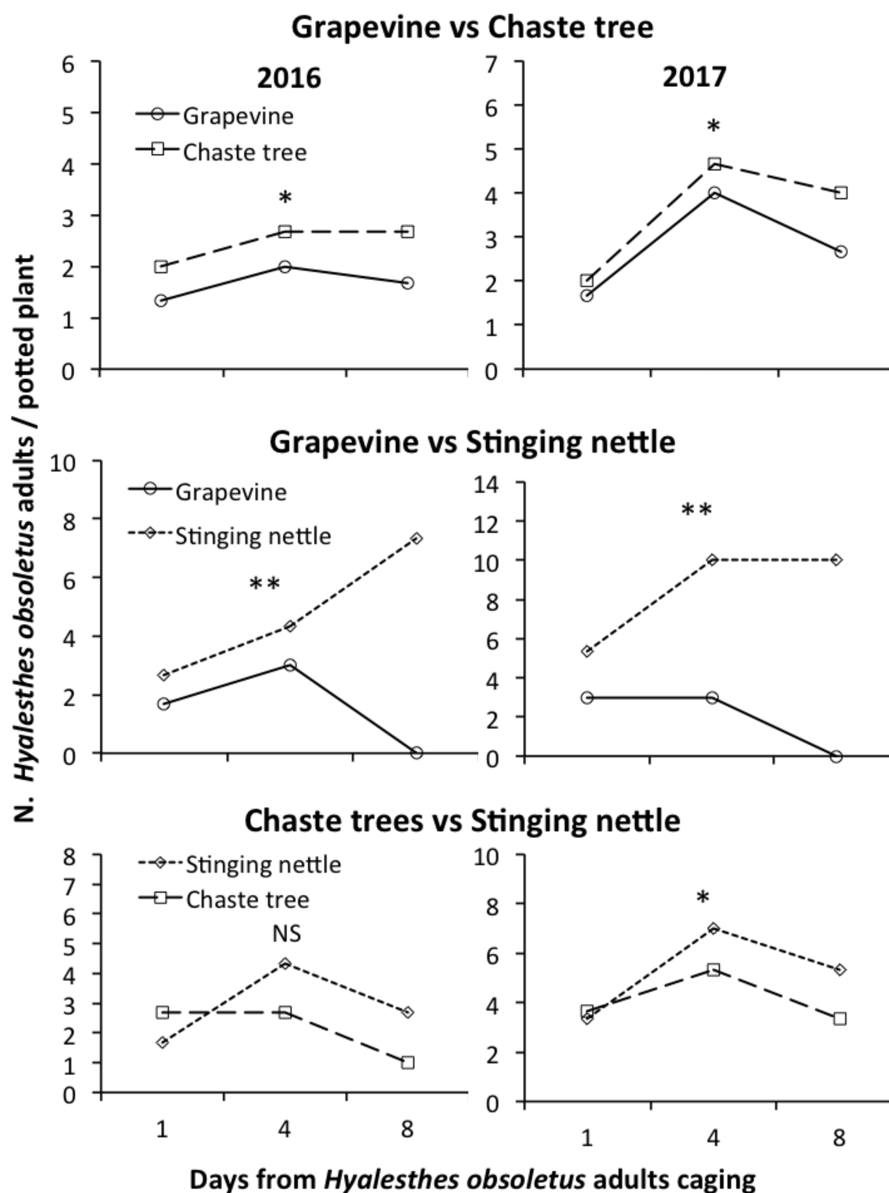

*Figure 10: Number of H. obsoletus individuals out of 60 choosing the different plants in two-choice test with potted plants. NS, * and ** indicate, respectively, not significant and significant differences according to a paired-sample t test (α = 0.05 and 0.01)*

In the open field, captures of *H. obsoletus* from stinging nettle plants along a ditch were significantly influenced by time (i.e., days from nettle mowing), plants and distance from *H. obsoletus* adults' source (Table 1).

Table 1: Results of ANOVA on the captures of *Hyalesthes obsoletus* recorded in the field on three potted plants (i.e., grapevine, chaste tree and stinging nettle) at two different times from sting-nettle mowing (7 and 14 days) and at three different distances from the different distances from the ditch source of the *H. obsoletus* adults (5 m, 10 m, 20 m).

Source of variation	**F**	**df**	**P**
Time	19.88	1, 90	< 0.0001
Plant	59.50	2, 90	< 0.0001
Distance	50.04	2, 90	< 0.0001
Time × plant	9.11	2, 90	< 0.0001
Time × distance	2.28	4, 90	0.10
Plant × distance	19.00	4, 90	< 0.0001
Time × plant × distance	3.55	4, 90	0.010

In particular, captures were higher the second than the first week from nettle mowing. On stinging nettle, the captures were significantly higher than on the other two plants (Table 2). Although no individuals were captured on grapevine, the differences with respect to chaste tree were not statistically significant (Table 2).

Table 2: Attractiveness of chaste tree and grapevine for *Hyalesthes obsoletus* from stinging nettle: average capture recorded on the three plants and at the three different distances from the ditch where stinging nettle was mowed. SED, standard error of the differences between two means; LSD 5%, least significant difference between two means at p = 0.05; df, degrees of freedom associated with LSDs and SEDs

Plant	Mean	Mean [log (x +1)]	Distance	Mean	Mean [log (x +1)]
Stinging nettle	5.72	(0.50)	5 m	5.64	(0.48)
Chaste tree	1.00	(0.13)	10 m	0.94	(0.12)
Grapevine	0.00	(0.00)	20 m	0.14	(0.03)
Stinging nettle vs chaste tree			5 m vs 10 m		
SED		(0.15)	SED		(0.15)
LSD 5%		(0.30)	LSD 5%		(0.31)
df		34	df		34
Chaste tree vs grapevine			10 m vs 20 m		
SED		(0.07)	SED		(0.08)

LSD 5%		(0.15)	LSD 5%		(0.16)
df		34	df		34

The captures decreased with distance from the ditch, that is, from the source of *H. obsoletus* adults, and were significantly higher at 5 m than both 10 m and 20 m (Table 2). The interactions time × plant, plant × distance and time × plant × distance was significant as the captures were influenced by time and distance only for stinging nettle and chaste tree. The captures on grapevines were always zero (Table 1).

4.3. Egg laying of *Hyalesthes obsoletus* from stinging nettle on chaste tree

Based on the nymphs observed in February of the next year, *H. obsoletus* females laid eggs on potted plants in 10 of 12 cages. Nymphs were recorded on the roots of both stinging nettle and chaste tree, showing indirectly that females had laid eggs on both plants, but a significantly higher number was observed on the former ($t = 3.36$, $df = 9$; $p = 0.009$) (Figure 4).

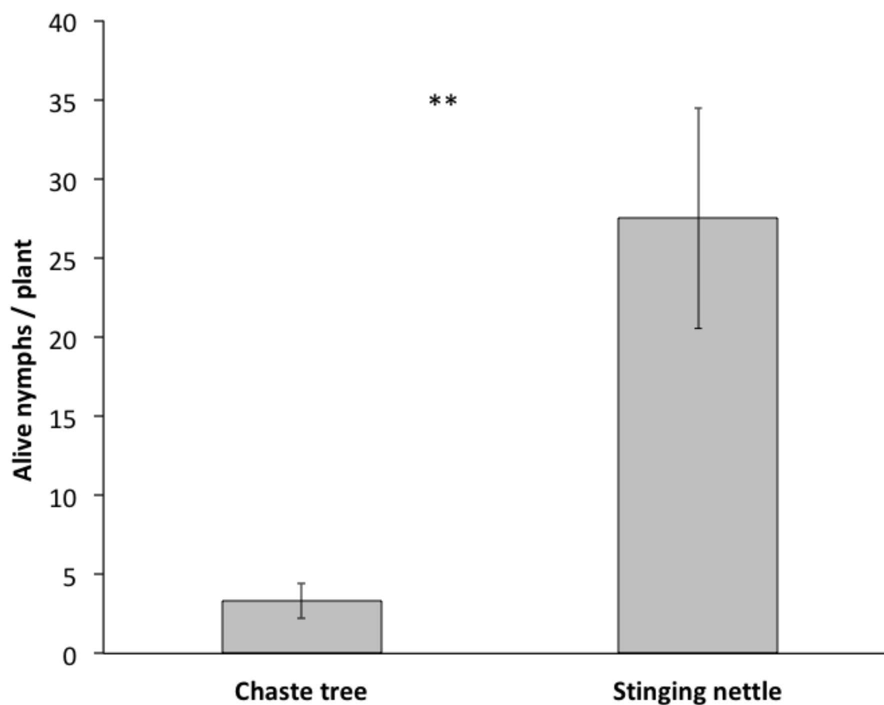

*Figure 11: Alive nymphs of Hyalesthes obsoletus observed on the roots of the two plant species in the February following the oviposition period in late summer of the previous year. ** = significant differences according to a paired-sample t test (α = 0.01)*

4.4. Transmission trials

The PCR analyses for amplification of the stamp gene, performed on the total nucleic acids extracted from the chaste tree plants used in the transmission trials, showed the presence of '*Ca*. P. solani' in 2 plants (TBS6 and TBS7) of 16 (12.5%). No amplification was observed in the other 14 chaste tree plants, on which insects were maintained and on the eight control plants (without insects) (Table 3). The molecular analyses performed on the insect individuals collected from plants TBS6 and TBS7 revealed that 5 individuals of 16 (31.3%) and 6 of 18 (33.3%) respectively were infected by '*Ca*. P. solani'. *H. obsoletus* adults, collected from the 14 chaste tree plants negative to phytoplasma presence, were found to be infected with incidences between 1 and 50% (Table 3). Nucleotide sequence analyses of the stamp gene showed that chaste tree plants and insect individuals feeding on them harbored the same '*Ca*. P. solani' strain, characterized by the stamp gene sequence variant St5 (i.e., strain GGY, NCBI GenBank Accession Number FN813256; Pierro, Passera, Panattoni, Rizzo, et al., 2018b). The PCR analyses performed on chaste tree leaves collected in October 2018 (1 year after the transmission trials), showed all 24 chaste tree plants, including TBS6 and TBS7 (positive in 2017), were not infected (Table 3).

Table 3: Transmission trials: results with *Hyalesthes obsoletus* adults collected on stinging nettle bordering BN-infected vineyards caged on healthy chaste tree potted plants

Origin	**Plants**			**Insects**			
	#	**BNp-infected (strain)**		**Number**			**BNp strain**
		Oct 17	**Oct 18**	**Released**	**Collected**	**BNp-infected**	
Verona	TVR1	-	-	30	27	10 (37%)	
	TVR2	-	-	30	13	3 (23%)	
	TVR3	-	-	30	21	10 (48%)	
	TVR4	-	-	30	20	4 (20%)	
	TVR5	-	-	30	15	4 (27%)	
	TVR6	-	-	30	19	3 (16%)	
	TVR7	-	-	30	16	4 (25%)	
	TVR8	-	-	30	15	1 (7%)	
Brescia	TBS1	-	-	30	17	4 (23%)	
	TBS2	-	-	30	18	3 (17%)	
	TBS3	-	-	30	17	8 (47%)	
	TBS4	-	-	30	20	2 (10%)	
	TBS5	-	-	30	1	0	
	TBS6	+ (St5)	-	30	16	5 (31%)	St5
	TBS7	+ (St5)	-	30	18	6 (33%)	St5
	TBS8	-	-	30	20	10 (50%)	
Control	T1	-	-				
	T2	-	-				
	T3	-	-				

T4	-	-
T5	-	-
T6	-	-
T7	-	-
T8	-	-

5. Discussion

Survival of *H. obsoletus* adults from stinging nettle was significantly improved on the plants on which the nymphs developed (i.e., stinging nettle), more than on grapevine and chaste tree. This occurrence was previously observed for *H. obsoletus* from stinging nettle or bindweed that had better survival on the origin plant than on the other (Kessler et al., 2011; Maixner, Albert, & Johannesen, 2014; Mori et al., 2008a). Survival on chaste tree was significantly better than on grapevine in one of the two study years. However, the differences were not so high as expected from that chaste tree (unlike grapevine) is a true host of the planthopper (Sharon et al., 2015). Our study also indirectly confirmed that *H. obsoletus* can complete its life cycle on chaste tree because nymphs were observed in February on the roots of potted chaste tree plants on which planthopper adults had been caged and able to lay eggs in the previous summer. In the field, *H. obsoletus* adults from stinging nettle were more attracted by stinging nettle than chaste tree and not captured on grapevine. Semi-field experiments confirmed both the scarce attractiveness of grapevine and the preference for stinging nettle than chaste tree. With reference to the two true host plants, namely stinging nettle and chaste tree, preference for the former may be associated with the origin of adults used for the experiments, all collected from stinging nettle plants. Based on this result, the higher attractiveness of chaste tree in comparison with other plants observed in the olfactometer studies by Sharon et al. (2005) may have been influenced by the collection of most of the adults on chaste tree. The evidence that chaste tree resulted significantly more attractive than grapevine would suggest its use as trap plant at vineyard borders. However, because the infected *H. obsoletus* adults that colonize vineyards in northern Italy move mostly from stinging nettle and for this planthopper population, the nettle was more attractive than chaste tree, the use of healthy potted plants of stinging nettle as trap plants would be preferable. Our two-choice olfactometric studies showed no significant preference by *H. obsoletus* for either of the two plants, even if fewer adults were observed on grapevine than stinging nettle.

Results of the transmission trials conducted in the study demonstrated that chaste tree can harbor '*Ca*. P. solani' and that infectious *H. obsoletus* adults from stinging nettle can

spread inoculum into chaste tree. This evidence is in agreement with the results obtained by Kosovac et al. (2016), who demonstrated that naturally occurring chaste tree in vineyard agro-ecosystems in Montenegro may be infected by '*Ca*. P. solani'. The '*Ca*. P. solani' strain St5, transmitted with *H. obsoletus* originating from stinging nettle to chaste tree in the present study, is known to be associated only with bindweed as a source plant, *H. obsoletus* from bindweed as a vector and grapevine across Europe (Pierro, Passera, Panattoni, Rizzo, et al., 2018b). Moreover, strain St5 groups within the bindweed-related stamp phylogenetic Cluster b-II along with strains St1, St2 and St30, were previously found associated with chaste tree or transmitted to grapevine by chaste tree associated *H. obsoletus* (Kosovac et al., 2016). Thus, this is the first report of strain St5 transmitted to chaste tree by *H. obsoletus* from stinging nettle. As chaste tree constitutes an important reservoir for *H. obsoletus*-mediated transmission of BN phytoplasma to grapevine (Kosovac et al., 2016), our findings that chaste tree can host the '*Ca*. P. solani' strain St5, largely prevalent in the Franciacorta area, open a new intriguing scenario on its possible role in BN epidemiology in northern Italy. These results are in disagreement with Sharon et al. (2005, 2015), who showed that, even if chaste tree is a preferred host plant of *H. obsoletus*, chaste tree did not harbor '*Ca*. P. solani'. Interestingly, '*Ca*. P. solani'-infected insect individuals were found on 15 of 16 chaste tree plants used in transmission trials, *H. obsoletus* was only able to transmit the pathogen in two cases. This could be explained considering the short survival of insect adults on chaste tree; in fact, the insect populations decreased dramatically in 4–6 days after release. However, adults of *H. obsoletus* from stinging nettle survive on grapevine no better than on chaste tree and still are able to inoculate the BN phytoplasma.

Moreover, the success of transmission trials can depend on the phytoplasma strain and its titre within the insect adults. For example, it is reasonable to hypothesize that '*Ca.* P. solani' strains not transmitted to chaste tree in the present study could be (as expected) those that are strictly associated with stinging nettle (*stamp* clusters a1 and a2). The result that chaste tree plants, found positive for phytoplasma presence in October 2017, were phytoplasma-free in October 2018 can be explained by natural recovery from infection, as reported for a broad range of poly- annual plants infected by phytoplasmas (Osler, Carraro, Loi, & Refatti, 1993; Romanazzi et al., 2009), increased by abiotic stresses because of the overgrowth of chaste trees in pots under controlled conditions, which is not convenient in terms of spacing.

According to Sharon et al. (2005, 2015), showing that chaste tree is a preferred host plant of *H. obsoletus* and does not harbor '*Ca*. P. solani, in Israel a 'push & pull' strategy was

suggested to reduce the population of *H. obsoletus* in a vineyard by using chaste tree as a trap plant (Zahavi et al., 2007). On the contrary, based on the findings of this and previous research work (Kosovac et al., 2016), it is doubtful that chaste tree can be used in the containment of the BN spread in Europe by using it as an attractant to *H. obsoletus* because it can also act as a reservoir of '*Ca*. P. solani'. However, volatiles from both chaste tree and stinging nettle could be used in the context of 'push & pull strategies' (Riolo et al., 2017). In conclusion, these results enhanced our knowledge about the role of *V. agnus-castus* as host plant of *H. obsoletus* and 'Candidatus Phytoplasma solani' in north Italy. Further studies are needed to determine the actual role of chaste tree in the BN epidemiology.

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Chapter. 3: Entomopathogenic nematodes and fungi acting for the control of *Hyalesthes obsoletus* (Hemiptera: Auchenorrhyncha: Cixiidae), the main insect vector of ‘*Candidatus* Phytoplasma solani’

1- Summary

Hyalesthes obsoletus Signoret (Hemiptera: Auchenorrhyncha: Cixiidae) is a univoltine, polyphagous planthopper that completes its life cycle, including the subterranean nymph cryptic stage, on herbaceous weeds. In vineyards, it can transmit '*Candidatus* Phytoplasma solani', an obligate parasitic bacterium associated with Bois noir (BN) disease of grapevine, from its host plants to grapevine when occasionally feeding on the latter. The main disease management strategies are based on vector(s) control. Insecticide treatments on grapevine canopy are completely inefficient on *H. obsoletus*, due to its life cycle; consequently, control of this planthopper focuses on the nymphs living on the roots of their host plants. Such practices, based on herbicide application and/or weed management, can reduce vector density in the vineyard but can impact the environment or may not be applicable, highlighting the necessity for alternative strategies. In this study, the efficacy of entomopathogenic nematodes (EPNs; *Steinernema carpocapsae*, *S. feltiae*, *Heterorhabditis bacteriophora*) and fungi (EPFs; *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea*, *Lecanicillium muscarium*) against *H. obsoletus* nymphs (EPNs) and adults (EPNs and EPFs) was assessed under laboratory and greenhouse conditions. The majority of examined EPNs and EPFs were able to kill *H. obsoletus* exhibiting a range of effectiveness. *S. carpocapsae* (among EPNs) and *I. fumosorosea* (among EPFs) were found to be the most effective biocontrol agents in all trials carried out. Advantages and limitations of such promising biocontrol agents were discussed. Ecological competency and conditions that can impede or enhance the EPNs and EPFs performance should be investigated to optimize their performance under field conditions.

2- Introduction

Hyalesthes obsoletus Signoret (Hemiptera: Auchenorrhyncha: Cixiidae) is a polyphagous planthopper able to transmit '*Candidatus* Phytoplasma solani' (CaPsol) to plants (Quaglino et al. 2013), including *Vitis vinifera* L. (Maixner 1994). CaPsol, a plant obligate parasitic bacterium, is associated with Bois noir (BN), a disease belonging to the complex of grapevine yellows which had high economic impact on viticulture in Europe in the last decades (Angelini et al. 2018). Although alternative insect vectors of CaPsol to grapevine were recently reported (Cvrkovic et al. 2014; Quaglino et al. 2019), BN epidemiology is principally determined by the life cycle of its main vector *H. obsoletus* (Mori et al. 2013).

H. obsoletus is a palaearctic, univoltine species that, in Europe, completes its life cycle mainly on bindweed (*Convolvulus arvensis* L.) and nettle (*Urtica dioica* L.) (Langer and Maixner 2004) but also on other host plants (Kosovac et al. 2019; Moussa et al. 2019). In summertime, females produce eggs on the root collar of host plants. After egg hatching, the nymphs migrate into the soil to the roots from which they can acquire CaPsol. After a latency period, *H. obsoletus* becomes able to transmit CaPsol to plants for the duration of its life. Overwintering occurs as second-third instar nymphs in the soil. In early spring, fourth and fifth instar nymphs migrate to the soil surface. Adults emerge from end of May till end of June and they fly from beginning of July to end of August, based on climate, host plant, and region (Cargnus et al. 2012; Maixner and Johannesen 2014; Alma et al. 2015). During their flights, *H. obsoletus* adults can occasionally feed on grapevine and, if infected, transmit CaPsol. However, due to their limited feeding activity on grapevine and the short lifespan of the adult stage, they cannot transmit CaPsol from vine to vine. Grapevine is therefore a dead-end host for the pathogen (Bressan et al. 2007).

No effective control measures directly targeting phytoplasmas are available. The main strategies to manage the spreading of phytoplasma-associated diseases are based on preventive measures, including the control of vectors before their emergence from the ground (Bianco et al. 2019). Due to its cryptic life cycle and polyphagous feeding habit, insecticide treatments on grapevine canopy are completely inefficient against *H. obsoletus*. Thus, strategies for its control focus on depriving the nymphs of their feeding substrate, the host plant roots. Before adult emergence, bindweed and nettle can be suppressed by planting of ground covering rosette plants, repeated mowing or weeding (Maixner and Mori 2013; Mori et al. 2014a). Since *H. obsoletus* presence depends on the distribution of its natural plant hosts both within and outside the vineyards, such strategies are limited by restrictions on the use of herbicides in uncultivated areas, as well as mechanical weeding on ditches and embankments because of soil landslide. In Israel, *H. obsoletus* populations within vineyards are successfully limited by a push and pull strategy using chaste tree (*Vitex agnus-castus* L.) (Sharon et al. 2015), but such a strategy cannot be employed in Europe where this plant hosts both *H. obsoletus* and CaPsol (Moussa et al. 2019).

Considering these limitations, a promising approach to control the vector populations could be based on the utilization of biocontrol agents such as entomopathogenic nematodes (EPNs) and fungi (EPFs). In particular, several Steinernematidae and Heterorhabditidae EPNs have been reported as effective biocontrol agents against a broad range of insects with a cryptic life cycle like *H. obsoletus* (Grewal et al. 2005; Lacey and Georgis 2012; Guerrero

and Pardey 2019). EPNs efficacy depends on their survival for a long time without their host targets in the soil, and their ability to find the hosts by ambush (i.e., *Steinernema carpocapsae*) or cruising (i.e., *Heterorhabditis bacteriophora*) strategy (Kaya et al. 1993; Grewal et al. 1994;). Concerning EPFs, they are reported as important antagonists of soil-dwelling insect pests adapted to live in agricultural soils, such as the grapevine phylloxera in vineyards (Kirchmair et al. 2004). Interestingly, the EPF *Metharizium anisopliae* showed a great efficacy against *H. obsoletus* adults under laboratory conditions (Langer et al., 2005).

In this study, the efficacy of different EPNs and EPFs against nymphs and adults of *H. obsoletus* were assessed under laboratory and greenhouse conditions to develop effective and innovative approaches to control the main vector of CaPsol.

3- Materials and Methods

3.1. Hyalesthes obsoletus collection

Nymphs of *H. obsoletus* were collected on two dates immediately before the different experiments. The collection for lab bioassay was done in the middle of May 2019; nymphs were obtained from pots of stinging nettle plants at the rearing facility in Julius Kühn-Institute in Siebeldingen, Germany. Collection for the field trials was performed in late May 2019 from roots of nettle plants growing at the borders of a highly BN-affected vineyard in Mosel area (49.9198°N, 7.0627°E), Germany. Nymphs were placed in falcon tubes filled with the same soil in which they were collected and sent to the lab for trials. Nymphs identity was confirmed based on the taxonomic key by Stöckmann et al. (2013).

Adults of *H. obsoletus* were collected from bindweed and stinging nettle plants using a sweep net and mouth aspirator from mid-June till the end of July 2019, based on the trial requirements, in the vicinity of highly BN-affected vineyards in Mosel area (49.9198°N, 7.0627°E, and 49.1928°N, 8.0830°E), Germany. Collected adults were placed in collapsible insect mesh cages with shoots of nettle plants as food source, transferred to the lab, and identified by taxonomic key (Bertin et al. 2010). *H. obsoletus* adults were subject to immediate use in bioassays and greenhouse efficacy trials.

3.2. Entomopathogenic nematodes

Three EPNs were applied in both bioassays and greenhouse trials against *H. obsoletus* nymphs and adults. In detail, the utilized EPNs (*Steinernema carpocapsae, S. feltiae, Heterorhabditis bacteriophora*, and a combination (1:1) of *S. feltiae* and *H. bacteriophora*)

were purchased from E-nema® Company (Schwentinental, Germany) (Table 1) and maintained at 4 °C. Immediately before using, each EPN (supplied in powder) was suspended in tap water and tested for its viability by counting the infective juveniles (IJs) under stereomicroscope. IJs without a response to stimulators were considered dead (Lacey 1997). All EPNs showed a viability higher than 95%. Before the application, each EPN was serially diluted in tap water to reach the required concentration for bioassay (200 IJs ml^{-1}) and greenhouse trials (400 IJs ml^{-1}) (Guerrero and Pardey 2019).

3.3. Entomopathogenic fungi

Three distinct EPF isolates of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosoroseus* (provided by Dr. Dietrich Stephan - Institute for Biological Control, Julius Kühn-Institut, Darmstadt, Germany), were applied in preliminary screening against *H. obsoletus* adults. In addition to these isolates, one commercial isolate of *M. anisopliae* and one of *Lecanicillium muscarium* were purchased from Koppert Biological Systems Company (Verona, Italy) and applied in greenhouse trials against *H. obsoletus* adults (Table 1). EPFs were cultivated in Petri dishes on malt extract peptone agar (30 g of malt extract, 3 g of peptone, 15 g of agar in 1 l of distilled water) previously autoclaved at 121 °C for 10 min. After inoculation, EPFs were grown for 5 days at room temperature (22 °C) and for 6 days at 4 °C. For each EPF isolate, fungal conidia were recovered from the mycelium, suspended in distilled water and counted (three times per isolate) at 400X magnification through a haemocytometer. For each EPF isolate, stock solutions of conidial suspension were prepared at the concentrations of 10^6, 10^8 and 10^{10} conidia ml^{-1} and stored at 4 °C until use.

3.4. Entomopathogenic nematodes: laboratory bioassays

Bioassay to evaluate the efficacy of EPNs against *H. obsoletus* nymphs was conducted in 12-well cell culture plates with lids. According to the protocols of Kaya and Stock (1997) and Lewis (2000), in each well, filled with 1 g of autoclaved sand, 1 ml of EPN suspension (200 IJs ml^{-1}) was applied and one nymph was placed; control plates were treated with distilled water before placing the nymphs. Bioassay to evaluate the efficacy of EPNs against *H. obsoletus* adults was conducted in plastic Petri dishes (9 cm diameter) padded with filter paper. Following Lewis (2000) procedures, in each dish, fresh shoots of stinging nettle (5 cm) were placed as a food source together with 12 adults of *H. obsoletus*, and 1 ml of EPN suspension (200 IJs ml^{-1}) was applied using a hand-held sprayer. Control dishes were sprayed

with distilled water before placing the adults. The edges of Petri dishes were dried with tissue paper to prevent the adults sticking to water droplets. Three replicates of each plate (nymphs)/Petri dish (adults) were made per EPN as well as the control. All plates/dishes were placed in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6 days. Mortality readings were taken daily for 6 consecutive days. To confirm that insect mortality was caused by the activity of the EPNs, the presence of EPN was evaluated by dissecting nymphs and adult insect bodies under a 40X magnifying stereomicroscope. This was done after rinsing single dead nymphs and adults in a conical flask filled with 20 ml of distilled water, for removing nematodes from their surface. Nymphs and adults were then placed on a moist filter paper padded plastic Petri dish and maintained at 25 °C for 3 days.

3.5. ***Entomopathogenic nematodes*: greenhouse trials**

To evaluate the efficacy of EPNs against *H. obsoletus* nymphs and adults, greenhouse trials were conducted on potted stinging nettle (*Urtica dioca* L.) and faba bean (*Vicia faba* L.) plants, respectively. The nettle plants (diameter about 0.1 m and height approximately 0.4 m) were grown in 3 l pots, while the bean plants (diameter about 0.1 m and height approximately 0.25 m) in 1 l pots and placed in transparent plastic ventilated cages; all plants were in good vegetative condition and did not show symptoms of biotic and abiotic stresses. In each pot with nettle, 5 ml of EPN suspension (400 IJs ml^{-1}) was applied to the soil using a hand-held sprayer, and a total of 20 nymphs were placed after treatment. The control potted plants were treated with 5 ml of distilled water before placing the nymphs. On each caged bean plant, 10 ml of EPN suspension (400 IJs ml^{-1}, with 0.02% Tween 80) was applied using a hand-held sprayer, and 15 *H. obsoletus* adults were released. The control caged plants were treated with 10 ml of distilled water with 0.02% Tween 80 before releasing insect adults. Three replicates, arranged in randomized blocks, were made per EPN as well as the control. Potted plants were kept in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod). Mortality was recorded 6 days after treatment. As described above for the bioassay, mortality due to nematodes infection was confirmed by dissecting the dead nymph and adult insect bodies under a 40X magnifying stereomicroscope.

3.6. **Entomopathogenic fungi: laboratory bioassays**

Initially, the nine EPF isolates provided by the Institute for Biological Control - Julius Kühn-Institute (JKI) (Table 1) were screened for their entomopathogenic activity against *H.*

obsoletus adults in plastic Petri dishes (8.5 cm diameter), filled to a depth of 5 mm with a mixture of plaster of Paris and charcoal (10:1) and moistened with distilled water (Green 1964; Maixner 2005). In each Petri dish, fresh bindweed (*Convolvulus arvensis* L.) shoots (5 cm) were placed as a food source, 1 ml of EPF conidia suspension (10^8 conidia ml^{-1}) was applied using a hand-held sprayer, and 12 adults were released. The control dishes were treated with distilled water before the release of insects. Three replicates were conducted per EPF isolate as well as the control. Petri dishes were kept in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod). Mortality readings were taken after 6 days. Single dead insects from each EPF isolate were placed in malt extract peptone agar plates kept for 3 days at room temperature (22 °C) and for 5 days at 4 °C. Mortality due to EPF infection was confirmed through the observation of EPF mycelium growth on the insect body. Subsequently, EPF isolates, found active against *H. obsoletus* in the initial screening test, were employed in a bioassay applying conidial suspension at different concentrations (10^6, 10^8, 10^{10} conidia ml^{-1}). The bioassays were conducted in plastic Petri dishes as described above for the initial screening test. Three replicates were conducted per concentration per EPF isolate as well as the control. Petri dishes were kept in controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6 days. Mortality readings were taken daily for 6 consecutive days. Single dead insects from each bioassay were placed in malt extract peptone agar plates and checked to confirm EPF-related mortality as described above.

3.7. Entomopathogenic fungi: greenhouse trials

Greenhouse trials were conducted on potted stinging nettle plants, placed singly in transparent plastic ventilated cages, to evaluate the efficacy against *H. obsoletus* adults of the previously selected JKI EPF isolates along with two commercial EPFs (*M. anisopliae* and *L. muscarium*). On each caged plant, 1 ml of EPF suspension (10^8 conidia ml^{-1}) was applied on the potted plants using a hand-held sprayer, and 15 *H. obsoletus* adults were released. The control caged plants were treated with 1 ml of distilled water before the release of insects. Three replicates were made per EPF as well as the control. Caged potted plants were kept in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6 days. Mortality readings were taken after 6 days. EPF-related mortality was confirmed by observing the EPFs mycelium growth directly on insect bodies on stinging nettle or placing single dead insects in malt extract peptone agar plates as described above.

3.8. Statistical analyses

In the bioassays, the median lethal time (LT_{50}) for EPNs against nymphs and adults was calculated from daily mortality data. The median lethal concentration (LC_{50}) of EPFs against adults was log_{10} transformed and calculated from the data obtained on the 4th day post-inoculation (dpi). LT_{50} and LC_{50} results with their fiducial confidence limits were obtained by package ecotox (Hlina et al. in preparation) based on the probit analysis. In the screening (EPFs) and greenhouse trials (EPNs and EPFs), mortality data of nymphs and adults were transformed in percentage (%) and tested for normality and equality of variance with the Shapiro-Wilk test followed by Levene's test of homogeneity of variance. Greenhouse trials data were subjected to the ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc for multiple comparisons. Graphical representations of the statistical analysis results were produced using ggplot2 (Wickham 2016). All the analyses were done using R (version 3.6.2) (R Core Team 2019).

4- Results

4.1. Biocontrol efficacy of entomopathogenic nematodes against *H. obsoletus* nymphs and adults

In the bioassay, *S. carpocapsae* was the most effective with a LT_{50} value of 3.24- and 3.69-days post-inoculation (dpi) for *H. obsoletus* nymphs and adults, respectively (Fig. 12a, b). *S. feltiae* was the second most effective with a LT_{50} of 3.49 and 4.05 dpi for nymphs and adults, respectively (Fig. 12c, d). The mixture of *H. bacteriophora* and *S. feltiae* showed a LT_{50} of 3.65 and 4.61 dpi for nymphs and adults, respectively (Fig. 12e, f). *H. bacteriophora* (Hf) was the least effective with a LT_{50} of 4.34 and 5.06 dpi for nymphs for adults (Fig. 12g, h) (Table . In the greenhouse trials, results of ANOVA [nymphs ($F_{4,10} = 82.55$; $p < 0.001$); adults ($F_{4,10} = 87.96$; $p < 0.001$)], followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* nymphs and adults treated with the different EPNs were significantly higher than the non-treated control. Among EPNs, *S. carpocapsae* showed the highest efficacy against *H. obsoletus* nymphs (average mortality rate 86.67%) and adults (81.67%), while *H. bacteriophora* the lowest (56.67% vs nymphs; 55% vs adults) (Fig. 13a, b; table 5). Stereomicroscope analyses confirmed that the mortality of all *H. obsoletus* nymphs and adults, both in bioassay and greenhouse trials, was caused by EPNs (Supplementary Material Fig. 14a, b).

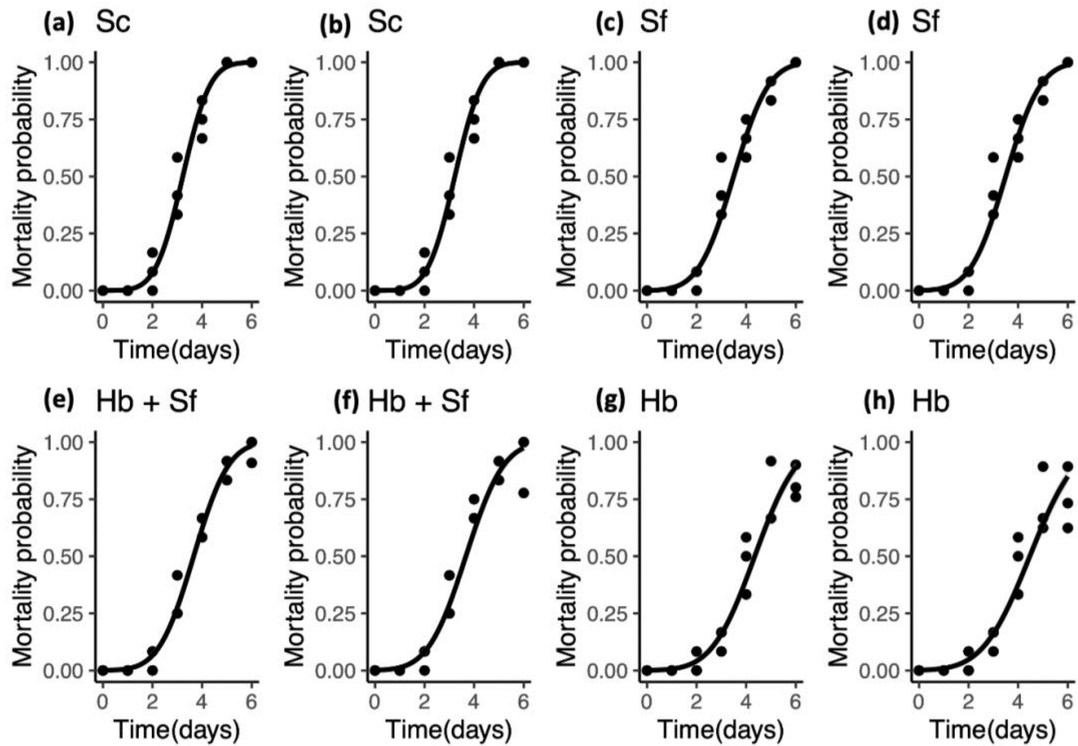

*Figure 12 Median lethal time (LT_{50}) of different EPNs against nymphs and adults of H. obsoletus. Black dots represent the observations. Black curves were computed using the glm smoothing method within ecotox. Shaded grey areas represent the 95% confidence intervals. S. carpocapsae (Sc) vs H. obsoletus nymphs (**a**) and adults (**b**); S. feltiae (Sf) vs nymphs (**c**) and adults (**d**); H. bacteriophora + S. feltiae (Hb + Sf) vs nymphs (**e**) and adults (**f**); H. bacteriophora (Hb) vs nymphs (**g**) and adults (**h**).*

Table 4 Median lethal time (LT_{50}) of different EPNs against nymphs and adults of *H. obsoletus*. Means represent the accumulated mortality rate per treatment. Hb (*H. bacteriophora*), Sf + Hb (*S. feltiae + H. bacteriophora*), Sc (*S. carpocapsae*), Sf (*S. feltiae*); dpi (days post inoculation).

Treatment	Nymphs		Adults	
	Accumulated % mortality mean ± SE	**LT_{50} (dpi) [a]**	**Accumulated % mortality mean ± SE**	**LT_{50} (dpi) [a]**
Hb	31.57 ± 7.62	4.34 (4.11 – 4.59)	23.48 ± 5.66	5.06 (4.77 – 5.42)
Sf + Hb	43.25 ± 8.84	3.65 (3.50 – 3.80)	24.14 ± 5.15	4.61 (4.25 – 5.03)
Sc	46.82 ± 9.46	3.24 (3.06 – 3.37)	40.08 ± 8.44	3.69 (3.46 – 3.90)
Sf	40.83 ± 8.78	3.49 (3.30 – 3.68)	34.92 ± 8.21	4.05 (3.82 – 4.29)

[a] 95% lower and upper fiducial limits are shown in parentheses.

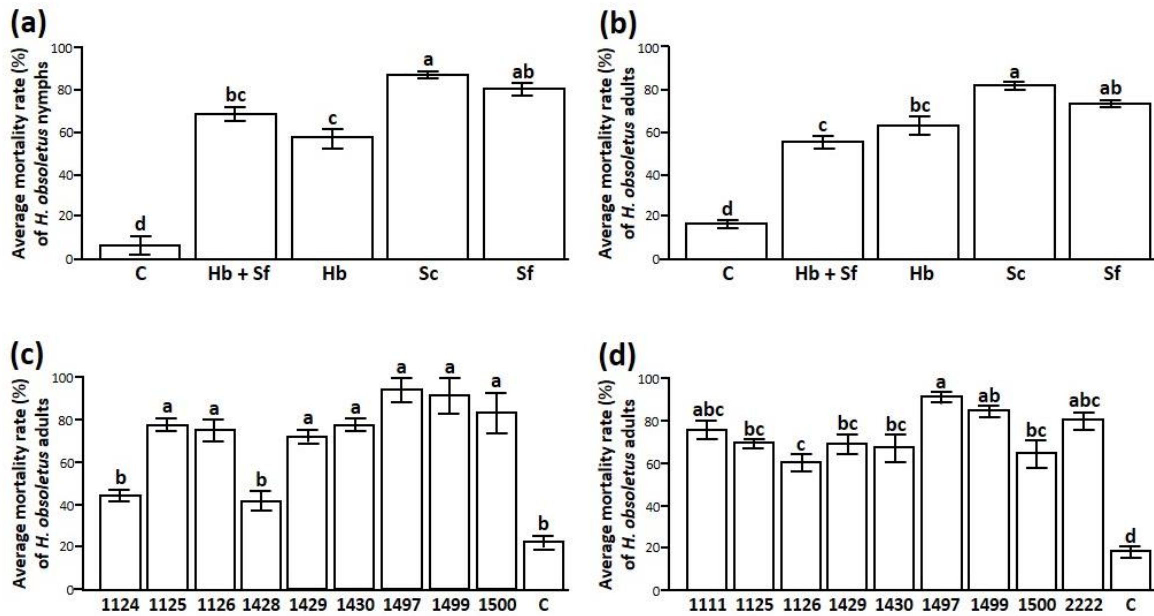

*Figure 13: Average mortality rate of Hyalesthes obsoletus nymphs and adults treated with EPNs and EPFs. EPNs greenhouse trials vs H. obsoletus nymphs (**a**) and adults (**b**); EPFs initial screening test vs H. obsoletus adults (**c**); EPFs greenhouse trials vs H. obsoletus adults (**d**). On each bar: letters (a-d) indicate significant differences (p < 0.05) based on ANOVA followed by Tukey's HSD test; bars indicate the standard errors (SE). Acronyms in (**a**) and (**b**): C (non-treated control); Hb + Sf (H. bacteriophora + S. feltiae); Hb (H. bacteriophora); Sc (S. carpocapsae); Sf (S. feltiae). Acronyms in (**c**) and (**d**): 1124, 1125, 1126 (B. bassiana strains JKI-BI-1124, JKI-BI-1125, JKI-BI-1126); 1428, 1429, 1430 (M. anisopliae strains JKI-BI-1428, JKI-BI-1429, JKI-BI-1430); 1497, 1499, 1500 (I. fumosorosea strains JKI-BI-1497, JKI-BI-1499, JKI-BI-1500); 1111 (M. anisopliae strain 1111); 2222 (L. muscarium strain 2222); C (non-treated control).*

Table 5 Average mortality rate of *Hyalesthes obsoletus* nymphs and adults treated with EPNs. Sc (*S. carpocapsae*), Sf (*S. feltiae*), Sf + Hb (*S. feltiae* + *H. bacteriophora*), Hb (*H. bacteriophora*).

Treatment	Nymphs		Adults	
	Mean ± SE	**Group**	**Mean ± SE**	**Group**
Sc	86.67 ± 1.67	a	81.67 ± 1.66	a
Sf	80.00 ± 2.89	ab	73.33 ± 1.66	ab
Sf+Hb	68.33 ± 3.33	bc	63.00 ± 4.41	bc
Hb	56.67 ± 4.41	c	55.00 ± 2.89	c
Control	6.67 ± 4.41	d	16.67 ± 1.67	d

Means followed by similar letters in the group column are not significantly different according to Tukey's HSD test ($p \geq 0.05$)

4.2. Biocontrol efficacy of entomopathogenic fungi against *H. obsoletus* adults

In the initial screening, results of ANOVA ($F_{9,20} = 20.39$; $p < 0.001$), followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* adults were significantly higher than the non-treated control in all EPFs treatments, except *B. bassiana* strain 1124 and *M. anisopliae* 1428 (Fig. 13c). In the bioassay, the seven effective EPFs were employed to define their proper concentration leading to 50% of mortality of *H. obsoletus* adults. *I. fumosorosea* strains 1497 and 1499 were found to be the most virulent against *H. obsoletus* adults with a LC_{50} (log_{10} concentration) of 6.076 and 6.459 conidia ml^{-1}, respectively. *M. anisopliae* strains 1429 and 1430 showed a LC_{50} of 8.207 and 8.506 conidia ml^{-1}, respectively. *B. bassiana* strains1125 and 1126 were the least virulent with a LC_{50} of 8.893 and 9.226 conidia ml^{-1}, respectively (Fig. 15; Table 6).

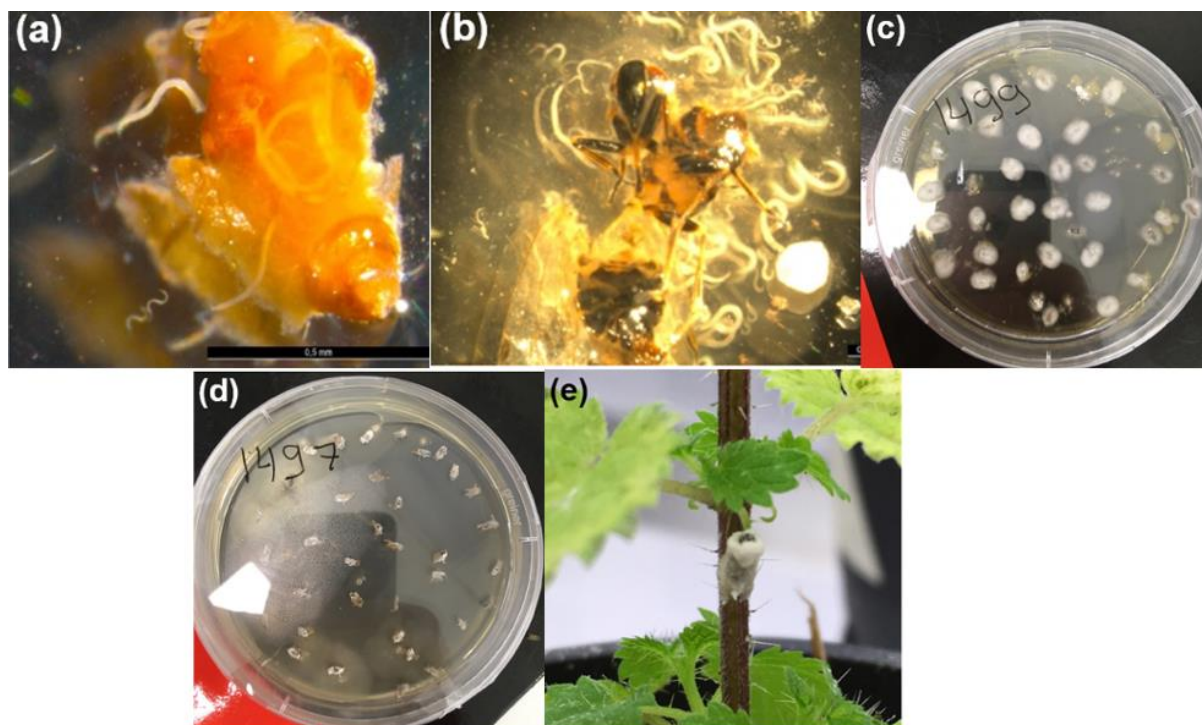

*Figure 14: Observation of EPNs and EPFs from dead H. obsoletus nymphs and adults. S. carpocapsae infecting nymphs in bioassay (**a**) and adults in greenhouse trials (**b**); I. fumosorosea infecting adults in initial screening (strain 1499) (**c**), bioassay (strain 1497) (**d**), and greenhouse trials directly on stinging nettle (**e**).*

In the greenhouse trials, results of ANOVA ($F_{9,20} = 23.87$, $p < 0.001$), followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* adults treated with the different EPFs were significantly higher than the non-treated control. Among EPFs, *I. fumosorosea* strain 1497 showed the highest efficacy against *H. obsoletus* adults (average mortality rate 91.1%). This percentage was not significantly different in comparison to that

obtained by *I. fumosorosea* strain 1499 (84.45%), *M. anisopliae* strain 1111 (75.56%), and *L. muscarium* strain 2222 (80%). On the contrary, it was significantly different in comparison to that obtained by *M. anisopliae* strains 1429 (68.89%) and 1430 (66.67%), and *B. bassiana* strains 1125 (68.89%). *B. bassiana* strain 1126 showed the lowest efficacy (60%) (Fig. 13d; Table 7). EPF mycelium growth was observed on the body of all dead insect adults confirming that the mortality was due to EPF infection in initial screening, bioassay and greenhouse trials (Supplementary Material Fig. 14c, d, e).

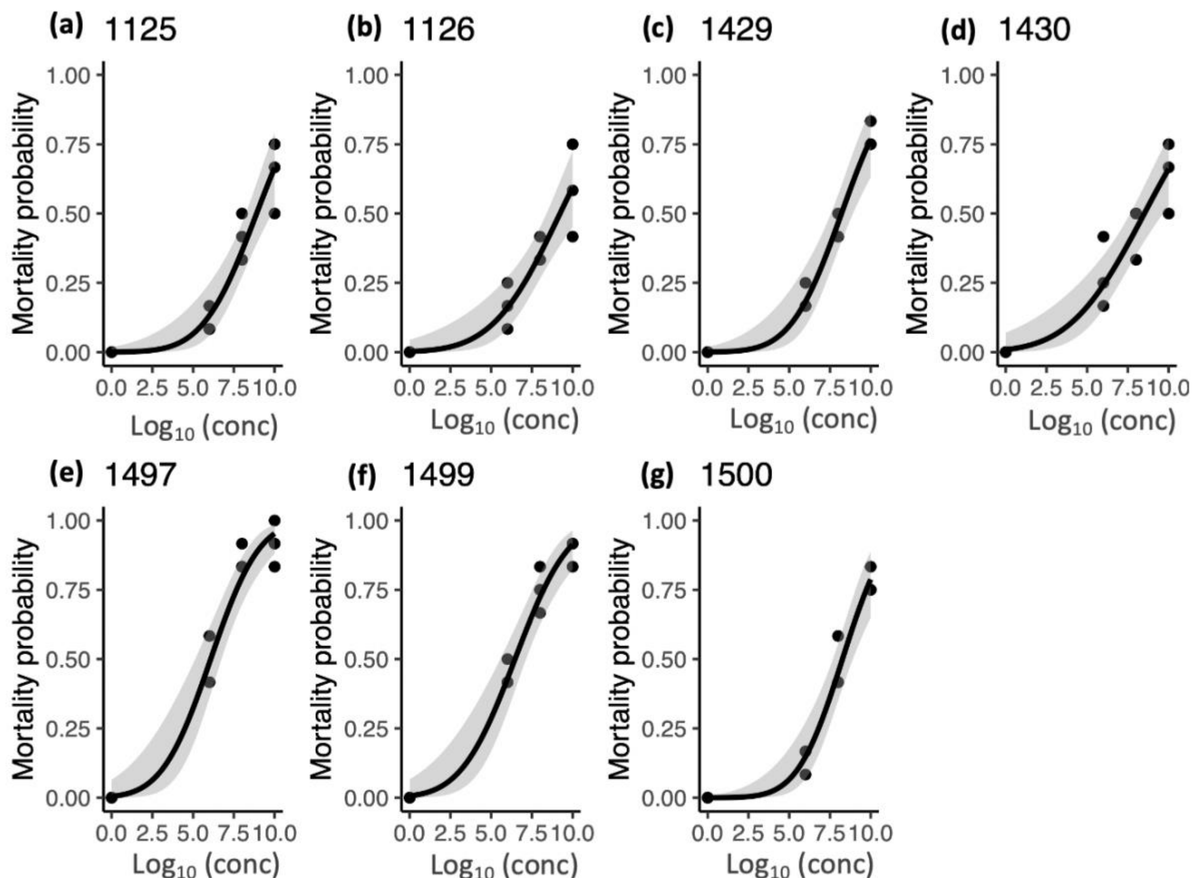

*Figure 15: Median lethal concentration (LC_{50}) of EPFs against H. obsoletus adults. Black dots represent the observations. Black curves were computed using the glm smoothing method within ecotox. Shaded grey areas represent the 95% confidence intervals. B. bassiana strains JKI-BI-1125 (1125) (**a**), JKI-BI-1126 (1126) (**b**); M. anisopliae strains JKI-BI-1429 (1429) (**c**), JKI-BI-1430 (1430) (**d**); I. fumosorosea strains JKI-BI-1497 (1497) (**e**), JKI-BI-1499 (1499) (**f**), JKI-BI-1500 (1500) (**g**).*

Table 6: LC50 of EPFs against Hyalesthes obsoletus adults calculated 4 days after treatment. Means represent the mortality rate per cage.

EPFs	Strain	log_{10} conc	% mortality mean ± SE	log_{10} LC_{50} [a]
B. bassiana	JKI-BI-1125	6	11.11 ± 2.77	8.893 (8.43 - 9.46)
		8	41.67 ± 4.81	
		10	63.89 ± 7.84	
	JKI-BI-1126	6	16.66 ± 4.81	9.23 (8.59 – 10.15)
		8	36.11 ± 2.78	
		10	58.33 ± 9.68	
M. anisopliae	JKI-BI-1429	6	19.44 ± 2.77	8.21 (7.99 – 8.43)
		8	44.44 ± 2.77	
		10	77.78 ± 2.78	
	JKI-BI-1430	6	27.78 ± 7.35	8.51 (7.78 – 9.41)
		8	44.44 ± 5.56	
		10	63.89 ± 7.35	
I. fumosorosea	JKI-BI-1497	6	47.22 ± 5.55	6.37 (5.91 – 7.29)
		8	86.11 ± 2.78	
		10	91.67 ± 4.81	
	JKI-BI-1499	6	44.44 ± 2.78	6.46 (5.99 – 6.85)
		8	75.00 ± 4.80	
		10	88.89 ± 2.78	
	JKI-BI-1500	6	13.89 ± 2.78	8.27 (7.99 – 8.55)
		8	47.22 ± 5.56	
		10	77.78 ± 2.78	

[a] 95% lower and upper fiducial limits are shown in parentheses

Table 7 Mean mortality rate of Hyalesthes obsoletus adults treated with EPFs in greenhouse.

EPFs	Strain	% mortality mean ± SE	Group
Control	-	17.78 ± 2.20	d
B. bassiana	JKI-BI-1125	68.89 ± 2.20	bc
	JKI-BI-1126	60.00 ± 3.85	c
M. anisopliae	JKI-BI-1429	68.89 ± 4.40	bc
	JKI-BI-1430	66.67 ± 6.67	bc
I. fumosorosea	JKI-BI-1497	91.10 ± 2.20	a
	JKI-BI-1499	84.45 ± 2.20	ab
	JKI-BI-1500	64.40 ± 5.88	bc
M. anisopliae	JKI-BI-1111	75.56 ± 4.40	abc
L. muscarium	JKI-BI-2222	80.00 ± 3.85	abc

Means followed by similar letters in the group column are not significantly different according to Tukey's HSD ($p \geq 0.05$)

5- Discussion

Due to the complex life cycle of *Hyalesthes obsoletus*, most strategies to control its populations in the vineyard agro-ecosystem are not effective or can impact the environment (Maixner and Mori 2013). In the last years, biocontrol has been proposed and frequently utilized as sustainable strategy to control plant pathogen insect vectors (Kumar 2016; Abdel-Razek et al. 2017; Abd El-Ghany et al. 2018). Entomopathogenic nematodes (EPNs) and fungi (EPFs) have been largely employed as effective biocontrol agents against insects with a cryptic life cycle, including phytoplasma vectors (Grewal et al.2005; Lacey and Georgis 2012; Guerrero and Pardey 2019), making this approach promising also for the main vector of '*Candidatus* Phytoplasma solani' to grapevine, *H. obsoletus*.

The results obtained in this study demonstrated that all the examined EPNs are able to kill *H. obsoletus* nymphs and adults and that the EPFs, except *Beauveria bassiana* strain 1124 and *Metharizium anisoploae* strain 1428, are able to control the adults in both laboratory bioassays and greenhouse trials, exhibiting a range of effectiveness related to their virulence against the target insect. In all conducted trials, *Steinernema carpocapsae* and *Isaria fumosorosea* were found to be the most effective biocontrol agents of *H. obsoletus* among the examined EPNs and EPFs, respectively.

Concerning *Steinernema* spp., our findings are in agreement with those from previous study showing that, in laboratory bioassay performed against the vine mealy bug *Planococcus ficus*, the EPN *Steinernema yirgalemense* moved 15 cm vertically downward, and infected its insect target through an ambush strategy inducing a mortality of 95% (Le Vieux et al. 2013). Another study demonstrated that the combination of *S. yirgalemense* with specific adjuvants increased its biocontrol activity against the vine mealy bug on grapevine leaves in both laboratory and semi-field conditions (Platt et al. 2019). Such evidence fortifies the possibility of applying *Steinernema* spp. in the open field against both subterranean forms and adults of *H. obsoletus*. Among tested EPNs, also *Heterorhabditis bacteriophora* showed a high efficacy in *H. obsoletus* biocontrol through a cruising strategy. Interestingly, this EPN was reported to be effective against the nymphs of *Haplaxius crudus*, the insect vector of '*Candidatus* Phytoplasma palmae' associated with Palm Lethal Yellowing disease in Florida (Guerrero and Pardey 2019), and of *Aeneolamia* spp., a putative vector of genetically distinct phytoplasmas (Pérez et al. 2018). Moreover, previous study reported that *H. bacteriophora* strongly reduced the survival of the root-form of grapevine phylloxera (English-Loeb et al. 1999). This evidence highlighted that, in vineyard

agroecosystems, treatments based on the application of *H. bacteriophora* could be effective against multiple insect pests. Based on all these evidences, it would be interesting to apply a combination of *S. carpocapsae*, found here as more effective against *H. obsoletus* nymphs and adults, and *H. bacteriophora*, reported in previous studies as the most effective EPN against phytoplasma insect vectors and grapevine insect pests with a cryptic life stage.

Concerning *Isaria fumosorosea*, found here as the most effective EPF against *H. obsoletus*, previous studies showed its biocontrol activity against various nymphal stages of the green leafhopper *Empoasca decipiens* Paoli under laboratory and greenhouse conditions (Tonou et al. 2003; Kodjo et al. 2011). Similar efficacy was found by treatments with *Metarhizium anisopliae* (strain Ma43) and *Beauveria bassiana* (strain Bba113) (Tonou et al. 2003; Kodjo et al. 2011). For all these EPFs, percentage of mortality and LC_{50} values reported against *E. decipiens* were comparable to those observed in this study against *H. obsoletus* adults. Moreover, promising results obtained in the present study with two strains of *M. anisopliae* confirmed its entomopathogenic activity against *H. obsoletus* adults under laboratory conditions (Langer et al. 2005). Interestingly, *Beauveria bassiana*, two strains of which showed a great biocontrol activity against *H. obsoletus* adults in the present work, was found naturally infecting and causing visual symptoms on *H. obsoletus* adults in Georgia (Caucasus region) (Chkhaidze et al. 2017). Moreover, *B. bassiana* showed an efficacy in biocontrol of young stages and adults of *Scaphoideus titanus* Ball, the insect vector of Flavescence dorée phytoplasma, in semi-field and field trials (Mori et al. 2014b). All these evidences underlined that *B. bassiana* can control both the insect vectors of phytoplasmas associated with the main grapevine yellows diseases; thus, *B. bassiana* strains represent really promising EPFs for application in vineyards.

Effectiveness of EPNs and EPFs, as well as other living organisms used as biocontrol agents, depends on a range of climatic and environmental parameters allowing their liveliness and entomopathogenic activity. In particular, it is crucial that the target insect stage is present when climatic parameters are optimal for EPNs and/or EPFs (Lacey and Georgis 2012; Wang and Wang 2017). In the case of *H. obsoletus*, it is known that the duration of the cryptic (subterranean) phase of its life cycle, involving the nymph stages, is dependent on the degree day units that can be estimated based on forecasting models measuring the accumulated heat units (Maixner and Mori 2013). Such models allow understanding of the life cycle of the insect as well as narrowing the spraying window of products for plant protection, including EPNs and EPFs. In particular, the spraying window should prioritize two important aspects: (i) the ecological competency of EPNs as well as EPFs; (ii) the proper timing for application

against the different stages and instars of *H. obsoletus*. In Europe and Mediterranean area, considering the life cycle of *H. obsoletus* and the environmental conditions suitable for EPNs and EPFs utilized in the present study, it should be recommended to apply EPNs and EPFs on *H. obsoletus* host plants in the open field from mid of September to October and/or in early spring to optimize the activity of each biocontrol agent and avoid resistance in the insect target populations. Moreover, given their ability to colonize the soil after their inoculation (Meyling and Eilenberg 2006; Denno et al. 2008), EPNs and EPFs could reduce the *H. obsoletus* population density for a long time. According to our result EPFs could be applied also with foliar application from end of May till end of June against newly hatched adults before grapevine infestation. Optimized application of entomopathogenic nematodes (on the soil) and fungi (on the plants) can increase the control of *H. obsoletus* nymphs and adults, respectively.

In conclusion, the majority of EPNs and EPFs utilized in the present study showed a considerable biocontrol activity against *H. obsoletus* nymphs and adults in laboratory bioassays and greenhouse trials. The ecological competency of both EPNs and EPFs, the conditions that can impede or enhance their performance, the barriers that can block infection from taking place on the target host, and the possible actions on non-target species should be carefully investigated for a better understanding of their potential performance under field conditions.

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Chapter. 4: Identification and ecology of alternative insect vectors of ‘*Candidatus* Phytoplasma solani’ to grapevine

1- Summary

Bois noir, a disease of the grapevine yellows complex, is associated with '*Candidatus*Phytoplasma solani' and transmitted to grapevines in open fields by the cixiids *Hyalesthes obsoletus* and *Reptalus panzeri*. In vine-growing areas where the population density of these vectors is low within the vineyard, the occurrence of bois noir implies the existence of alternative vectors. The aim of this study was to identify alternative vectors through screening of the Auchenorrhyncha community, phytoplasma typing by *stamp* gene sequence analyses, and transmission trials. During field activities, conducted in Northern Italy in a vineyard where the bois noir incidence was extremely high, nine potential alternative insect vectors were identified according to high abundance in the vineyard agro-ecosystem, high infection rate, and harbouring phytoplasma strains characterized by *stamp* gene sequence variants found also in symptomatic grapevines. Transmission trials coupled with molecular analyses showed that at least eight species (*Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Philaenus spumarius*, and *Psammotettix alienus/confinis*) are alternative vectors of '*Candidatus* Phytoplasma solani' to grapevines. These novel findings highlight that bois noir epidemiology in vineyard agro-ecosystems is more complex than previously known, opening up new perspectives in the disease management.

2- Introduction

Bois noir (BN), a disease of the grapevine yellows (GY) complex, causes serious crop losses in wine-making grape varieties in the Euro-Mediterranean area and in other vine-growing countries. BN is associated with strains of '*Candidatus* Phytoplasma solani' (CaPsol) (subgroup 16SrXII-A), a phloem-limited cell wall-less bacterium of the Mollicutes class (Choueiri *et al.*, 2002; Gajardo *et al.*, 2009; Duduk *et al.*, 2010; Quaglino *et al.*, 2013; Mirchenari, Massah and Zirak, 2015). *Hyalesthes obsoletus* Signoret (Hemiptera, Cixiidae), a polyphagous insect feeding mainly on bindweed (*Convolvulus arvensis* L.), nettle (*Urtica dioica* L.), chaste tree (*Vitex agnus-castus* L.), and stinking hawk's beard (*Crepis foetida* L.), is the principal vector of CaPsol strains to grapevine (Langer and Maixner, 2004; Kosovac *et al.*, 2016, 2019). In the last years, the spreading of CaPsol in vineyards where *H. obsoletus* was absent suggested the existence of additional vectors.

Recently, a study conducted in Serbia demonstrated the capability of *Reptalus panzeri* Löw to transmit CaPsol to grapevine (Cvrković *et al.*, 2014). On the other hand, *Macrosteles quadripunculatus* (Kirschbaum), *Anaceratagallia ribauti* Ossiannilsson, and *Reptalus quinquecostatus* (Dufour) were found able to transmit CaPsol in experimental conditions but no evidence of transmission of the pathogen to grapevine is available yet (Batlle *et al.*, 2008; Riedle-Bauer, Sára and Regner, 2008; Chuche *et al.*, 2016). Furthermore, numerous CaPsol-harbouring planthopper (Cixiidae) and leafhopper (Cicadellidae) species were found within or around BN-affected vineyards (Oliveri *et al.*, 2015; ŠafářoVá *et al.*, 2018). Considering such evidences, the identification of which insect species can effectively transmit the phytoplasma to grapevine is of paramount importance to formulate effective control strategies to reduce the BN incidence.

Based on *tufB* gene sequence analysis, three CaPsol *tuf*-types, identified in both BN-affected grapevines and non-crop host plants, were associated with two distinct CaPsol ecological cycles related to bindweed (*tuf*-type b) and nettle [*tuf*-type a and ab (formerly known as b2)] (Langer and Maixner, 2004; Aryan *et al.*, 2014; Atanasova *et al.*, 2015). A study conducted in Eastern Europe reported the direct epidemiological role of chaste trees as CaPsol source in the *H. obsoletus*-mediated transmission to grapevine (Kosovac *et al.*, 2016). Moreover, in the Balkan region, it was recently highlighted that *H. obsoletus* population related to stinking hawk's beard can acquire CaPsol *tuf*-type b from this source plant and transmit it to grapevine (Kosovac *et al.*, 2019). A larger genetic diversity among CaPsol strains was described by molecular characterization of less conserved genes (*i.e.*, *secY*, *vmp1*, and *stamp*) (Murolo and Romanazzi, 2015; Quaglino *et al.*, 2016). Interestingly, studies focused on *stamp* gene molecular markers improved the knowledge on CaPsol strain population structure and dynamics (ROBERTO Pierro *et al.*, 2018), revealing the phytoplasma transmission ways in vineyard agro-ecosystems (Mori *et al.*, 2015; Kosovac *et al.*, 2016). In detail, it was found that CaPsol strains grouped in diverse *stamp*-based phylogenetic clusters are associated with bindweed- and nettle-related ecological cycles (Aryan *et al.*, 2014; Atanasova *et al.*, 2015; Plavec *et al.*, 2015). Moreover, recent findings highlighted that in Tuscany (central Italy) BN is prevalently associated with a CaPsol strain never found before in grapevine but detected exclusively in other host plants (Roberto Pierro *et al.*, 2018). This reinforced the evidence of the existence of alternative BN epidemiological cycles that could include other insect vectors able to transmit CaPsol to grapevine. Thus, in the present study, we investigated the composition of Auchenorrhyncha community in vineyards where CaPsol is significantly spreading, identified putative insect vectors and conducted transmission trials,

along with molecular analyses to investigate the capability of prevalent species of Cixiidae and Cicadellidae to transmit CaPsol to grapevine.

3- Materials and methods

The survey was conducted in Franciacorta grape-growing area, a gently rolling hilly zone in Lombardy Region (Northern Italy) bordering Lake Iseo. The Franciacorta is the most important Italian area for the production of sparkling wines with bottle fermentation. The grapes (more than 1,200 ha) are Chardonnay, Pinot noir, Pinot blanc and Erbamat (autoctonous variety). The BN incidence was investigated since 2012 in 30 vineyards by symptoms observations, molecular CaPsol investigation and spatial distribution analysis of vectors and symptomatic grapevines. In one of these, the population density of *H. obsoletus* was very low within the vineyard and the adults were localized only on nettle along the borders, while few individuals of *R. panzeri* were observed in the nearby forest. The BN incidence in this vineyard was extremely high (around 30% of symptomatic grapevines in 2012 and more than 50% in 2015) with scattered distribution of symptomatic grapevines no correlated with known vector spatial distributions. To identify alternative insect vectors, molecular investigation on Auchenorrhyncha community and symptomatic grapevines (in 2013 and 2014) and transmission trials (in 2015–2017) were conducted.

3.1. Characteristics of target vineyard

The investigation on insect vectors of CaPsol was conducted in a 10-year-old Chardonnay organic vineyard divided into two parts bordering a forest composed of broadleaf species (mainly *Castanea sativa* Mill., *Fraxinus ornus* L., *Quercus pubescens* Willd.) (N 45°35′38.12″, E 10°09′34.32″). In the both parts of the vineyard, the rows were north-south oriented and grapevines, on Kober 5BB rootstock, were trained using the Guyot system (distance between rows 2 m, plant distance along the row 0.8 m, for a total of 3876 vines). Ground cover was typical of Northern Italy with spontaneous grasses and broadleaf species. Among the agricultural practices adopted, spring weeding on the row, mowing between rows and two insecticide treatments with pyrethrum (applied at the end of June) against *Scaphoideus titanus* Ball should be mentioned because they could interfere with vineyard colonization by insects.

3.2. Insect and grapevine samples collection

During 2013 and 2014, insects were monitored and captured every week, from May to September, by yellow sticky traps (placed within, around the borders and in the neighbourhood of the vineyard in a regular grid), sweep entomological net and pooter. The traps (21 cm × 40 cm, SuperColor Giallo®, Serbios) were positioned in the canopy of the grapevines on the support wire and on grass 0.5 m above the ground with poles. All captures were stored in ethanol 90% and identified by stereomicroscope based on phenotypic characters (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Holzinger, Kammerlander and Nickel, 2003; Biedermann and Niedringhaus, 2009). The species of genus *Psammotettix* (Dahlbom) were considered together due to difficulties in classifying the live specimens without body dissection. Based on their size and number of captures, individuals of the same taxonomic group were pooled (1–3 specimens per pool) for further molecular analyses. Leaf petioles of symptomatic grapevine plants were also collected and stored at −30 °C. Observation of BN symptoms was made each year by the same two people. They inspected both sides of the plants in order to exclude other causes of similar symptoms (e.g. partial broken canes, *Stictocephala bisonia* Kopp and Yonke activity).

3.3. Molecular identification and characterization of '*Ca*. P. solani' in insects and grapevines

Total nucleic acids were extracted from 1150 insect pools (621 in 2013 and 529 in 2014) and leaf petioles of 60 grapevines (30 in 2013 and 2014) using the CTAB-based protocols described by Marzachì *et al* (1998) and Angelini *et al*.(2001), respectively. Specific detection of CaPsol was conducted by direct PCR using StampF/StampR0 primer pair followed by nested PCR with the StampF1/StampR1 primer pair, using mixtures and PCR conditions as described by Fabre *et al*.(2011). Total nucleic acids from periwinkle (*Catharanthus roseus* L. G. (Don)) plants infected by phytoplasma strains EY1 ('*Ca*. P. ulmi'), STOL ('*Ca*. P. solani'), and AY1 ('*Ca*. P. asteris') were used as reference controls. Total nucleic acids from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls. The presence of the nested PCR products was verified through electrophoresis on 1% agarose gel and visualized under UV transilluminator. Fifty-eight and 54 *stamp* PCR products (StampF1/StampR1), amplified from insect specimens and grapevine samples, respectively, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences

were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pair in the software BioEdit, version 7.2.6 (Hall, 1999). Nucleotide sequences of *stamp* gene, amplified from the CaPsol strains detected in the examined grapevine samples and insect specimens, were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analysed by Sequence Identity Matrix to estimate their genetic diversity. *Stamp* sequence variants, identified in the study, were aligned and compared with representative sequences of previously defined sequence variants (Pierro *et al.*, 2018); a nucleotide sequence identity of 100% was necessary for the attribution to such sequence variants.

3.4. '*Ca*. P. solani' phylogenetic analysis

Stamp gene nucleotide sequences of CaPsol representative strains of GuSt (*stamp*) sequence variants, identified in this and in previous studies (Pierro *et al.*, 2018), were aligned and used for generating unrooted phylogenetic trees by Neighbor-Joining method performed using the Jukes-Cantor model and bootstrap replicated 1000 times in the MEGA6 software (Tamura *et al.*, 2013). All positions with less than 95.0% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

3.5. Transmission trials

Based on the results obtained from Auchenorrhyncha community description and molecular analysis of CaPsol strains, insects to be tested in transmission trials for their CaPsol vectoring activity to grapevine were selected using the following three criteria simultaneously satisfied in at least 2013 and/or 2014: (i) high abundance (>25 individuals collected in the vineyard over the vegetative season); (ii) high infection rate (>10% of CaPsol-infected insect pools); (iii) the harbouring of CaPsol strain characterized by *stamp* sequence variant found also in grapevine.

A total of 550 individuals of the 10 selected insect species were captured in the examined vineyard on three sampling days in 2015 (June 11 and 25; July 7) and five days in 2016 (June 28; July 6 and 21; August 3 and 28). The specimens of each insect species were captured, on each sampling date, using sweep net and pooter on the canopy of symptomatic grapevines and on the grassing near them. The captured insects were kept in jars for transport to the laboratory for their classification. Collected insects were kept alive and in the

conditions required for survival and good fitness maintenance. After the classification, the insects were forced to feed on asymptomatic and PCR-tested phytoplasma-free potted grapevines (cv. Chardonnay) previously treated with hot water. Forty-four transmission trials were conducted in a greenhouse under controlled conditions [25 ± 3 °C, 70 ± 5 RH 16:8 (L:D) daily light cycle], located in Verona province (45°20′13.72″N; 11°13′03.28″E), and left till the end of adult survival. After this period, the plants were kept in an insect-free greenhouse in both years. On each date, one phytoplasma-free grapevine plant was maintained without insects as control. Dead insects were stored at −30 °C. CaPsol was detected by nested PCR-based amplification of *stamp* gene (Fabre, Danet and Foissac, 2011) and by SYBR Green real-time amplification assay (Galetto, Bosco and Marzachi, 2005) using as templates the total nucleic acids extracted from both the individual insect specimens and the petioles of grapevine plants collected in October 2015 and July 2016 for the trials performed in 2015, and in October 2016 and July 2017 for the trials performed in 2016. In real-time amplification assay, only amplified PCR products showing a Tm of 81.5 ± 0.2 °C and a Ct < 37 were associated with the presence of CaPsol, as previously described (Mori *et al.*, 2015). CaPsol strains detected in insects and plants were characterized through nucleotide sequence analyses of *stamp* amplicons as described above.

3.6. Plants eaten by the insects

In order to identify the plant species on which the insects have had their last meal, a molecular characterization of the gut contents has been performed for 154 insect specimens captured in the examined vineyard in 2016 simultaneously to those captured for transmission trials. A fragment (750 bp) of *rbcL* gene, coding for the plant plastid ribulose-bisphosphate carboxylase large subunit, was amplified by PCR using primers rbcL1F/rbcL724R as previously described (Taberlet *et al.*, 1991, 2007). Nucleotide sequences of *rbcL* gene, amplified from insect specimens, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics). Nucleotide sequences were assembled by the Contig Assembling Program, trimmed to the annealing sites of primer pair in the software BioEdit, compared to GeneBank through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast).

4- Results

4.1. Auchenorrhyncha community description

During field activities, 1018 and 896 adult insects were collected in 2013 and 2014, respectively. Stereomicroscope analysis, based on observation of morphological characters, allowed the identification of 48 distinct taxonomic groups, 44 defined at species level and four at genus level, belonging to a total of nine different families (Table 1). The most represented family was Cicadellidae, as 30 out of 48 taxonomic groups belong to this family, adding up to 377 individuals out of the 1018 captured in 2013 (37%) and 377 out of the 896 captured in 2014 (42%). The other most relevant families were Delphacidae (representing 17.7% and 18.5% of insects captured in 2013 and 2014, respectively), Cixiidae (representing 11% and 13.5% of insects captured in 2013 and 2014, respectively) (around the borders), and Aphrophoridae (representing 9.1% and 10.1% of insects captured in 2013 and 2014, respectively), mostly represented by the species *Laodelphax striatella*, *H. obsoletus*, *Philaenus spumarius*, respectively. Cercopidae (most of which belong to the *Cercopis vulnerata* species), Dictyopharidae (with *Dictyophara europaea* species) and Flatidae (with *Metcalfa pruinosa* species) families were highly represented with 24% and 15% of captured insects in 2013 and 2014, respectively. The remaining two families, Caliscelidae and Membracidae, had the lowest abundance (less than 1%) among captured insects. The grapevine leafhoppers *Empoasca vitis* (Göthe) and *Zygina rhamni* Ferrari were not considered, as their population density was very low.

4.2. Identification and molecular characterization of '*Ca*. P. solani' in insects and grapevines

PCR-based amplification of *stamp* gene revealed the presence of CaPsol in 63 out of 621 analyzed insect pools from 2013 and 82 out of 529 pools from 2014, belonging to 19 species in 2013 and 18 species in 2014, 12 of which are common to both years (Table 8). The amplification of *stamp* gene also revealed the presence of CaPsol in 54 grapevine leaf samples (29 out of 30 in 2013, and 25 out of 30 in 2014).

Table 8 Captures and infection rate of CaPsol potential insect vectors in the examined vineyard in 2013 and 2014.

Family	**Subfamily**	**Insect**	**2013**				**2014**			
			Number of captured specimens	**Number of pools**	**Number of CaPsol infected pools**	**Proportion of infected pools**	**Number of captured specimens**	**Number of pools**	**Number of CaPsol infected pools**	**Proportion of infected pools**
Cixiidae	Cixiinae	*Cixius spp*. Latreille, 1804	2	2	—	—	—	—	—	—
Cixiidae	Cixiinae	*Hyalesthes obsoletus* Sign., 1865	100	44	13	30	113	43	10	23
Cixiidae	Cixiinae	*Hyalesthes scotti*Ferrari, 1882	8	6	—	—	4	2	—	—
Cixiidae	Cixiinae	*Reptalus spp*. Emeljanov, 1971	2	2	—	—	4	4	—	—
Delphacidae	Asiracinae	*Asiraca clavicornis (*F., 1794)	6	6	2	33	—	—	—	—
Delphacidae	Delphacinae	*Dicranotropis hamata* (Boh., 1847)	31	15	2	13	12	7	1	14
Delphacidae	Delphacinae	*Laodelphax striatella* (Fall., 1826)	121	56	6	11	71	30	2	7
Delphacidae	Delphacinae	*Toya propinqua*(Fieb., 1866)	20	9	—	—	81	24	2	8
Delphacidae	Kelisiinae	*Kelisia guttulifera*(Kbm., 1868)	2	2	—	—	—	—	—	—
Delphacidae	Stenocraninae	*Stenocranus major*(Kbm., 1868)	—	—	—	—	2	2	—	—
Dictyopharidae	Dictyopharinae	*Dictyophara europaea* (L., 1767)	47	47	—	—	59	59	12	20
Flatidae	Flatinae	*Metcalfa pruinosa*(Say, 1830)	68	35	9	26	79	41	1	2
Cercopidae	Cercopinae	*Cercopis vulnerata*Rossi, 1807	120	120	2	2	—	—	—	—
Cercopidae	Cercopinae	*Haematoloma dorsatum* (Ahr., 1812)	12	7	—	—	—	—	—	—
Aphrophoridae	Aphrophorinae	*Philaenus spumarius* (L., 1758)	93	28	2	7	91	53	14	26
Membracidae	Centrotinae	*Centrotus cornutus* (L., 1758)	1	1	—	—	—	—	—	—
Membracidae	Smiliinae	*Stictocephala bisonia* Kopp & Yon, 1977	1	1	—	—	3	3	1	33
Cicadellidae	Agallinae	*Anaceratagallia*	—	—	—	—	5	4	—	—

		ribauti (Oss., 1938)								
Cicadellidae	Aphrodinae	*Aphrodes makarovi* Zachv., 1948	29	21	5	24	2	2	—	—
Cicadellidae	Cicadellinae	*Cicadella viridis*(L., 1758)	43	35	6	17	142	123	21	17
Cicadellidae	Cicadellinae	*Evacanthus acuminatus* (F., 1794)	3	3	2	67	—	—	—	—
Cicadellidae	Deltocephalinae	*Anoplotettix fuscovenosus*(Ferr., 1882)	13	12	1	8	—	—	—	—
Cicadellidae	Deltocephalinae	*Arthaldeus striifrons* (Kbm., 1868)	2	1	—	—	2	1	—	—
Cicadellidae	Deltocephalinae	*Aconurella prolixa*(Leth., 1885)	1	1	—	—	6	3	—	—
Cicadellidae	Deltocephalinae	*Allygidius furcatus*(Ferr., 1882)	25	24	2	8	33	9	1	10
Cicadellidae	Deltocephalinae	*Euscelis incisus*(Kbm., 1858)	30	15	2	13	11	10	1	10
Cicadellidae	Deltocephalinae	*Euscelidius variegatus* (Kbm., 1858)	58	31	3	10	21	20	1	5
Cicadellidae	Deltocephalinae	*Fieberiella florii*(Stål, 1864)	5	5	1	20	—	—	—	—
Cicadellidae	Deltocephalinae	*Goniagnathus brevis* (H.-S., 1835)	5	5	—	—	—	—	—	—
Cicadellidae	Deltocephalinae	*Hishimonus hamatus* Kuoh, 1976	4	4	—	—	13	13	1	8
Cicadellidae	Deltocephalinae	*Japananus hyalinus* (Osb., 1900)	2	2	—	—	10	5	1	20
Cicadellidae	Deltocephalinae	*Jassargus flori*(Fieb., 1869)	—	—	—	—	17	9	—	—
Cicadellidae	Deltocephalinae	*Macrosteles cristatus* (Rib., 1927)	5	4	—	—	1	1	—	—
Cicadellidae	Deltocephalinae	*Mocydia crocea*(H.-S., 1837)	4	2	—	—	5	3	—	—
Cicadellidae	Deltocephalinae	*Mocydiopsis spp*. Rib. 1939	2	2	—	—	1	1	—	—
Cicadellidae	Deltocephalinae	*Neoaliturus fenestratus* (H.-S., 1834)	5	2	1	50	—	—	—	—
Cicadellidae	Deltocephalinae	*Ophiola sp*. Edwards, 1922	—	—	—	—	1	1	—	—
Cicadellidae	Deltocephalinae	*Orientus ishidae*(Mats., 1902)	—	—	—	—	8	8	6	75
Cicadellidae	Deltocephalinae	*Psammotettix alienus/confinis*(Dhlb, 1850)	30	12	—	—	81	34	4	12
Cicadellidae	Deltocephalinae	*Scaphoideus titanus* (Ball, 1932)	4	1	1	100	8	8	2	25

Cicadellidae	Deltocephalinae	*Thamnotettix zelleri* (Kbm., 1868)	5	5	1	20	9	5	1	20
Cicadellidae	Idiocerinae	*Balcanocerus larvatus* (H.-S., 1835)	5	5	—	—	—	—	—	—
Cicadellidae	Macropsinae	*Hephathus nanus*(H.-S., 1835)	70	25	—	—	1	1	—	—
Cicadellidae	Macropsinae	*Macropsis fuscula*(Zett., 1828)	13	6	—	—	—	—	—	—
Cicadellidae	Megophthalminae	*Megophthalmus scanicus* (Fall. 1806)	4	4	2	50	—	—	—	—
Cicadellidae	Typhlocybinae	*Dikraneura variata*Hardy, 1850	1	1	—	—	—	—	—	—
Cicadellidae	Typhlocybinae	*Typhlocyba quercus* (F., 1777)	9	5	—	—	—	—	—	—
Caliscelidae	Caliscelinae	*Caliscelis bonellii*(Latreille, 1807)	7	7	—	—	—	—	—	—
	Total	1018	621	63	10	896	529	82	16	

Table 9 Stamp sequence variants of CaPsol identified in symptomatic grapevines and potential insect vectors.

Species	Year	CaPsol infected pools	Sequenced	CaPsol strain (*stamp* gene sequence variant)					
				GuSt1 (St5)	**GuSt2 (St11)**	**GuSt3 (St19)**	**GuSt4 (St21)**	**GuSt5 (St30)**	**GuSt6 (St36)**
Vitis vinifera L., 1753	2013	29	29	15	5	4	—	5	—
	2014	25	25	12	7	4	—	2	—
Hyalesthes obsoletus Sign., 1865	2013	13	5	4	—	—	—	1	—
	2014	10	4	1	1	—	1	1	—
Asiraca clavicornis (F., 1794)	2013	2	1	1	—	—	—	—	—
Dicranotropis hamata (Boh., 1847)	2013	2	1	—	1	—	—	—	—
	2014	1	1	1	—	—	—	—	—
Laodelphax striatella (Fall., 1826)	2013	6	3	3	—	—	—	—	—
	2014	2	1	1	—	—	—	—	—
Toya propinqua (Fieb., 1866)	2014	2	1	1	—	—	—	—	—
Dictyophara europaea (L., 1767)	2014	12	5	2	—	—	—	2	1
Metcalfa pruinosa (Say, 1830)	2013	9	1	1	—	—	—	—	—
	2014	1	—	—	—	—	—	—	—
Cercopis vulnerata Rossi, 1807	2013	2	2	2	—	—	—	—	—
Philaenus spumarius (L., 1758)	2013	2	2	—	—	2	—	—	—
	2014	14	4	3	—	—	—	1	—
Stictocephala bisonia Kopp & Yon, 1977	2014	1	—	—	—	—	—	—	—

Aphrodes makarovi Zachv., 1948	2013	5	3	3	—	—	—	—	—
Cicadella viridis (L., 1758)	2013	6	2	2	—	—	—	—	—
	2014	21	5	3	—	—	—	2	—
Evacanthus acuminatus (F., 1794)	2013	2	1	—	—	—	—	—	1
Allygidius furcatus (Ferr., 1882)	2013	2	1	1	—	—	—	—	—
	2014	1	1	—	—	—	—	—	1
Anoplotettix fuscovenosus (Ferr., 1882)	2013	1	1	—	—	—	—	—	1
Euscelis incisus (Kbm., 1858)	2013	2	1	1	—	—	—	—	—
	2014	1	1	—	—	1	—	—	—
Euscelidius variegatus (Kbm., 1858)	2013	3	2	2	—	—	—	—	—
	2014	1	1	1	—	—	—	—	—
Fieberiella florii (Stål, 1864)	2013	1	1	—	—	—	—	—	1
Hishimonus hamatus Kuoh, 1976	2014	1	1	—	—	—	—	1	—
Japananus hyalinus (Osb., 1900)	2014	1	1	1	—	—	—	—	—
Neoaliturus fenestratus (H.-S., 1834)	2013	1	—	—	—	—	—	—	—
Orientus ishidae (Mats., 1902)	2014	6	2	1	—	1	—	—	—
Psammotettix alienus/confinis (Dhlb, 1850)	2014	4	3	2	—	—	—	1	—
Scaphoideus titanus Ball, 1932	2013	1	—	—	—	—	—	—	—
	2014	2	—	—	—	—	—	—	—
Thamnotettix zelleri (Kbm., 1868)	2013	1	—	—	—	—	—	—	—
	2014	1	—	—	—	—	—	—	—
Megophthalmus scanicus (Fall. 1806)	2013	2	—	—	—	—	—	—	—

Table 10 Identification of CaPsol insect vectors by transmission trials to grapevine and molecular analyses in 2015 and 2016. Sympt, potted grapevines with typical BN symptoms (yellowing and downwards rolling of leaves and lack of cane lignifications).

Species	**Trial year**	**Number of insects**				**Number of grapevines**		
		Total	**Infected**	**Sequenced**	**CaPsol strain (number)**	**Total**	**Infected (CaPsol strain)**	
							October (same year)	**July (following year)**
***Hyalesthes obsoletus* Sign., 1865**	2015	15	8	8	St5 (8)	2	2 (St5)	2 (St5)
	2016	24	22	4	St5 (3); St19 (1)	2	—	1 (St5) Sympt
***Dicranotropis hamata* (Boh., 1847)**	2015	120	5	3	St5 (3)	3	—	2 (St5)
	2016	4	—	—	—	1	—	1
***Laodelphax striatella* (Fall., 1826)**	2015	46	—	—	—	2	—	—
	2016	16	12	6	St5 (4); St11 (1); St19 (1)	2	—	1 (St5)
***Dictyophara europaea* (L., 1767)**	2016	26	25	5	St5 (5)	3	—	1 (St5)
***Philaenus spumarius* (L., 1758)**	2016	20	18	3	St5 (3)	4	—	2 (St5)
***Aphrodes makarovi* Zachv., 1948**	2016	5	4	2	St5 (2)	2	—	1 (St5)
***Cicadella viridis* (L., 1758)**	2015	16	—	—	—	1	—	—
	2016	15	13	—	—	1	—	—
***Euscelis incisus* (Kbm., 1858)**	2015	12	4	4	St5 (3); St19 (1)	3	—	3 (St5)
	2016	20	18	12	St5 (11); St19 (1)	3	—	3 (St5) Sympt
***Euscelidius variegatus* (Kbm., 1858)**	2015	10	—	—	—	3	—	—
	2016	52	51	12	St5 (12)	5	1 (St5)	2 (St5)
***Psammotettix alienus/confinis* (Dhlb, 1850)**	2015	100	—	—	—	3	—	—
	2016	49	43	16	St5 (14); St11 (1); St19 (1)	4	—	2 (St5)
No insect (control)	2015	—	—	—	—	3	—	—
	2016	—	—	—	—	5	—	—

Table 11 Identification of the plants present in the gut of the insect species tested in CaPsol transmission trials by *rbcL* gene amplification and sequence analysis.

Species	Number of insects		Plant in the insect gut		
	Analyzed	*rbcL* PCR-positive	Number of insects	GenBank closest relative plant (Acc. No.)	% Identity
Hyalesthes obsoletus Sign., 1865	23	1	1	*Vitis vinifera* (MG946878)	99
Dicranotropis hamata (Boh., 1847)	8	2	1	*Urtica dioica* (MG946931)	99
			1	*Vitis vinifera* (AJ419718)	97
Laodelphax striatella (Fall., 1826)	10	—	—	—	—
Dictyophara europaea (L., 1767)	10	6	3	*Vitis vinifera* (MG946878)	99
			1	*Crepis elongata* (JQ933285)	98
			1	*Urtica dioica* (MG946931)	98
			1	*Pisum sativum* (MG917089)	99
Philaenus spumarius (L., 1758)	16	11	7	*Vitis vinifera* (MG946878)	99
			1	*Vicia cracca* (KP699058)	98
			1	*Potentilla hebiichigo* (MG742490)	96
			1	*Urtica dioica* (MG946931)	94
			1	*Daucus pumilus* (KX832312)	98
Aphrodes makarovi Zachv., 1948	1	1	1	*Peltophorum pterocarpum* (AM234243)	95
Cicadella viridis (L., 1758)	24	8	5	*Vitis vinifera* (MG946878)	98
			1	*Crepis capillaris* (KM360738)	98
			1	*Laportea interrupta* (KM586531)	87
			1	*Pisum sativum* (MG917089)	95
Euscelis incisus (Kbm., 1858)	22	20	10	*Vitis vinifera* (MG946878)	99
			2	*Vicia spp.* (KP699053)	98
			1	*Potentilla spp.* (MG742490)	96
			1	*Crepis spp.* (KF602078)	93
			1	*Pisum sativum* (MG859922)	95
			1	*Corylus spp.* (MF996573)	99
			1	*Xanthoceras sorbifolium* (KP088923)	81
			1	*Ternstroemia gymnanthera* (MF179490)	96
			1	*Musa coccinea* (MH603431)	96
			1	*Nicotiana sylvestris* (KM025249)	98
Euscelidius variegatus (Kbm., 1858)	17	10	6	*Vitis vinifera* (MG946878)	98
			2	*Nicotiana spp.* (KU199713)	98
			1	*Berberidopsis corallina* (EU002274)	96
			1	*Quiina glaziovii* (JX664069)	78
Psammotettix alienus/confinis (Dhlb, 1850)	23	—	—	—	—

Table 12: Host plants, hosting/vectoring CaPsol, occurrence, and biology of alternative CaPsol vectors.

Species	Host plant	Survival on grapevine	Host/vector of CaPsol	Abundance, development cycle, adult presence in Northern Italian vineyards
Dicranotropis hamata (Boh., 1847)	Poaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	not available	Host (Sanna *et al.*, 2016)	Uncommon – 2 generations/year. Overwintering as nymphal stage. Adult presence: mid-April - end of October(Nickel and Remane, 2002; Holzinger, Kammerlander and Nickel, 2003; Biedermann and Niedringhaus, 2009)
Laodelphax striatella (Fall., 1826)	Poaceae, Juncaceae, Cyperaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	not available	host(Sabaté, Laviña and Batlle, 2003)	Common – 2 generations/year. Overwintering as egg stage. Adult presence: beginning of June - end of November(Nickel and Remane, 2002; Holzinger, Kammerlander and Nickel, 2003; Biedermann and Niedringhaus, 2009)
Dictyophara europaea (L., 1767)	Polyphagous(Nickel and Remane, 2002)	2–6 days(Filippin *et al.*, 2009)	Host (Filippin *et al.*, 2009; Cvrković *et al.*, 2011)	Common – 1 generation/year. Overwintering as egg stage. Adult presence: end of June - beginning of October(Krstic *et al.*, 2016)
Philaenus spumarius (L., 1758)	Polyphagous(Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	>10 days (Cornara *et al.*, 2016)	Host (Cvrković *et al.*, 2011)	Common – 1 generation/year. Overwintering as egg stage. Adult presence: May - beginning of November (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)
Aphrodes makarovi Zachv., 1948	*Urtica dioica*, *Taraxacum, Cirsium*(Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	not available	Host (Sanna *et al.*, 2016)	Uncommon – 1 generation/year. Overwintering as egg stage. Adult presence: End of May - beginning of November (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)
Euscelis incisus (Kbm., 1858)	Poaceae, Fabaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	3 days (Alma *et al.*, 2001)	vector(Brčák, 1979)	Common – 3 generations/year. Overwintering as nymphal stage. Adult presence: February - mid of November (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)
Euscelidius variegatus (Kbm., 1858)	Poaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	3 days (Alma *et al.*, 2001)	vector(Laviña, Sabaté and Battle, 2006)	Common – 3 generations/year. Overwintering as adult stage. Adult presence: Mid of April – October (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)
Psammotettix alienus (Dhlb, 1850)	Poaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	not available	host(Cvrković *et al.*, 2011)	Common – 2/3 generations/year. Overwintering as egg stage. Adult presence: Mid of June - beginning of October (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)
Psammotettix confinis (Dhlb, 1850)	Poaceae (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)	not available	—	Common – 2/3 generations/year. Overwintering as egg stage. Adult presence: Mid of June - beginning of October (Ribaut, Le Quesne and De Jong, 1952; Ossiannilsson, 1981; Nickel and Remane, 2002)

The 112 *stamp* fragments (StampF1/StampR1) of the expected size, amplified in 2013 and 2014 (58 from insects and 54 from grapevines) were sequenced (Table 9). Based on the nucleotide sequence identity, six *stamp* sequence variants (here named GuSt1 to GuSt6) were identified. CaPsol strains characterized by *stamp* sequence variants GuSt1, GuSt2, GuSt3 and GuSt5 were identified in both insects and grapevines, while GuSt4 and GuSt6 were found only in insects (Table 9). Comparison of such sequence variants (GuSt1 to GuSt6) with the previously published dataset (Pierro *et al.*, 2018) revealed that they were identical to the previously described sequence variants St5 [representative strain GGY, GenBank Accession Number (Acc. No.) FN813256], St11 (representative strain 19–25, Acc. No. FN813267), St19 (representative strain CrHo13_1183, Acc. No. KJ469719), St21 (representative strain Aa21, Acc. No. KJ145380), St30 (representative strain Vv24, Acc. No. KC703022) and St36 (representative strain Carv2, Acc. No. KT184880), respectively (Table 9). In detail, in 2013 and 2014, insects contained CaPsol strains characterized by *stamp* sequence variants St5 (63.8%), St11 (3.5%), St19 (6.9%), St21 (1.7%), St30 (15.5%) and St36 (8.6%). Grapevines harboured CaPsol strains characterized by *stamp* sequence variants St5 (50%), St11 (22.2%), St19 (14.8%) and St30 (13%) (Table 9).

The alignment of *stamp* nucleotide sequences of CaPsol strains representative of the GuSt sequence variants identified in Gussago (GuSt1 to GuSts6) and those previously described (St1 to St58)(Pierro *et al.*, 2018) was used for generating a phylogenetic tree in which three main clusters (b-I, b-II, b-III) and two subclusters (a1 and a2) were observed. CaPsol strains identified in the present study are found in four of these groups, as no strain clusters within the b-I cluster. CaPsol strains sharing the *stamp* sequence variant St19 (12 strains) grouped in the nettle-related subcluster a2; those with sequence variant St11 (14 strains) grouped in the nettle-related subcluster a1; those with sequence variants St5, St21 and St30 (81 strains) grouped in the bindweed-related cluster b-II; those with sequence variant St36 (five strains) in the bindweed-related cluster b-III (Fig. 16, 17).

4.3. Identification of '*Ca*. P. solani' insect vectors by transmission trials

Based on the criteria applied to select potential CaPsol insect vectors to be tested, transmission trials were conducted in 2015 and/or 2016 on the insect species *Aphrodes makarovi* Zachvatkin, *Cicadella viridis* L., *Dicranotropis hamata* Boheman, *Dictyophara europaea* Spinola, *Euscelis incisus* Kirschbaum, *Euscelidius variegatus* Kirschbaum, *Hyalesthes obsoletus*, *Laodelphax striatella*, *Philaenus spumarius* and *Psammotettix*

alienus/confinis (Table 10). Due to the fact that dead specimens of both *P. alienus* and *P. confinis* were found in the cage after the transmission period, it is not possible to distinguish the species transmission ability. In the 2015 transmission trials, *stamp* gene amplification and SYBR Green real-time amplification assay allowed the detection of CaPsol in *D. hamata*, *E. incisus* and *H. obsoletus*specimens, and in leaf samples (collected one year after the transmission trials, July 2016) of the grapevines on which each of these insect species was forced to feed. Only grapevines hosting *H. obsoletus* were found infected by CaPsol as early as October 2015, the end of the growing season in which transmission trials were conducted. No amplification was obtained from insect specimens of *C. viridis*, *E. variegatus*, *L. striatella*, or *P. alienus/confinis* nor from the leaf samples of the grapevines on which each of these insect species was forced to feed (Table 10).

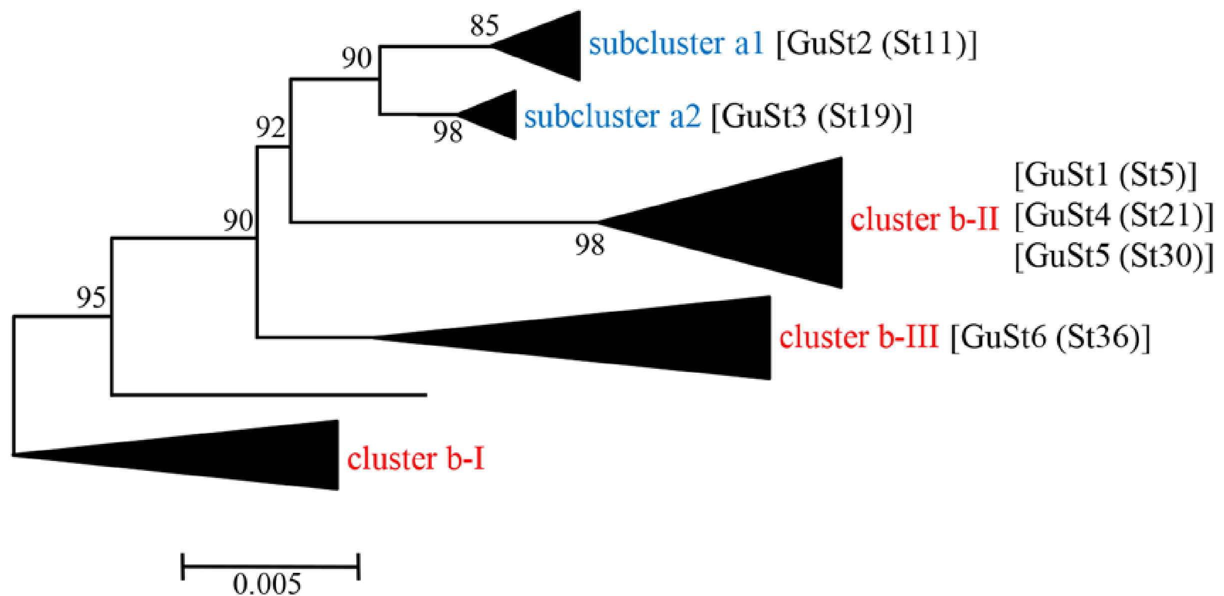

Figure 16: Unrooted phylogenetic tree inferred from stamp gene nucleotide sequences of BNp strains representative of stamp sequence variants identified in this study (Table 9) and previously described (Pierro et al., 2018). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.33585582 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 64 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 495 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

In the 2016 transmission trials, *stamp* gene amplification and SYBR Green real-time amplification allowed the detection of CaPsol in *A. makarovi*, *D. europaea*, *E. incisus*, *E. variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius* and *P. alienus/confinis* specimens, and in

leaf samples (collected one year after the transmission trials, July 2017) of the grapevines on which each of these insect species was forced to feed.

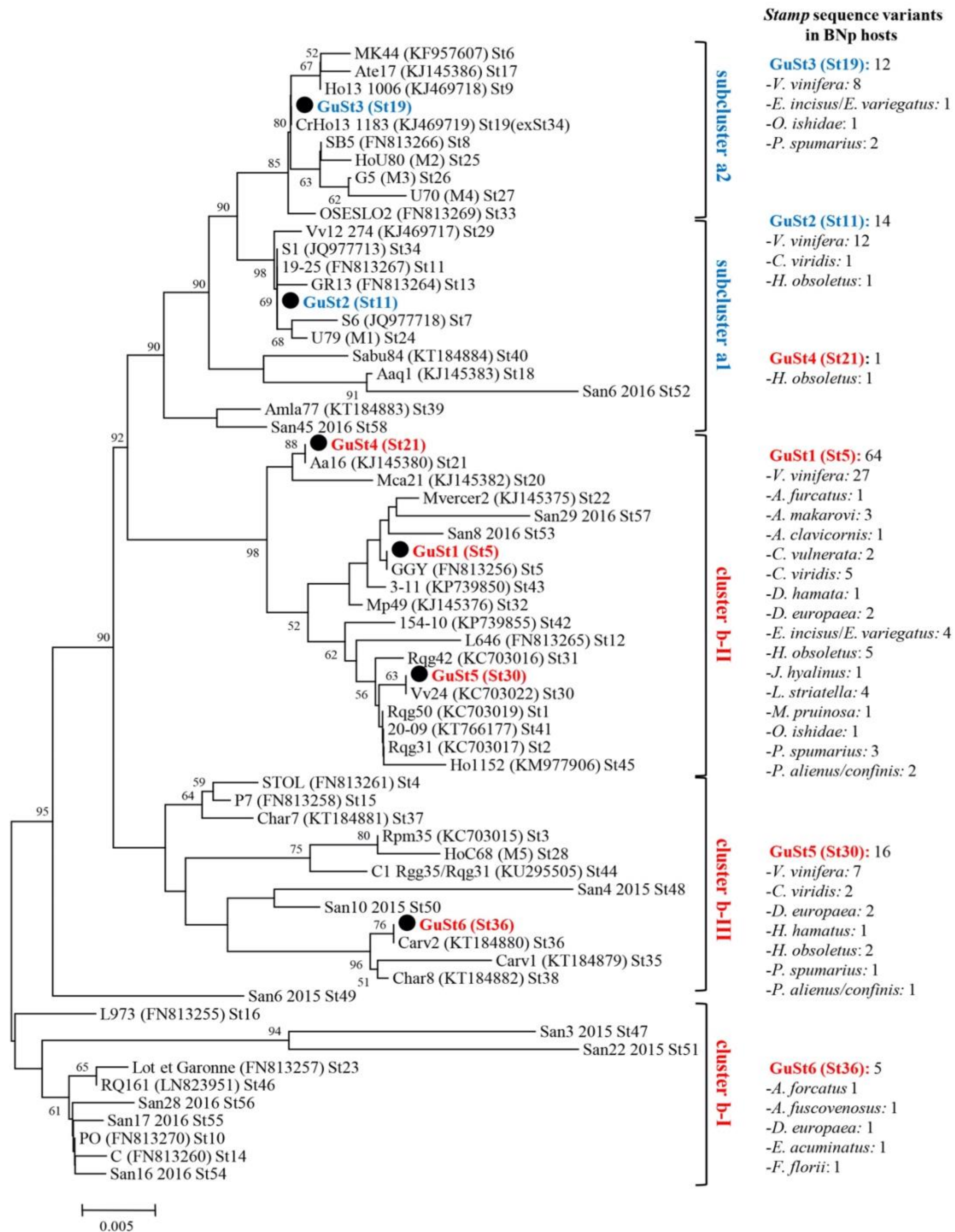

Figure 17 Stamp sequence variants in BNp hosts grouped in phylogenetic clusters

Only one grapevine plant hosting *E. variegatus* was found to be infected by CaPsol as early as October 2016, the end of the growing season in which transmission trials were conducted. No amplification was obtained from insect specimens of *D. hamata*; despite this, the

grapevine plant on which this insect was forced to feed was found to be infected. On the contrary, CaPsol was detected in *C. viridis* specimens but no amplification was obtained from the grapevine plant on which this insect was forced to feed. Furthermore, in both years no amplification was obtained from control plants, kept in controlled conditions without insects (Table 10). Moreover, *stamp* gene nucleotide sequences were obtained from 75 representative CaPsol-harbouring insect specimens forced to feed on the 24 grapevine plants that were found to be CaPsol-infected after the transmission trials.

Sequence identity analysis evidenced that (i) all 24 infected grapevines harboured CaPsol strains characterized by the same *stamp* sequence variant (St5); (ii) in each of the nine insect species forced to feed on these 24 grapevines (all species used in the transmission bioassay except *C. viridis*) the prevalent CaPsol strains were characterized by the same *stamp* sequence variant (St5, 68 out of 75 specimens); CaPsol strains characterized by the *stamp* sequence variant St19 and St11 were identified in five and two out of 75 insect specimens, respectively; (iii) at least one insect specimen that was forced to feed on each of the 24 infected grapevines harboured CaPsol strain characterized by the *stamp* sequence variant St5 (Table 10). Yellowing and downwards rolling of leaves and lack of cane lignification were observed in September 2018 in one and two grapevines where *H. obsoletus* and *E. incisus* were confined, respectively.

4.4. Plants eaten by alternative insect vectors

rbcL gene was amplified in 59 out of 154 insects of eight species. No amplification was obtained for *L. striatella* and *P. alienus/confinis*. BLAST analysis of *rbcL* nucleotide sequences showed that, with the exception of *A. makarovi*, grapevine was the most prevalent plant on which all the insects have had their last meal. Moreover, specimens of the species *D. hamata*, *D. europaea*, *E. incisus*, and *P. spumarius* had their last meal on nettle and/or *Crepis*spp. (Table 11).

5- Discussion

In Euro-Mediterranean regions the main insect vector of '*Ca*. P. solani' (CaPsol) is *Hyalesthes obsoletus* (Langer and Maixner, 2004; Kosovac *et al.*, 2016). Management of its host plants in the vineyards and surrounding areas is therefore considered crucial for BN control (Mori *et al.*, 2012, 2016). The existence of additional vectors has been theorized from the observations that BN incidence is not always correlated to high densities or presence

of *H. obsoletus* populations (Batlle, Martinez and Laviña, 2000; Chuche *et al.*, 2016; ŠafářoVá *et al.*, 2018). Thus, several studies, conducted to discover alternative insect vectors, detected more than 35 insect species harbouring CaPsol, 16 of which were found to be able to transmit this phytoplasma (Batlle *et al.*, 2008; Riedle-Bauer, Sára and Regner, 2008; Oliveri *et al.*, 2015; Chuche *et al.*, 2016; ŠafářoVá *et al.*, 2018; Trivellone, 2019). Among these 16 species, only *Reptalus panzeri* and *Macrosteles quadripunctulatus* Fieber have been proven as vectors of CaPsol to grapevine plants in Serbia and Spain (Batlle *et al.*, 2008; Cvrković *et al.*, 2014). The small number of effective vectors compared to CaPsol-infected insects is related to the specific phytoplasma-vector recognition mechanism (Suzuki *et al.*, 2006; Weintraub and Beanland, 2006), which involves the binding of insect cytoskeleton proteins with the antigenic membrane protein encoded by the CaPsol *stamp* gene (Fabre, Danet and Foissac, 2011). Thus, *stamp*-based molecular typing of CaPsol strains has been employed to identify its insect vectors and transmission routes in this and previous studies (Chuche *et al.*, 2016; Kosovac *et al.*, 2016).

In order to optimize the experimental conditions allowing identification of CaPsol insect vectors, this study was conducted in a vineyard where BN incidence was extremely high without any correlation with *H. obsoletus* density and distribution, the ground cover had many CaPsol host plants, and the agro-ecosystem included a high plant biodiversity (presence of forest, grass and broadleaf species). In fact, probably due to this high biodiversity, many more insect species were found in the target area compared to a previous study conducted in Lombardy Region (Nicoli Aldini, 2001). The differences in insect population presence and density observed in 2013 and 2014 should be explained by diverse climate conditions (dry and cold in 2013, wet and hot in 2014) and consequently by grass cover management within and around the vineyard, such as cutting and/or mowing, that affect the Auchenorrhyncha population density as previously demonstrated (Mori *et al.*, 2016).

Within the investigated Auchenorrhyncha community, nine potential insect vectors, besides *H. obsoletus*, were identified according to high abundance in the vineyard agro-ecosystem, high CaPsol-infection rate, and harbouring CaPsol strains characterized by *stamp* gene sequence variants undistinguishable from those found in symptomatic grapevines.

Interestingly, except *D. hamata*, an extraordinary infection rate was found in the tested insects, in agreement with data obtained in previous study conducted on other phytoplasma insect vectors (Bosco *et al.*, 2014). The different temperatures registered in the two investigated years could also explain the diverse CaPsol-infection rates observed, as

reported for *Candidatus*Phytoplasma asteris (CYP) in *Chrysanthemum carinatum* and its vector *Macrosteles quadripunctulatus* (Murral *et al.*, 1996; Maggi *et al.*, 2014). In this CYP epidemics the mean latency period in the insect and in the host plant was faster at high temperatures than low ones as consequence of faster phytoplasma multiplication in the host plants and higher frequency of feeding bouts of vectors at higher temperatures (Maggi *et al.*, 2014).The difference in the infection rate between the two investigated years was probably due to the diverse insect collection period.

Transmission trials proved that eight insect species are vectors of CaPsol to grapevine. Among these, *Euscelidius variegatus* and *Euscelis incisus* were previously reported as able to transmit the pathogen to *in vitro* grapevine plantlets (Laviña, Sabaté and Battle, 2006) and to solanaceous plants (Brčák, 1979), respectively; the other six insect species (*Dicranotropis hamata, Laodelphax striatella*, *Dictyophara europaea, Philaenus spumarius, Aphrodes makarovi*, *Psammotettix alienus*/*confinis*) were found harbouring CaPsol in vineyards but there was no evidence of transmission to grapevine (Table 12). Moreover, *D. hamata* and *L. striatella* are related to the species *Javesella discolor* (Boheman 1847) (Delphacidae, Delphacinae) proved to be able to transmit CaPsol to artificial diet medium (Trivellone, 2019); *Psammotettix alienus*/*confinis* is related to the species *Psammotettix striatus*, previously proved to be able to transmit CaPsol to artificial diet medium (Sabaté, Laviña and Batlle, 2003); *A. makarovi* is related to the species *A. bicincta* proved to be able to transmit CaPsol to various plants in Europe (Brčák, 1979).

The eight alternative insect vectors were found largely infected by St5 CaPsol strain, prevalent in symptomatic grapevines in the examined vineyard. Even if St11 and St19 CaPsol strains were present in individuals used in transmission trials, the insects were able to vector exclusively the St5 CaPsol strain to adult grapevines. Interestingly, St5 CaPsol strain grouped in the bindweed-related *stamp* phylogenetic cluster b-II, and was extensively reported in vineyard agro-ecosystems in Italy, Austria, Germany, Macedonia, Serbia and Slovenia (Cvrković *et al.*, 2011; Aryan *et al.*, 2014; Atanasova *et al.*, 2015; Murolo and Romanazzi, 2015; Pierro *et al.*, 2018). It is reasonable to hypothesize that alternative insect vectors, identified in this study, can play a role in the transmission to grapevine of at least St5 CaPsol strain in Europe. Due to their wide geographical distribution, the alternative insect vectors could be involved in CaPsol spreading also in worldwide. Furthermore, St11 and St19 CaPsol strains, grouping in the nettle-related subclusters a1 and a2, respectively, were found in 37% of the examined grapevines but in only 10% of the analyzed insects. As no insects were able

to transmit such phytoplasma strains to grapevine in these experimental conditions, further investigation is necessary.

The potential significance in BN epidemiology of the insect species identified as new CaPsol vectors to grapevine is strictly related to their ecology. Considering their abundance, development cycle and adult presence in Northern Italian vineyards, known host plants, and feeding preference towards grapevine and/or common weeds in vineyards (Table 12), it is reasonable to hypothesize that *D. hamata*, *D. europaea*, *P. spumarius*, *E. incisus*, and *E. variegates* could play a role in the transmission of CaPsol to grapevine at least in the examined area. In fact, even some of them are known as oligophagous on Poaceae, in the present study these insects were found feeding on grapevine (Table11). Moreover, as previously reported, these insects can survive on grapevine for at least two days (Table 12). Regarding *P. spumarius*, a detailed study on its feeding habit on olive revealed no indication of any puncturing of phloem tissue (Cornara *et al.*, 2018). Nevertheless, since *P. spumarius* has been reported as a vector of the elm yellows phytoplasma (Marcone, 2017), in certain conditions and on certain hosts, the insect might divert its behavior from xylem to phloem feeding as some phloem feeding leafhoppers feed occasionally on xylem (Pompon *et al.*, 2011). Interestingly, the main host plants of *A. makarovi* are important reservoir of CaPsol; thus, also this insect could be involved in the CaPsol spreading to grapevine. Considering the lack of information about host plants and feeding preferences of *L. striatella* and *P. alienus*/*confinis*, further study is needed to investigate their potential on BN epidemiology. The novel findings acquired in this study evidenced that the BN epidemiology in vineyard agro-ecosystems is more complex than previously known. In fact, even if tested *H. obsoletus* specimens harboured principally the bindweed-related phytoplasma strains, found prevalent within the vineyard, they were captured by netting exclusively around the borders and in the vineyard neighbourhood. For that reason, it is reasonable to hypothesize that '*Ca*. P. solani' spreading to vineyard borders could be due to *H. obsoletus* and its transmission within the vineyard could be due to the feeding activity of the alternative vectors. Other studies are needed to investigate if the alternative insect vectors could be able to acquire and transmit CaPsol using the infected grapevines as source of inoculum. Furthermore, the polyvoltinism of these alternative insect vectors increases the probability of acquiring and transmitting the phytoplasma during the growing season. Accurate investigation on the ability of CaPsol acquisition related to developmental stage, the latency period, and transmission efficiency of the alternative vectors will be necessary to improve the knowledge of BN epidemiology. The BN management, based mainly on the

weeding of herbaceous plants hosting both the phytoplasma and the vectors, should consider that the eight alternative insect vectors, identified in the present study, live mainly on grasses (Table 12) and not on broad-leaves as *H. obsoletus* and *R. panzeri*

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Chapter. 5: Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

1- Summary

Bois noir is a grapevine disease causing severe yield loss in vineyards worldwide. It is associated with '*Candidatus* Phytoplasma solani', a phloem-limited bacterium transmitted by polyphagous insects. Due to its complex epidemiology, it is difficult to organize effective containment measures. This study aimed to describe the bacterial microbiota associated with '*Candidatus* Phytoplasma solani' infected and non-infected insect hosts and vectors to investigate if phytoplasma presence can shape the microbiota, with special attention for bacteria known as essential for insect survival, parasites, or phytoplasma antagonists. Alpha-diversity analysis showed a low microbiota diversity in these insects, in which few genera were highly abundant. Beta-diversity analysis revealed that the xylem- and phloem-feeding behavior influences the microbiota structure. Moreover, it highlighted that phytoplasma infection is associated with a restructuring of microbiota exclusively in Deltocephalinae insect vectors. Obtained data showed that '*Candidatus* Phytoplasma solani' may have adverse effects on the endosymbionts *Sulcia* and *Wolbachia*, suggesting a possible fitness modification in the insects. The phytoplasma-antagonistic *Dyella* was not found in any of the examined insect species. The results indicate an interesting perspective regarding the microbial signatures associated with xylem- and phloem-feeding insects, and determinants that could be relevant to establish whether an insect species can be a vector or not, opening up new avenues for developing microbial resource management-based approaches.

2- INTRODUCTION

Diseases that are transmitted by vectors are not only a threat to human health, but can also cause disastrous losses in agriculture, being a threat for livestock and plants upon which we depend for food. Most of the vectors that transmit diseases are arthropods, among which insects and mites can transmit a wide range of pathogens to a broad range of hosts (Ciancio 2016).

Among the plant pathogens that are transmitted by vectors, phytoplasmas deserve a specific mention due to their unique nature, being obligate bacterial pathogens with a broad host range that localize in the phloem of their host plant. However, they have a much stricter specificity when it comes to their insect vectors, as several molecular recognition stages are needed for the phytoplasmas to pass from the insect gut to the hemolymph and ultimately to the salivary glands of the vector, from where they can infect new plants (Namba 2019).

Each phytoplasma can have different vectors but all known vectors are insects belonging to the order Hemiptera, suborder Auchenorrhyncha and Sternorrhyncha, in particular leafhoppers (family Cicadellidae), planthoppers (superfamily Fulgoroidea), and psyllids (superfamily Psylloidea) (Weintraub and Beanland 2006; Alma *et al*. 2015). This study focuses on '*Candidatus* Phytoplasma solani', associated, among others, with grapevine Bois noir, the most widespread disease in the complex of grapevine yellows (Quaglino *et al*., 2013). This complex includes grapevine diseases, associated with genetically and biologically distinct phytoplasma species, that induce common symptoms (desiccation of inflorescences, berry shrivel, leaf discolorations, reduction of growth, and irregular ripening of wood), and cause serious economic damage and yield loss in vineyards (Belli *et al.* 2010; Angelini *et al*. 2018).

The epidemiological cycle associated to Bois noir is extremely complex and was recently discovered to include not only the most well-known vectors *Hyalesthes obsoletus* (Maixner 1994) and *Reptalus panzeri* (Cvrkovic *et al*. 2014), but also other eight species: *Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Phylaenus spumarius*, and *Psammotettix alienus*/*confinis* (Quaglino *et al*. 2019).

Since the cycle includes so many insects, all highly polyphagous, and a very broad range of secondary, wild hosts, it is difficult to organize effective prevention and containment measures (Bertaccini *et al.* 2014; Moussa *et al*. 2019; Quaglino *et al.* 2019) as the typical management strategies for phytoplasma diseases, based on the control of the vector with insecticides and the removal of infected plants (Bianco *et al*. 2011), are not effective against '*Ca.* P. solani' (Angelini *et al*. 2018). For this reason, other methods are being envisioned, including the use of Microbial Resource Management (MRM).

MRM is the proper management of the microbial resources available in a given ecosystem in order to solve a practical problem by directing the potential of microorganisms and, on the topic of control of insect vectors, some first steps have already been taken towards defining the composition and functionality of microbial communities associated with insects (Marzorati *et al.* 2006; Miller *et al.* 2006; Crotti *et al.* 2012).

Insects, like all other higher organisms, maintain several symbiotic interactions with their associated microbial community, which has a great influence on their fitness, evolution, and diversity (Margulis and Fester 1991; Ruby *et al*. 2004). The microbial community can contain beneficial symbionts, called mutualists, but also detrimental ones, which are parasites or pathogens, and the dynamic balance found in a microbial community can produce either a

positive or negative effect for the health of the host (Berg *et al.* 2014; Lebeis 2014). A MRM approach to control these insect vectors would therefore be performed by manipulating the microbial community of these insects to promote the effect of naturally present antagonistic microorganisms (Trivedi *et al.* 2016).

A negative prospect for this strategy is that, as the interactions between environment, host, and microbiota are very complex and influenced by several variables (Trivedi *et al.* 2015; Douglas 2015; Fonseca-García *et al.* 2016) more studies need to be conducted in the description of the bacterial community associated to these vectors before its manipulation can become a viable option. The positive prospect is that, since these phloem-feeding insects rely heavily on obligate bacterial symbionts to provide nutrients which are lacking in their unbalanced diet (Buchner 1965; Baumann 2005; Bourtzis and Miller 2006; Skidmore and Hansen 2017), it is hypothesized that these insects will be particularly susceptible to unbalances in their microbial community.

A main actor in these obligate mutualistic interactions is '*Candidatus* Sulcia muelleri', a bacterial species that greatly reduced its genome as it evolved as an obligate symbiont, and is documented to be strictly associated to leafhoppers and planthoppers, among other hosts (Moran *et al.* 2005; McCutcheon *et al.* 2009). This bacterial species is involved in the synthesis of several amino acids necessary for the insect host (McCutcheon and Moran 2007).

Other mutualistic bacteria involved in these interactions belong to the genera *Nasuia* and *Sodalis* (Kobiałka *et al*. 2018).

Another bacterial genus interesting for MRM approach is *Wolbachia*, ubiquitous endosymbionts associated with over 60% of known insect species, as well as other arthropods and nematodes (Hosokawa *et al.* 2010; Zug and Hammerstein 2012; Newton and Rice 2020). *Wolbachia* species are cytoplasmically inherited and known as reproductive parasites due to their ability to manipulate reproduction such as sperm-egg incompatibility (cytoplasmic incompatibility), parthenogenesis induction, male killing, and feminization, making it a possible biocontrol agent against the vectors (Werren 1997; Stouthamer *et al.* 1999; Werren *et al.* 2008; Brelsfoard and Dobson 2009; Chuche *et al*. 2017). Nevertheless, several studies showed that *Wolbachia* can act as mutualistic towards insect hosts, modulating nutrition and immune responses (Hosokawa *et al.* 2010; Iturbe-Ormaetxe *et al*. 2011; Newton and Rice 2020). Moreover, recent studies proposed that *Wolbachia* can act as biocontrol agent of insect-transmitted pathogens, including phytoplasmas, by increasing latency period and blocking pathogen transmission (Shaw *et al.* 2016; Chuche *et al*. 2017).

Dyella-like bacterium (DLB), gram-negative, aerobic, rod-shaped endophytic bacteria belonging to the family Rhodanobacteraceae, can be acquired by feeding and has shown a potential biocontrol activity against phytoplasmas and their cultivable relative *Spiroplasma melliferum* (Iasur-Kruh *et al.* 2017, 2018). The possible mechanisms of DLB antagonism towards phytoplasmas have been hypothesized to be (i) competition for nutrients or colonization niches, (ii) induction of plant systemic resistance, (iii) secretion of plant growth hormones, or (iv) secretion of phytoplasma growth inhibitory substances (Eljounaidi *et al.* 2016).

In this scenario, the current study aims to characterize through an Next Generation Sequencing (NGS) approach the bacterial community associated with selected '*Ca*. P. solani' insect hosts, both infected and non-infected by the phytoplasma, with the following goals: (i) describe the bacterial communities in different insect hosts of '*Ca*. P. solani'; (ii) determine whether the presence of '*Ca*. P. solani' affects the bacterial community, in particular if it can cause a dysbiosis (also called dysbacteriosis) or increase diversity; (iii) evaluate the presence of possible antagonists towards the insect (e.g. *Wolbachia* spp.) or phytoplasma (e.g. *Wolbachia* spp. and *Dyella*-like bacteria); (iv) investigate the effect of '*Ca*. P. solani' on the obligate endosymbiont '*Ca.* Sulcia' spp.. The selected insects are the main vector *H. obsoletus*, newly reported vectors (phloem-feeders: *A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, and *P. alienus*/*confinis*; xylem-feeder: *P. spumarius*), and *Cicadella viridis*, one of the most abundant insects living in Italian vineyard, harboring with high infection rate but not vectoring '*Ca*. P. solani' (Quaglino *et al*. 2019), and characterized by xylem-feeding activity. *C. viridis* was included in the study for comparing the microbiota associated with xylem- and phloem-feeders, and investigating the phytoplasma influence on the microbiota structure in comparison with vectors. *R. panzeri* was not among the selected vectors because it is not found in the studied area.

Achieving the previously mentioned aims regarding the description of the bacterial communities may help in devising MRM-based approaches to achieve the main objective of biological control of '*Ca*. P. solani' and its insect vectors.

3- MATERIALS AND METHODS

3.1. Insect collection

Specimens of the insect species *A. makarovi*, *C. viridis*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius*, and *P. alienus*/*confinis* were

captured by sweep entomological net in mid-July 2018 in the Chardonnay organic vineyard (Franciacorta, Lombardy Region, North Italy; N 45°35′38.12″, E 10°09′34.32″) where new insect vectors of '*Ca*. P. solani' had previously been identified (Quaglino *et al*. 2019). Insect individuals were stored in ethanol 90%, transferred to the lab for species identity confirmation by stereomicroscope based on the taxonomic keys of den Bieman *et al.* (2011), and maintained in absolute ethanol at 4°C till use.

3.2. Total nucleic acids extraction and suitability for amplification

Total nucleic acids (TNAs) were extracted from ethanol preserved insects (dried by filter paper) through homogenization in a CTAB-based buffer [2% w/v cetyltrimethylammonium-bromide (CTAB); 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HCl pH 8.0; 0.5% ascorbic acid]. After incubation at 60°C for 20 min, TNAs were separated with one volume of chloroform: isoamyl alcohol 24:1 v/v solution and precipitated with the addition of one volume of cold isopropanol. The TNAs pellet was then washed with ethanol 70%, air dried, dissolved in 30µL of TE buffer pH 8.0, and maintained at −20 °C until use (Moussa *et al.* 2019).

The suitability of the extracted TNAs for amplification was tested through a bacterial *16S rRNA* gene PCR assay using the universal primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') / 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). PCR reactions were conducted in Applied Biosystems 2720 thermocycler (Applied Biosystems, Monza, Milan) with the following conditions: 2 min at 95 °C; 35 cycles consisting of 1 min at 95 °C, 1 min 30 s at 50 °C and 2 min at 72 °C; 10 min at 72 °C. PCR reactions were performed in 25 µL volume containing 50 µM of each dNTP, 0.4 µM of each primer, 1.5 mM MgCl2, 1× polymerase buffer, 1 unit GoTaq polymerase enzyme (Promega, Milan, Italy). PCR mixture devoid of TNAs was employed as negative control. PCR products were analyzed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator. Only the samples that gave positive amplification with this reaction were considered for further analyses.

3.3. Molecular detection of '*Candidatus* Phytoplasma solani'

The presence of '*Ca*. P. solani' in collected insects was verified by species-specific nested PCR-based amplification of the *stamp* gene using the primer pair *Stamp*-F (5'-GTAGGTTTTGGATGTTTTAAG-3') / *Stamp*-R0 (5'-

AAATAAAAGAACAAGTATAGACGA-3'), followed by the primer pair *Stamp*-F1 (5'-TTCTTTAAACACACCAAGAC-3') / *Stamp*-R1 (5'-AAGCCAGAATTTAATCTAGC-3') (Fabre *et al*. 2011). PCR reactions were conducted in Applied Biosystems 2720 thermocycler with the following conditions: 4 min at 94 °C; 35 cycles consisting of 30 s at 94 °C, 30 s at 56 °C (direct PCR) or 52 °C (nested PCR) and 1 min 30 s at 72 °C; 7 min at 72 °C. PCR mixture devoid of TNAs was employed as negative control. PCR reaction mixtures and PCR products visualization were as described above for bacterial *16S rRNA* gene.

3.4. Illumina Mi Seq sequencing

Based on the molecular detection of '*Ca*. P. solani' and the requested TNAs quantity (at least 0.5 μg) / quality (ratio 260/280 nm ~2), TNAs extracted from 96 insect specimens were selected to undergo Illumina Mi Seq sequencing. These 96 samples were picked to ensure that at least five samples for each insect species were included in both the '*Ca*. P. solani'-infected and non-infected groups.

Next generation sequencing library preparations and Illumina Mi Seq sequencing were conducted by an external provider (Personal Genomics, Verona, Italy). The bacterial *16S rRNA* gene hypervariable region V4 libraries were prepared using the forward primer 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3'), and the amplification of sequences belonging to mitochondria was blocked using a PNA blocker (Lundberg *et al*. 2013). Metagenomic sequencing was performed using the Illumina Miseq 300PE sequencing technology. Obtained reads were deposited in the EMBL-ENA under the project number PRJEB38750.

3.5. Processing of high-throughput sequencing data

The raw sequencing reads were initially filtered, to remove low quality sequences, trim primers and Illumina adapters. The initial quality control of the reads was performed with FastQC v0.11.5. Primers were trimmed with the cutadapt tool version 1.14 (Martin 2011) while adapters were trimmed with Sickle version 1.33 (https://github.com/najoshi/sickle) and Scythe version 0.991 (https://github.com/vsbuffalo/scythe). The obtained reads were analyzed using the QIIME 2 pipeline (Bolyen *et al.* 2019) in order to assign them to OTUs. Allocation to OTUs and clustering were performed using uclust with a minimum similarity of 97% (default). Identified OTUs from representative sequences were aligned to Green-genes

(http://greengenes.lbl.gov/) using R-studio. Chloroplast and mitochondria (these constituted only 1-2% in some samples) were filtered as well as rare OTUs (i.e., singletons and OTUs < 10). The resulting OTU table was then used for the subsequent analyses.

3.6. Diversity and statistical analysis

After quality filtering and rarifying to 1600 sequences per sample, Alpha-diversity indices (Shannon index, ChaoI and observed OTU) were calculated to ensure that enough sequencing coverage had been achieved by using BiocManager package implemented in the R software (R Project 3.0.2; http://cran.rproject.org/). Observed, Chao1 (Chao 1984) and Shannon H' index (Shannon 1948) were considered for the aforementioned features. Alpha diversity indices were compared between different insect species groups ('*Ca*. P. solani' infected or non-infected). Shapiro test was performed for data normality followed by ANOVA in the case of Observed richness whereas Kruskal test was used for Chao1 and Shanon H' index. Welch t-test was carried out to compare between the infected and non-infected groups of individual species. Beta diversity was assessed by Bray-Curtis (Bray and Curtis 1957) distance matrices and visualized by principal coordinate analysis (PCoA). The PERMANOVA statistical analysis was performed to determine the significance of microbial community differences among the different insect species and infection status with controlled 10^5 permutations. Taxonomic abundance data was calculated using the percentage abundance of OTUs present in the core microbiota. Heat tree was used to plot all the OTUs present in the dataset using the 'metacoder' package. Taxonomic data were plotted using heat trees in which the size and color of tree parts correspond to reads for each taxon as the size of each taxon.

4- RESULTS

4.1. Insects collected and '*Ca*. P. solani' infection rate

A total of 400 insect individuals were captured. The most abundant species were *E. variegatus* (75 individuals), *P. alienus/confinis* (71) and *E. incisus* (59), while *L. striatella* (16), *D. hamata* (8) and *A. makarovi* (6) were scarcely present (Table 13). Bacterial 16S rDNA fragment 27F/1492R was amplified by the TNAs extracted from all insect specimens and not in the negative control, evidencing the TNAs suitability for further molecular analyses. PCR-based amplification of *stamp* gene identified the presence of '*Ca*. P. solani' in 127 out of 400 individuals.

Table 13. Insect species abundance, infection rate by '*Ca*. P. solani', and selected specimens for NGS analyses

Insect	**No. specimens collected**	**No. specimens CaPsol-infected**	**Infection rate (%)**	**No. specimens used for NGS (healthy/infected)**	**No. specimens analyzed after NGS (healthy/infected)**
Aphrodes makarovi	6	0	0	0	0
Cicadella viridis	39	13	33	8/6	8/5
Dicranotropis hamata	8	0	0	0	0
Dictyophara europaea	40	15	38	9/5	9/4
Euscelidius variegatus	75	32	43	7/7	7/5
Euscelis incisus	59	18	31	9/5	9/4
Hyalesthes obsoletus	47	21	45	7/7	7/5
Laodelphax striatella	16	0	0	0	0
Philaenus spumarius	39	16	41	7/7	7/3
Psammotettix alienus/confinis	71	12	17	6/6	6/4

Infection rate was >40% in *H. obsoletus*, *E. variegatus*, and *P. spumarius*, >30% in *D. europaea*, *C. viridis* and *E. incisus*, and >10% in *P. alienus/confinis*. The phytoplasma was not identified in the least abundant species *L. striatella*, *D. hamata* and *A. makarovi* (Table 13); these latter three species were thus not included in microbiota analyses. For each of the other seven insect species, the number of '*Ca*. P. solani'-infected and -non-infected specimens selected for microbiota analyses is reported in Table 13.

4.2. Bacterial diversity analysis

Poor quality sequences were obtained in twelve out of 96 insect specimens that were excluded from further analyses (Table 13). Sequencing of the V4 region of the *16S rRNA* gene on the '*Ca.* P. solani' infected and non-infected group produced, after filtering out organellar sequences and rare OTUs, a total of 527466 sequences belonging to 363 different OTUs. Out of all the obtained sequences, 228190 belong to '*Ca.* P.solani' infected group and 299276 to the non-infected group. Number of sequences and OTUs obtained from the '*Ca*. P. solani' infected and non-infected group are reported in Table 14.

Table 14. Number of reads and OTUs produced for infected and non-infected group of the different insect species

Species	Status	No. samples	Reads	OTUs
C. viridis	Infected	5	96729	202
	non-infected	8	143399	76
D. europaea	Infected	4	12803	29
	non-infected	9	25528	29
E. incisus	Infected	4	37284	198
	non-infected	9	33499	27
E. variegatus	Infected	5	34157	183
	non-infected	7	28210	36
H. obsoletus	Infected	5	30474	53
	non-infected	7	36010	29
P. spumarius	Infected	3	5609	40
	non-infected	7	12946	37
P. alienus/confinis	Infected	4	11134	43
	non-infected	6	19684	22

The alpha diversity indices of Observed, Chao1 and Shannon were used for this study as shown in Fig. 18. The observed OTUs were considered to show the absolute richness. The values of this parameter range from a minimum average of 17, found in non-infected *E. incisus* and *D. europaea*, to a maximum of 106, found in infected *E. incisus*. The corrected estimation of richness made through the Chao1 index are very close to the value of Observed for most samples, indicating that the sequencing has reached an adequate depth, having very few singletons and a low number of estimated undetected OTUs. The Shannon index, indicating the evenness of species distribution ranges from a minimum of 0.089 in non-infected *E. variegatus*, to a maximum of 2.25 in infected *E. incisus*. For *E. incisus, E. variegatus* and *H. obsoletus*, the number of Observed OTU and the Shannon index are significantly different between infected and non-infected samples, indicating that the presence of the phytoplasma has a strong effect on the alpha-diversity of the bacterial community in these species. For all other considered insect species, no statistically significant difference was found between these values for infected and non-infected groups. The bacterial distribution of the different insect species both infected and non-infected groups were characterized in terms of the relative taxonomic abundance. A total of 18 phyla, 46 classes, 58 orders, 89 families, 100 genera and 35 species (of which a total of 277 with an unidentified taxa).

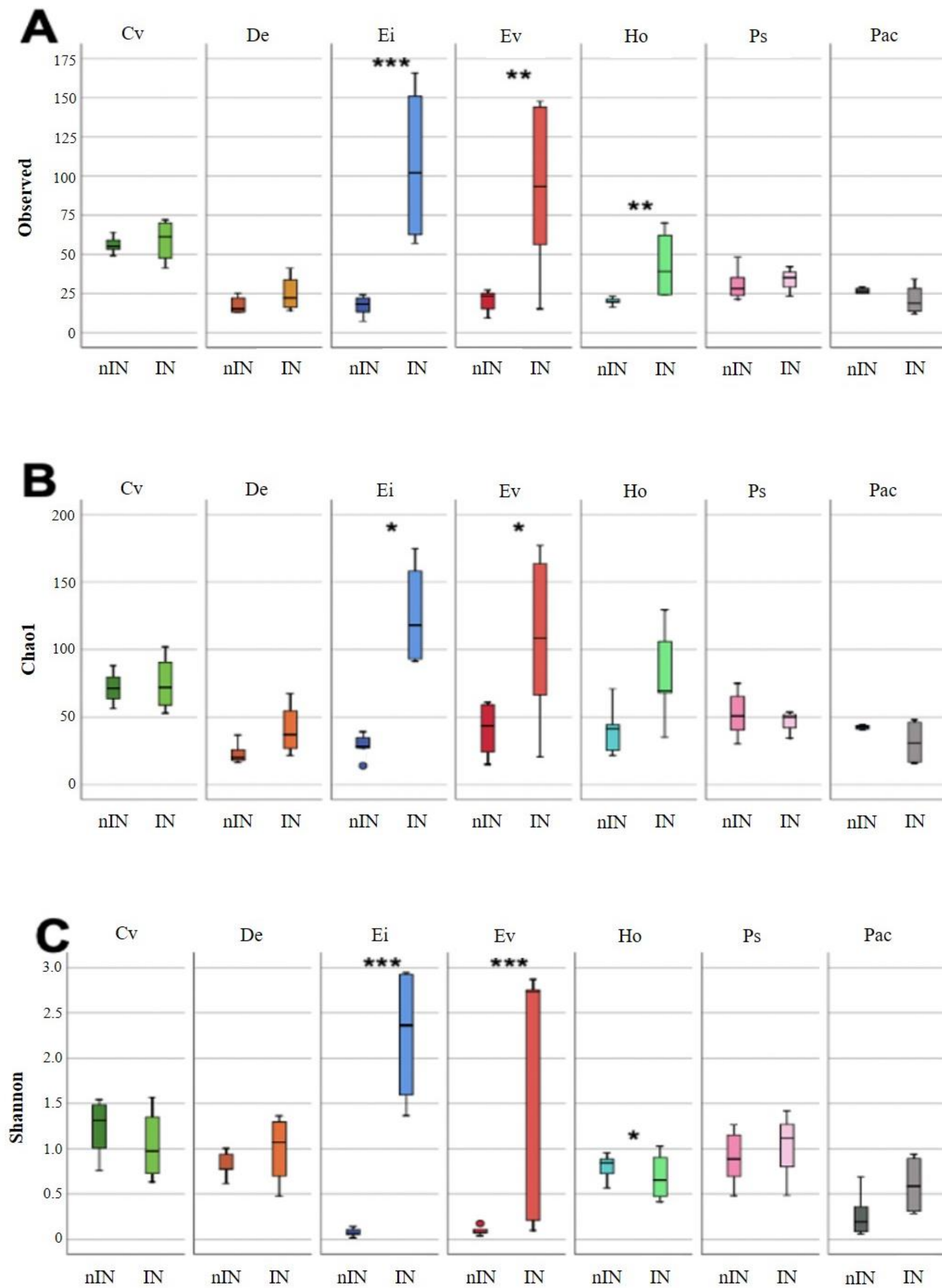

*Figure 18. OTU richness in insect microbiomes. Alpha diversity (Observed, Chao1, Shannon) comparison among 'Ca. P. solani' infected and non-infected insect species. Cv: Cicadella viridis; De: Dictyophara europaea; Ei: Euscelis incisus; Ev: Euscelidius variegatus; Ho: Hyalesthes obsoletus; Ps: Phylaenus spumarius; Pac: Psammotettix alienus/confinis. nIN: non-infected; IN: infected. Significance level: * < 0.05; ** < 0.01; *** < 0.001.*

4.3. Core microbiome

In order to highlight the existence of an identifiable common core microbiome, the group of members shared among the microbial community of the infected and non-infected

groups of the different insect species were identified. The bacterial communities in these insect populations are clearly distinct and do not share a common core as no single OTU is shared (i) among individuals of all insect species regardless of infection, (ii) among infected individuals regardless of insect species, (iii) among non-infected individuals regardless of insect species. Venn diagrams were used to represent the number of OTUs found exclusively in the infected group, non-infected group, or shared between the two groups (Fig. 2).

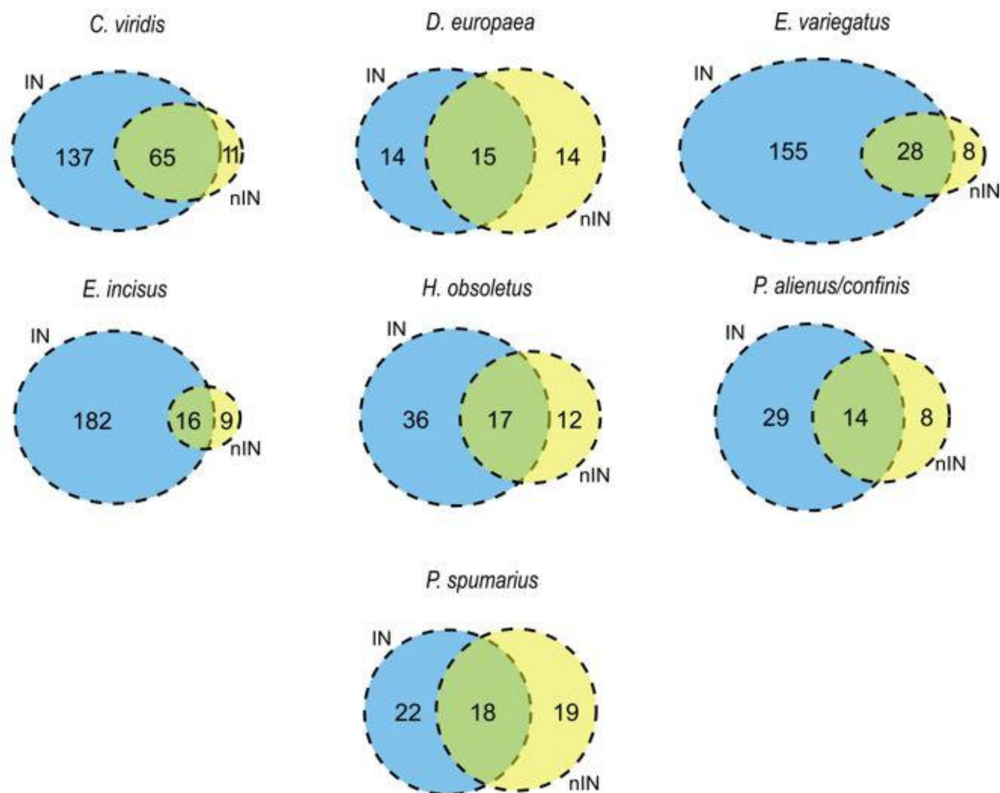

Figure 19. Venn diagrams showing the comparative distribution of OTUs in the different 'Ca. P. solani' infected and non-infected individuals within insect species. nIN: non-infected; IN: infected.

For most of the analyzed species, a common trend can be identified with infected individuals showing a much higher number of unique OTUs compared to non-infected samples. This difference is particularly pronounced in *E. incisus* and *E. variegatus* (Fig. 19 and 20). This is true for all species, except *D. europaea* and *P. spumarius*, for which the number of unique OTUs in infected and non-infected samples is very similar (Fig. 19). Interestingly, regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65 shared OTUs (Fig. 19).

Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between infected and non-infected in all species. Other relevant bacterial genera that are core

between infected and non-infected in particular species are *Cronobacter* and *Sodalis* (*C. viridis*), *Erwinia* (*P. spumarius*), *Propionibacterium* (*E. variegatus*), *Purcelliella* (*H. obsoletus*), and *Rickettsia* (*H. obsoletus*).

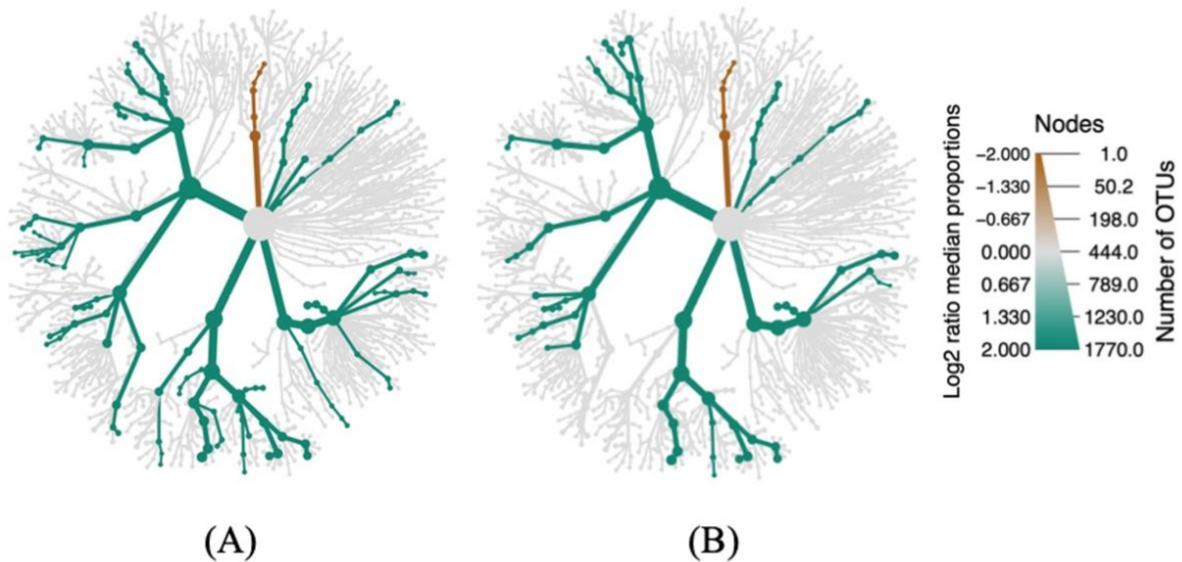

Figure 20. Differential heat tree showing differences in bacterial composition to the species level. The comparisons were made among the 'Ca. P. solani' infected and non-infected groups. A, E. incisus where the green color represents the microbial community of the infected group and the brown color represents the non-infected group. B, E. variegatus where the green color represents the microbial community of the infected group and the brown color represents the non-infected group. For each taxon, a Wilcoxon rank-sum test was used to test for differences.

4.4. Bacterial community structure

Venn diagram representation showed a qualitative difference among OTUs identified in infected and non-infected individuals in the species, without considering the vital quantitative aspect in describing the community structure. To compare the microbial community structure among the '*Ca.* P. solani' infected and non-infected individuals within and among insect species, principle coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 21).

The graph shows that the species is a major driver of diversity among the microbial communities, as each species tends to form a separate cluster. From this analysis, two groups of insects can be identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on species alone, with the single samples of infected and non-infected insects overlapping and mixing with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct clusters based on species for non-infected

samples, but the infected samples do form clusters based on species, distinct from the non-infected samples within the same species. These results were confirmed by an Adonis multivariate analysis of variance, showing that there are statistically significant differences between the structure of the community in infected and non-infected samples of *E. incisius* (p=0.001), *E. variegatus* (p=0.013) and *P. alienus/confinis* (p=0.006), while no significant differences were found in the other four species.

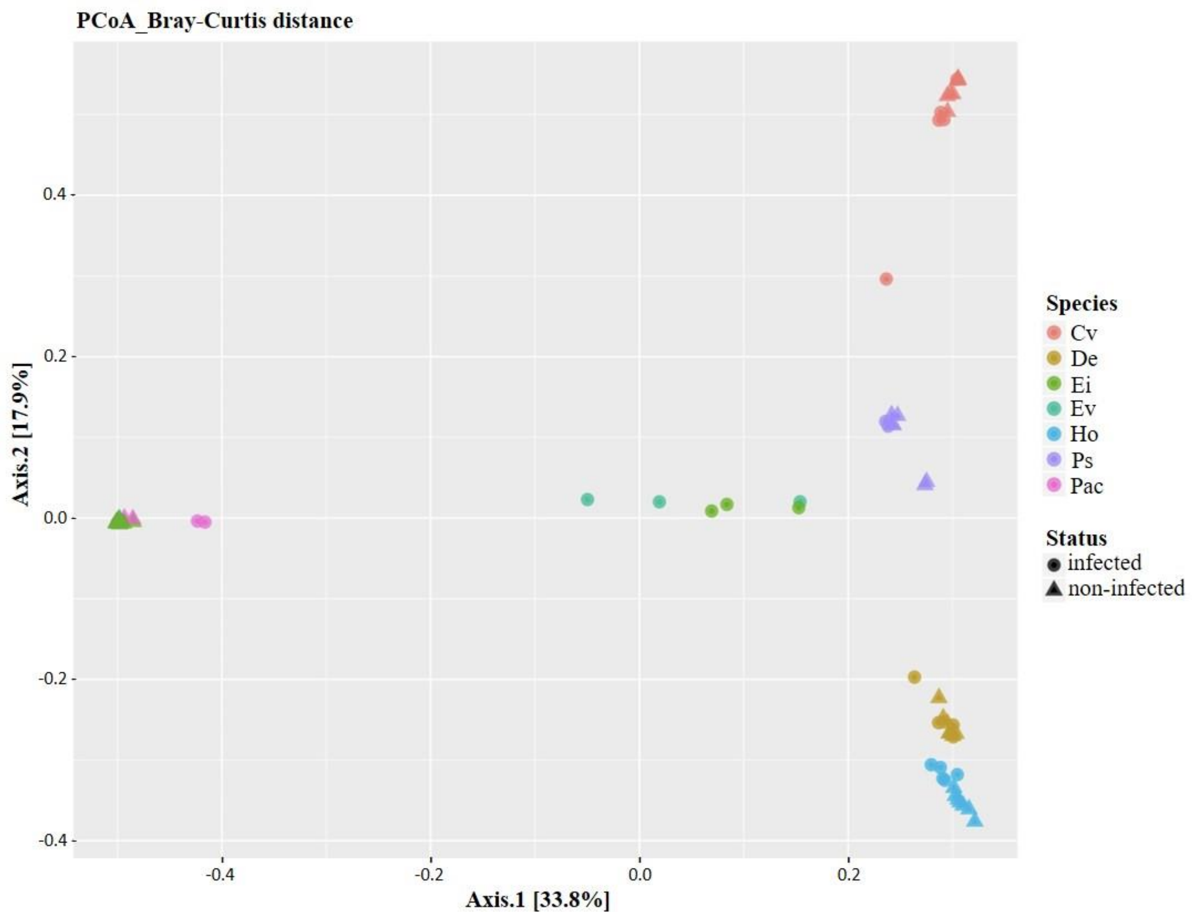

Figure 21. Beta-diversity. Graphs reporting the distribution of the samples according to beta-diversity calculated with a Bray-Curtis distance index. Different shape of the markers indicates different 'Ca. P. solani' infection status, different colors indicate different insect species, as indicated in the legend. Cv: Cicadella viridis; De: Dictyophara europaea; Ei: Euscelis incisus; Ev: Euscelidius variegatus; Ho: Hyalesthes obsoletus; Ps: Phylaenus spumarius; Pac: Psammotettix alienus/confinis.

4.5. Bacterial abundance and distribution

The composition in taxa of the microbial communities according to the different insect species as well as the different infection status are reported in the bar plots in Fig. 22. All detected OTUs could be assigned to one of ten phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Proteobacteria, or Tenericutes (Fig. 22A). In most analyzed samples, the most abundant

phylum is Bacteroidetes, which can compose up to 99% of the total community, as for the non-infected *E. variegatus*. This dominance of Bacteroidetes is seen in all non-infected samples, except for *H. obsoletus*, and also in some infected insect species: *D. europaea*, *P. spumarius*, and *P. alienus/confinis*. The second most abundant phylum in most samples is Proteobacteria: this phylum is the most abundant in *H. obsoletus*, both infected and non-infected, and is also highly abundant also in *C. viridis*, *D. europaea*, and *P. spumarius*, both in infected and non-infected samples. While mostly absent in non-infected samples, the phyla Actinobacteria and Firmicutes are found with higher abundance in the infected samples of *E. incisus*, and *E. variegatus*. Bacteria belonging to the phylum Cyanobacteria are found only in the infected samples of *E. incisus* and *E. variegatus*.

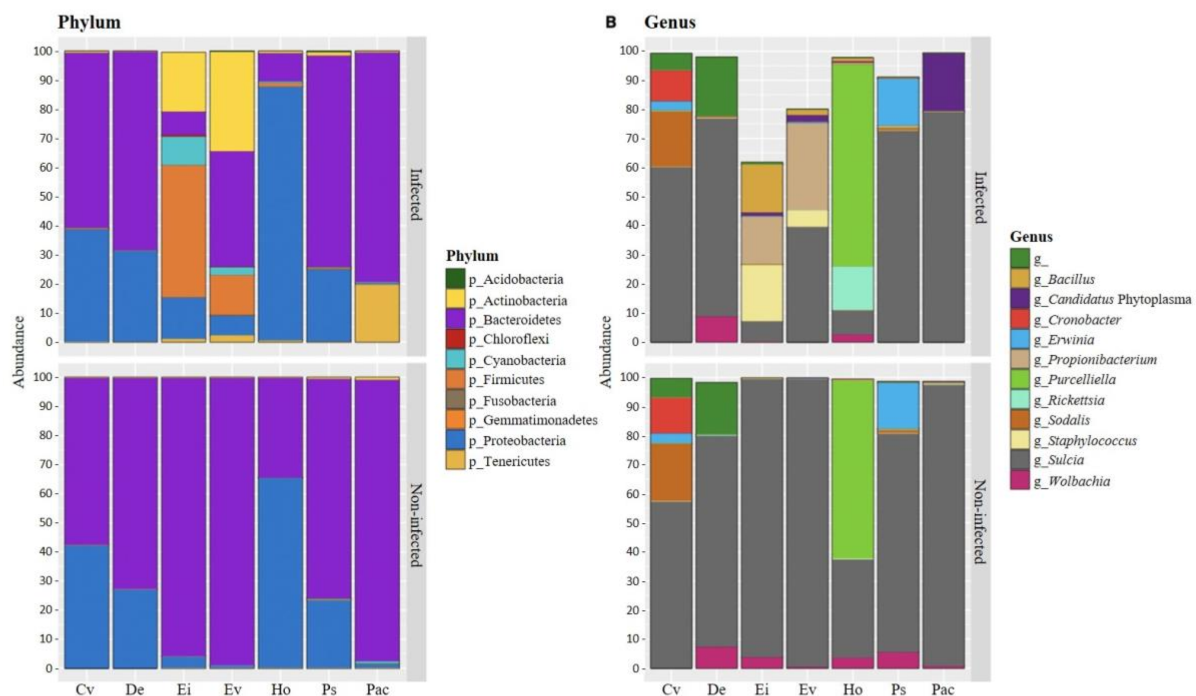

Figure 22. Relative abundance of operational taxonomic units at different levels: (A) phylum, (B) genus. Cv: Cicadella viridis; De: Dictyophara europaea; Ei: Euscelis incisus; Ev: Euscelidius variegatus; Ho: Hyalesthes obsoletus; Ps: Phylaenus spumarius; Pac: Psammotettix alienus/confinis.

In most of the examined insect species, the microbial community was composed of members of few genera, but at very high abundance. In fact, as it can be seen by comparison of Fig. 22A and 22B, almost the entire abundance of the Bacteroidetes phylum can be ascribed to the genus *Sulcia* alone. Likewise, the Tenericutes abundance is due uniquely to the presence of OTUs of '*Ca.* Phytoplasma' and, in *H. obsoletus*, the abundance of Proteobacteria overlaps with the abundance of *Purcelliella*. In contrast, the microbiota of *E. incisus* shows many more genera, but the 12 most abundant ones only cover 60% of the

abundance, while the rest are less abundant genera. Regarding '*Ca*. Phytoplasma' OTUs, they are found exclusively in infected individuals of all species, but with high abundance (>1%) only in *E. incisus*, *E. variegatus*, and *P. alienus/confinis* (Table 15).

Table 15. Top Relative abundance (%) of 'Ca. Phytoplasma', Sulcia and Wolbachia OTUs

Insect	**Status**	**'*Ca*. Phytoplasma'**	***Sulcia***	***Wolbachia***
C. viridis	Non-infected	0.00	57.49	0.00
	Infected	0.04	59.29	0.00
D. europaea	Non-infected	0.00	76.05	8.24
	Infected	0.08	69.36	9.69
E. incisus	Non-infected	0.00	95.78	3.91
	Infected	1.41	6.88	0.00
E. variegatus	Non-infected	0.00	98.62	0.56
	Infected	3.33	41.42	0.00
H. obsoletus	Non-infected	0.00	32.99	3.26
	Infected	0.87	7.35	2.33
P. spumarius	Non-infected	0.00	74.10	5.65
	Infected	0.08	71.84	0.02
P. alienus/confinis	Non-infected	0.00	96.26	0.87
	Infected	21.48	78.34	0.16

Comparing the infected and non-infected abundance of different genera, it emerges that for some species there are no changes, or very little changes, in the structure of the bacterial community in the presence of absence of '*Ca.* Phytoplasma solani': *C. viridis*, *D. europaea*, and *P. spumarius* (Fig. 22B). For the main host, *H. obsoletus*, the community itself does not seem to undergo great variations in quality, with the addition of only *Rickettsia* in infected samples, but the relative abundance of the members of the community are vastly different. Similarly, for *P. alienus/confinis* the community only shows the addition of '*Ca.* Phytoplasma' between healthy and infected samples, but the abundance of OTUs belonging to this genus is very high, suggesting a strong interaction between this plant pathogen, the host, and the microbial community already present in the host. For the remaining examined species (*E. incisus*, *E. variegatus*) the infection by the phytoplasma is accompanied by a radical change in the microbial community (Fig. 22B).

Regarding the bacterial genera that were of particular interest in this study, it can be seen that (i) the '*Ca.* Phytoplasma'-antagonistic *Dyella* is not found in any of the examined insect species. (ii) *Wolbachia* is found in non-infected specimens of all vector species, but with high abundance (>1%) only in *D. europaea*, *E. incisus*, *H. obsoletus*, and *P. spumarius*;

in all examined vector species, with the exception of *D. europaea*, the abundance of this genus is reduced in the infected samples, to the degree of disappearing entirely from the community for *E. incisus* and *E. variegatus*. *Wolbachia* is not found in *C. viridis* regardless of phytoplasma infection (Table 3). (iii) The mutualistic symbiont *Sulcia* makes up for the majority of the microbiota in non-infected specimens of all insects, except *H. obsoletus*. Within phloem-feeders, it showed an abundance >95% in *E. incisus*, *E. variegatus* and *P. alienus/confinis*, ~75% in *D. europaea*, and ~30% in *H. obsoletus*. Within xylem-feeders (*C. viridis* and *P. spumarius*), it showed an abundance <75%. With the exception of the xylem-feeders and *D. europaea*, its abundance is greatly reduced in infected samples, compared to non-infected samples of the same species (Table 15).

5- DISCUSSION

Until recently, *H. obsoletus* and *R. panzeri* were believed to be the only insect vectors of '*Ca*. P. solani' to grapevine, but recent researches allowed the identification of several new insect vectors (Quaglino *et al.* 2019). The insect survey and molecular identification of '*Ca*. P. solani', conducted in this study, confirmed the presence of abundant populations and the unusually high infection rate (>10%) in 2018 for the main vector *H. obsoletus* and for a majority of the newly reported insect species (Quaglino *et al.* 2019). If the scenario of containing Bois noir disease in vineyards was already bleak due to the high polyphagia of the established insect vectors, the addition of several more vectors is leading to the idea that there are no options to implement any traditional containment strategy against this disease, its pathogen, or vectors. A comprehensive and thorough investigation of the bacterial diversity in '*Ca*. P. solani' insect vectors is essential for understanding how this pathogen interacts with its hosts and their microbiota, possibly leading to the development of effective prevention and treatment strategies based on the management of the bacterial community in the vectors.

This study analyzes the bacterial community present in insects associated to '*Ca.* P. solani' collected in vineyards in northern Italy, including the main vector *H. obsoletus*, five newly reported vector species (*D. europaea, E. incisus*, *E. variegatus*, *P. spumarius*, and *P. alienus/confinis*) and a species that is known to host the phytoplasma but not to transmit it, *C. viridis*. In addition to investigating and describing the bacterial community found in these insects, both when they're infected with '*Ca*. P. solani' and when they aren't, the study focuses on the presence of specific genera of bacteria that have been reported as potentially essential for the survival of the insect (genus *Sulcia*), as potential parasites of the vectors

(genus *Wolbachia*), or as antagonistic towards the phytoplasma (genus *Wolbachia* and *Dyella*).

In comparison with previous studies on the topic of the bacterial communities associated to insect vectors of '*Ca.* P. solani', this study uses a more modern technique than those previously employed [LH-PCR, DGGE (Gonella *et al.* 2011); sequencing with Roche 454 (Iasur-Kruh *et al.* 2017)] and extends the range of investigation to more vectors, instead of analyzing just *H. obsoletus*.

Starting from the parameters of alpha- diversity, it is found that in these insects the microbial communities do not have a high diversity, showing a low number of different OTUs that dominate the whole community. This is particularly true for the non-infected samples that showed less than 20 different OTUs for most of the analyzed species. This result is in accordance with what was previously presented regarding the bacterial communities of phloem-/xylem-feeding insects, and it is hypothesized that this is due to their extremely specialized diet which (i) requires specific metabolic processes to implement the insect's own and ensure survival and (ii) comes from a compartment of the plant that is colonized only by very specialized bacteria and therefore acts as a low-diversity reservoir from which the insects ingest bacteria (Colman *et al.* 2012; Jing *et al.* 2014; Overholt *et al.* 2015).

For most species there is no difference in the alpha-diversity parameters between '*Ca.* P. solani' infected and non-infected specimens, indicating that the presence of the pathogen does not lead to a major change in the qualitative composition of the community. Still, for *E. incisus* and *E. variegatus* a statistically significant increase was observed for all parameters in the infected specimens, compared to the non-infected.

The analysis of abundance of the different taxa in the insect species in general revealed microbial communities with low diversity, in which only a handful of genera were present with high abundance: *Bacillus* (Firmicutes), '*Candidatus* Phytoplasma' (Tenericutes), *Cronobacter* (Proteobacteria), *Erwinia* (Proteobacteria), *Propionibacterium* (Actinobacteria), *Purcelliella* (Proteobacteria), *Rickettsia* (Proteobacteria), *Sodalis* (Proteobacteria), *Staphylococcus* (Firmicutes), *Sulcia* (Bacteroidetes), and *Wolbachia* (Proteobacteria).

The results obtained on the description of the bacterial microbiota of *E. incisus* and *P. alienus/confinis* agree with what was previously reported by Kobialka *et al.* (2018), who indicated a microbial community dominated by *Sulcia* for these species.

Regarding *H. obsoletus*, our results that highlight the presence of the genera *Sulcia*, *Wolbachia*, and *Purcelliella* confirm the results previously obtained by Bressan *et al.* (2009) and Gonella *et al.* (2011) in northern Italy, but not those obtained by Iasur-Kruh *et al.* (2017)

in Israel. This latter study determined, using both classical and molecular microbiology methods, that the genus *Sulcia* was the most abundant in *H. obsoletus*, followed by *Pectobacterium*. These differences may be explained by several variables, such as the different techniques used, and the different geographical areas from which specimens were sampled, which leads to different climatic conditions and insect diet.

The results obtained on *C. viridis*, with a high abundance of the genera *Sulcia* and *Sodalis* are in accordance to those previously reported by Michalik *et al.* (2014). Intriguingly, these results also revealed that *C. viridis* has a unique microbiota compared to the other insect species analyzed: it has a more diverse composition, in which five different genera have a relevant level of abundance, and it's the only species in which we find a high abundance of the genera *Cronobacter* and *Sodalis*. These results suggest that either the higher diversity, leading to a more resilient bacterial community, or these specific genera of bacteria could play a role in determining the non-vector status of this insect. Further studies will be conducted to determine if these elements can indeed be important and relevant for the development of an MRM strategy to reduce the spread of Bois noir.

The results regarding the beta-diversity in each analyzed insect species, infected and non-infected, highlighted the presence of two different groups among the insect species: (i) insects for which the presence or absence of the phytoplasma did not cause a major restructuring of the bacterial community, including the species *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius*; and (ii) insects for which the presence of the phytoplasma, not related to its abundance, caused a major change in the bacterial community, including the species *E. incisus*, *E. variegatus*, and *P. alienus/confinis*. Interestingly, among the analyzed species, these three are the only ones belonging to the subfamily Deltocephalinae. The microbiota associated with members of this subfamily is usually characterized by the presence of two ancient mutualistic endosymbiotic bacterial genera: *Sulcia* and *Nasuia* (Kobiałka *et al*. 2018). However, several studies reported that the symbiotic systems of Deltocephalinae leafhoppers can be very diverse, driven by processes of symbiont acquisition and replacement, which can include both bacteria and fungi (Nishino *et al*. 2016; Brentassi *et al*. 2017; Kobiałka *et al*. 2018; Mao and Bennett 2020). In our datasets, no OTUs assigned to the genus *Nasuia* were detected. This result is not in accordance with what is reported by Kobiałka *et al*. (2018), which found *Nasuia* in *E. incisus* and *P. alienus/confinis*. On the other hand, a similar situation, in which *Nasuia* was not detected and *Sulcia* represented more than 95% of microbiota OTUs, was reported in *Dalbulus maidis* (subfamily Deltocephalinae) (Brentassi *et al*. 2017). *D. maidis* is the vector of '*Ca.* Phytoplasma asteris' (Raygoza and

Nault 1998), associated with maize bushy stunt disease, a phytoplasma strictly related to '*Ca.* P. solani' (Quaglino *et al.* 2013). Considering these data, it is reasonable to propose that the symbiotic systems in our insect populations are prevalently based on *Sulcia*. Furthermore, in North Italian vineyards, *Nasuia* was not identified in *Scaphoideus titanus* (subfamily Deltocephalinae), the insect vector of the phytoplasma associated with flavescence dorée disease of grapevine (Sacchi *et al.* 2008). This could suggest the hypothesis that, in North Italy, the environmental conditions of vineyard agroecosystems do not favor *Nasuia* as mutualistic endosymbiont of phytoplasma insect vectors. For the species *C. viridis*, *D. europaea*, *H. obsoletus* and *P. spumarius*, our results are in accordance with what was reported by Fagen *et al.* (2012) regarding the bacterial community of *Diaphorina citri*, the vector of another obligate plant pathogen '*Ca.* Liberibacter asiaticus': the microbiota of these insects was dominated by the same three or four genera regardless of the presence or abundance of the plant pathogen. But, as the presence of the phytoplasma does affect the microbial community in the other three analyzed species, it becomes evident that it's not the phytoplasma alone that determines a change in the microbial community, but rather an interaction between phytoplasma, insect host, and bacterial community. As expected from their common feeding behavior, the xylem-feeding species *C. viridis* and *P. spumarius* showed a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. Anyway, the aforementioned unicity of *C. viridis* microbiota is not due exclusively by its source diet, which is shared by *P. spumarius*. This reinforces the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Regarding the specific genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), interesting considerations can be made for *Sulcia* and *Wolbachia*, while *Dyella* was not found to be present in any of the analyzed specimens. This might be due to the time of sampling, as it was reported that the presence of *Dyella*-like bacteria increases in the late stage of the season (Iasur-Kruh *et al.* 2017).

In terms of abundance, the genus *Sulcia* was found to be the most abundant in all non-infected insect species except *H. obsoletus* where it was the second most abundant after *Purcelliella*. This result is in agreement with Moran *et al.* (2005) who showed that several Auchenorrhyncha insect lineages, including Cicadomorpha and Fulgomorpha, house a single phylotype bacterium called '*Candidatus* Sulcia muelleri'. In the infected groups there was a dramatic decrease in the genus *Sulcia*; except in the case of *C. viridis*, *D. europaea*, and *P. spumarius* where the reduction was quite low. This reduction in the abundance of *Sulcia* has

several possible explanations: the first is that the interaction between the phytoplasma and other members of the microbiota lead to a rise of secondary mutualists, disadvantaging the primary mutualists such as *Sulcia* (Heddi *et al.* 1998); a second hypothesis is related to the host's immune response: it was demonstrated by Galetto *et al.* (2018) that the insect, *E. variegatus* in that study, can activate a strong immune response when interacting with a phytoplasma that is not the one that it usually transmits. This immune reaction could change the bacterial community inside the host drastically, favoring more resistant bacteria, in particular Gram-positive species, as is seen in our study for *E. incisus* and *E. variegatus*. A third hypothesis is based on results obtained of *D. citri* and '*Candidatus* Liberibacter asiaticus' by Vyas *et al.* (2015): this study demonstrated that the phytopathogen could modulate free amino acids availability by interfering with hexamerin storage pathways by regulating expression of amino acid storage protein genes. Such evidence suggests that the reason why there is a dramatic reduction in genus *Sulcia*, which is heavily committed to amino acid production and encodes enzymes for synthesis of all amino acids required as animal nutrients, is simply due to the fact that an infected insect does not need such a high abundance of this bacterial genus. On the other hand, sap-feeding insects rely heavily on the contribution of their obligate symbionts to maintain their metabolism (McCutcheon and Moran 2007). For this reason, the loss of dominance by the beneficial *Sulcia* endosymbionts could instead prove to be detrimental to the insect's fitness. More data on the fitness of the infected and non-infected insects would be needed to give a correct interpretation of this result.

Genus *Wolbachia* tended to be present only in the non-infected specimens and was largely reduced in the infected insect species, except in the case of *D. europaea*, in which the abundance of *Wolbachia* was higher in the '*Ca*. P. solani' infected group. From these results, it becomes evident that the interaction is not just between the phytoplasma and *Wolbachia*, but that the insect species and the rest of the microbiota play a role in determining its outcome. Still, considering that co-presence of phytoplasma and *Wolbachia* was not observed in the majority of the insect species, in general it is reasonable to conclude that a negative interaction governs the relationship between phytoplasma and *Wolbachia*. It should be established whether phytoplasma infection affects the *Wolbachia* concentration or if the presence of *Wolbachia* confers protection either by reduction in pathogen load, or competition with the pathogen (Krstić *et al*. 2018).

6- CONCLUSION

This study described the bacterial communities associated with seven insect species hosting '*Ca*. P. solani' and found in vineyards in North Italy. The mutualistic endosymbiont *Sulcia* was found as the prevalent member of the microbiota in all insect individuals non-infected by the phytoplasma. The non-vector *C. viridis* carries unique bacterial signatures (i.e, *Sodalis*, *Cronobacter*) distinguishing its microbiota from that of vector insects, including its fellow xylem-feeder *P. spumarius*.

Beta-diversity analysis revealed that the xylem-feeding behavior of *C. viridis* and *P. spumarius* gave a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. Anyway, the aforementioned unicity of *C. viridis* reinforces the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Analyses highlighted that, in North Italy, phytoplasma infection (not related to its abundance) is associated with major change due to an increase of diversity in the microbiota structure exclusively in *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, the only species, among the analyzed ones, belonging to the subfamily Deltocephalinae.

Considering the specific bacterial genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), obtained data showed that '*Ca*. P. solani' may have an adverse effect on the presence of *Sulcia* as well as *Wolbachia*, while *Dyella* was not found. Further studies are necessary to elucidate whether observed differences (reduction of *Sulcia* and *Wolbachia,* and increase of bacterial diversity) in phytoplasma infected insects are associated with fitness increase or decrease.

The results of this study indicate an interesting perspective regarding the microbial signatures that could be relevant to determine whether an insect species can be a vector or not, opening up new avenues for developing MRM-based approaches to contain BN spreading.

7- REFERENCES

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Chapter. 6: Grafting of recovered grapevine shoots exhibits preventive and curative effects against grapevine Bois noir

1- Summary

The aim of the present study was to evaluate if grafting of recovered material on symptomatic and symptomless grapevines can have curative and preventive effects against Bois noir. Symptom observation and molecular detection of '*Candidatus* Phytoplasma solani' were conducted on grafted and non-grafted grapevines. Percentage of symptomatic and infected plants was significantly reduced in grafted symptomatic ones, indicating that grafting of recovered shoots can have a curative effect.

2- Introduction

Phytoplasmas are cell wall-less pleomorphic biotrophic plant-pathogenic prokaryotes belonging to the class Mollicutes, closely related to Gram-positive bacteria (Bertaccini & Lee *et al.*, 2018) . They are obligate parasites, classified in the provisional genus '*Candidatus* Phytoplasma' that contains about 40 distinct species based on the sequence identity of 16S rRNA gene and other biological characteristics (IRPCM, 2004). During their evolution, phytoplasma genome underwent to a drastic reduction that resulted in the loss of several metabolic pathways and a stringent host dependence for several essential metabolites (Oshima *et al.*, 2013). Phytoplasmas live only in isotonic environments such as the phloem of plants, the haemocoel and the salivary glands of insects (mainly Cicadellidae and Cixiidae) (Weintraub and Beanland, 2006). Phytoplasmas are associated with several diseases with great economic impact on different economic crops such as grapevine, coconut palm, fruit trees, ornamental plants, and vegetables (Bertaccini *et al.*, 2014).

Phytoplasma-associated grapevine yellows (GYs) are among the most devastating grapevine diseases all over the world and can cause severe yield reductions. Until now, more than 10 taxonomically distinct phytoplasmas have been connected with the same typical GY symptoms (Dermastia *et al.*, 2017; Wei *et al.*, 2017; Tessitori *et al.*, 2018; Zambon *et al.*, 2018a). Interactions between grapevines and GY phytoplasmas remain unclear. However, it seems that the damage caused by GY phytoplasmas to grapevine is greater than might be expected from their relatively low concentration in the phloem and their uneven distribution throughout the plant (Dermastia, 2017). Bois noir (BN), a disease of the grapevine yellows (GY) complex, causes serious crop losses in grapevine growing areas in the Euro-Mediterranean area and in other vine-growing countries (Quaglino *et al.*, 2019). BN is associated with strains of '*Candidatus* Phytoplasma solani' (CaPsol) (subgroup 16SrXII-A) (Quaglino *et al.*, 2013), which is mainly transmitted to grapevine by the polyphagous leafhopper *Hyalesthes obsoletus* Signoret (Hemiptera, Cixiidae) (Maixner, 1994; Sforza *et*

al., 1998). BN epidemiology is very complex due to the existence of multiple alternative vectors and plant hosts representing natural inoculum source (Cimerman *et al.*, 2009; Fialová *et al.*, 2009; Pacifico *et al.*, 2009; Quaglino *et al.*, 2017; Quaglino *et al.*, 2019). Due to this multifaceted ecology, it is extremely difficult to develop efficient control strategies for BN. As no effective control measures directly targeting phytoplasmas are available, preventive measures are applied including the sanitary status check, hot water treatment of propagation material, and control of vectors before their emergence from the ground (Bertaccini et al., 2014; Bianco et al., 2019).

Recovery phenomenon is the spontaneous disappearance of GY symptoms from the canopy of diseased vines (Osler *et al.*, 1993). In recovered vines, symptoms remission, frequently associated with the absence of the phytoplasma, is maintained for a minimum of three consecutive years (Maixner, 2006). Temporary symptom remission (less than three years) is due to seasonal alternation. Several studies showed that recovery can be induced by stressing the plants through uprooting followed by immediate transplanting (Osler *et al.*, 1993; Romanazzi and Murolo, 2008), pruning or pollarding (Borgo and Angelini, 2002; Zorloni *et al.*, 2002), and treating the plants with resistance inducers (Romanazzi *et al.*, 2009, 2013).

Grafting is a special type of asexual plant propagation, in which parts of two plants (scion and rootstock) are joined to grow together and form a new plant more tolerant to biotic and abiotic factors (Meimandi *et al.*,2020). Propagation by grafting have been used in fruit trees for thousands of years and recently even in vegetables, not only for improving yield and fruit quality (López-Marín *et al.*, 2017; Kullaj, 2018; Arvanitoyannis *et al.*, 2005; Flores *et al.*, 2010) but also for the management of soilborne and foliar pathogens (Keatinge *et al.,* 2014; Lee *et al.*, 2010). The most common grafting methods are hole insertion grafting, tongue approach grafting, cleft grafting, splice grafting, pin grafting, tube grafting, side grafting, and bark grafting. Despite all the advantages some problems of grafting still exist; for instance, labor and technique essential for grafting process (Kumar and Sanket, 2017), the price of automatized grafting machines and grafted plant materials (Tsaballa *et al.*, 2013).

In the present study, grafting of materials collected from recovered grapevines was conducted in field trials with the aim to evaluate its preventive and curative potentials against BN.

3- Materials and Methods

3.1. Vineyard features

Grafting trials were carried out in a highly BN-affected 10-year-old organic vineyard, cultivated with Chardonnay cultivar, and located in Gussago, Franciacorta (BS) (N 45°35′38.12″, E 10°09′34.32″). The vineyard consists of 19 rows with 17 poles, and sixteen grapevines between poles. The vineyard has been monitored through visual inspection of GY symptoms and molecular detection of '*Ca*. P. solani' (CaPsol) from 2012 to 2016. BN incidence map was utilized for selecting diseased and healthy vines for grafting trials.

3.2. Selected recovered grapevines

In a Chardonnay vineyard located in Erbusco, Franciacorta (BS) (45°35'55.2"N, 9°57'38.9"E), the selection of grafting materials was obtained from BN-recovered grapevines. The vineyard was monitored for BN spontaneous recovery from 2011 to 2016. The BN-diseased grapevines that showed no symptoms and CaPsol infection for at least three consecutive years were considered as recovered (Maixner, 2006). Material from four recovered vines (F6P18V4, F13P29V1, F13P28V4 and F13P24V3) was collected for grafting trials.

3.3. Grafting

The recovered grapevine materials were grafted using the cleft grafting technique in April 2017. The vineyard was divided into four blocks. Symptomatic and symptomless vines in each block were grafted with plant material from one recovered vine previously selected (see above). In details, plant material from the recovered vine F6P18V4 was grafted in the block from row 1 to 4; plant material from vine F13P28V1 in the block from row 5 to 8; plant material from vine F13P28V4 in the block from row 9 to 12; and plant material from vine F13P24V3 in the block from row 13 to 19. To evaluate the curative effect of grafting with recovered material against BN, 50 symptomatic grapevines were grafted and compared with 50 symptomatic grapevines not grafted. To evaluate the preventive effect of grafting with recovered material against new BN infection, 50 symptomless phytoplasma free grapevines were grafted and compared with 50 symptomless grapevines not grafted. Grafting success was confirmed by checking the attachment percentage during vineyard monitoring for GY symptoms in September 2017.

3.4. Symptom observation and CaPsol detection

The grafting curative and preventive effects against BN was evaluated for three consecutive years, from 2017 to 2019, by field surveys for GY symptom observation and molecular analyses for CaPsol identification conducted on grafted and not-grafted grapevines. Field survey for GY symptom observation and leaf petiole collection from grafted and not-grafted plants were carried out in September from 2017 to 2019. Molecular analyses performed to detect the presence of CaPsol included the total nucleic acid extraction by a CTAB-based method (Angelini et al., 2001), followed by the CaPsol-specific nested-PCR amplification of *stamp* gene (Fabre et al., 2011). PCR products were electrophoresed on 1% agarose gel, stained by Midori Green, and visualized under UV transilluminator.

3.5. Statistical analysis

Levine's homogeneity of variance test was carried out to check differences in BN incidence (symptom observation) and CaPsol infection among the four blocks. Percentage of symptomatic and CaPsol-infected grapevines in grafted and not-grafted vines was compared by T-student test ($p < 0.05$) and one-way ANOVA followed by post-hoc Tukey test ($p < 0.05$), carried out using the software SPSS version 22 (IBM).

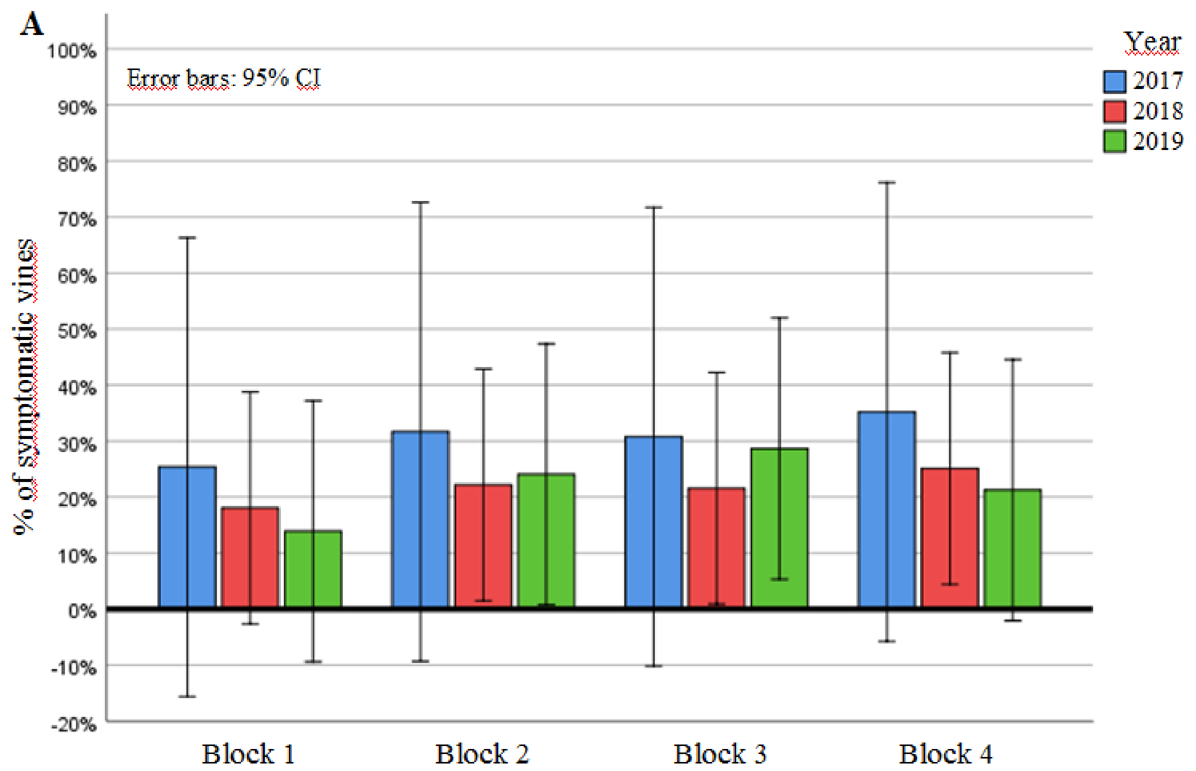
4- Results

4.1. BN incidence and CaPsol infection between blocks

Grafting attachment had high efficacy in both symptomatic (98%) and asymptomatic (86%) grafted vines (Table 16). Levine's homogeneity of variance test showed that no significant differences ($p > 0.05$) were found in BN incidence (percentage of symptomatic vines) and CaPsol infection rate (percentage of CaPsol-infected vines) between the four blocks from 2017 to 2019, excluding the presence of block-specific factor(s) affecting the distribution of the disease (Figure 23).

Table 16. Number of plants examined from 2016 to 2019

Plant group	N. of plants			
	2016	**2017**	**2018**	**2019**
Symptomatic grafted	50	49	49	46
Symptomatic non-grafted	50	49	42	42
Symptomless grafted	50	43	43	43
Symptomless non-grafted	50	50	49	48



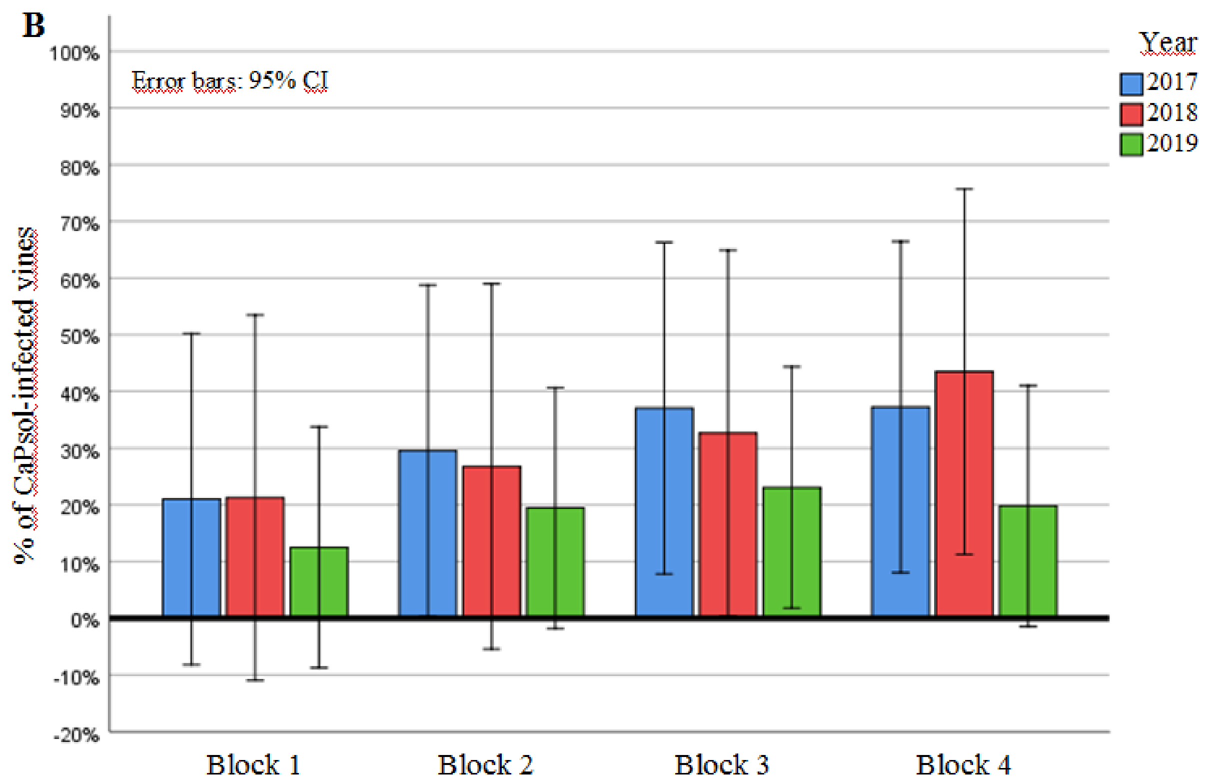


Figure 23. Percentage of symptomatic (A) and CaPsol-infected (B) grapevines in the four experimental blocks from 2017 to 2019

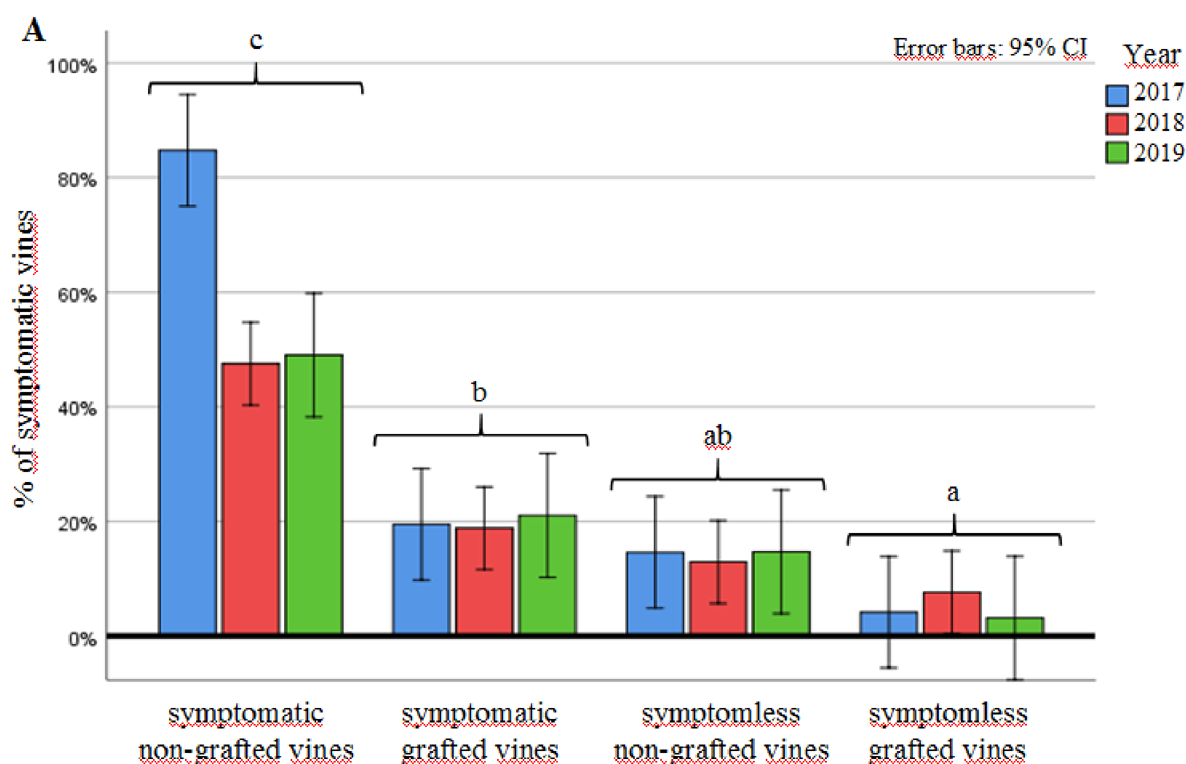
4.2. Symptoms observation and CaPsol infection on grafted and non-grafted vines

Percentages of GY symptomatic and CaPsol-infected vines found within grafted/non-grafted symptomatic/symptomless plant groups from 2017 to 2019 are reported in Table 17.

Table 17. Symptoms presence and '*Ca*. P. solani' infection percentage from 2017-2019

Plant group	**% Symptomatic plants**			**% Infected plants**		
	2017	**2018**	**2019**	**2017**	**2018**	**2019**
Symptomatic grafted	20,41	18,37	21,73	20	26,53	15,22
Symptomatic non-grafted	75,51	47,62	51,06	68,29	71,43	46,81
Symptomless grafted	2,33	8	4	12,5	8	6
Symptomless non-grafted	14	12,24	14,58	23,26	14,29	6,25

The analysis of variance showed that there is a significant difference in BN incidence (percentage of symptomatic plants) and CaPsol infection rate (percentage of CaPsol-infected plants) between the different plant groups ($p < 0.05$). Tuckey test showed a significant difference between the percentage of symptomatic and CaPsol-infected plants in the symptomatic non-grafted plant group (higher percentage) compared to the other plant groups. No significant difference was found between the symptomless non-grafted and grafted plant groups (Figure 24).



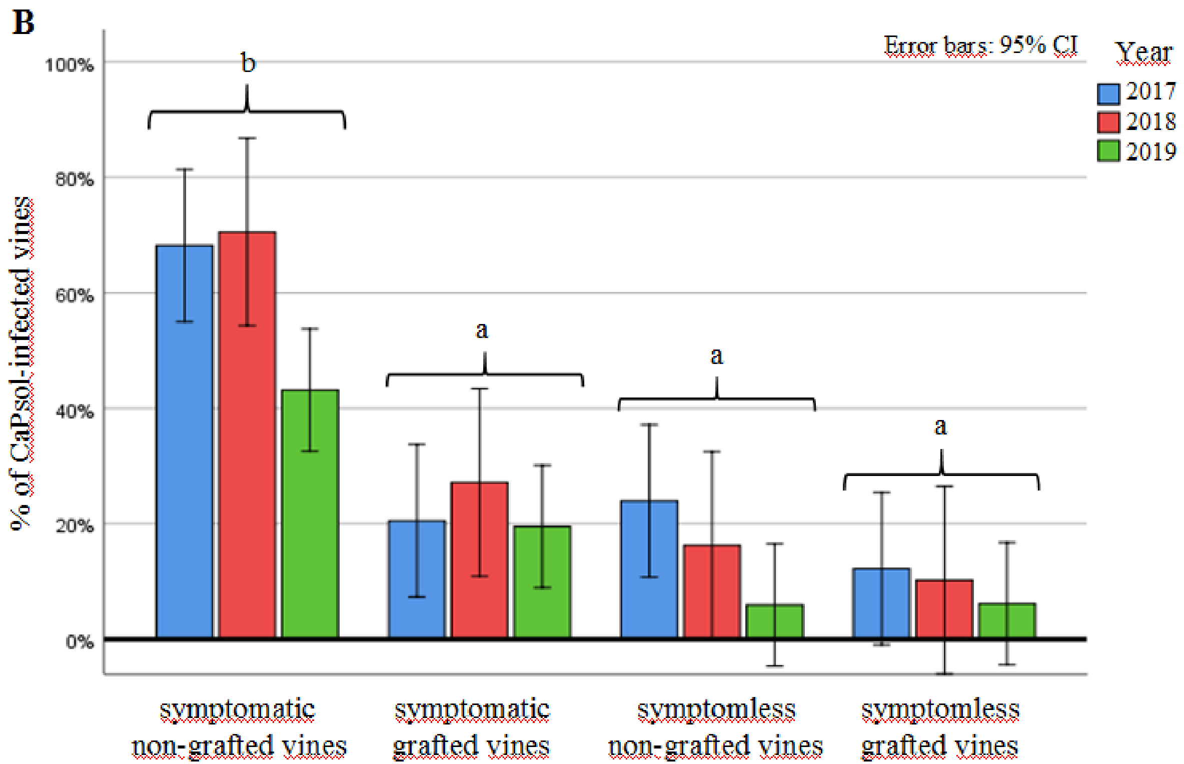


Figure 24 Percentage of symptomatic and CaPsol-infected grapevines observed in the four plant groups (symptomatic/symptomless grafted/non-grafted) from 2017 to 2019

4.3. Recovery rate

Considering the vines showing symptoms in 2016, the percentage of recovered grapevine plants (not showing symptoms for three consecutive years) was significantly ($p < 0.05$) higher (60.86%) in symptomatic grafted vines compared to symptomatic non-grafted vines (12.76%) (Figure 25).

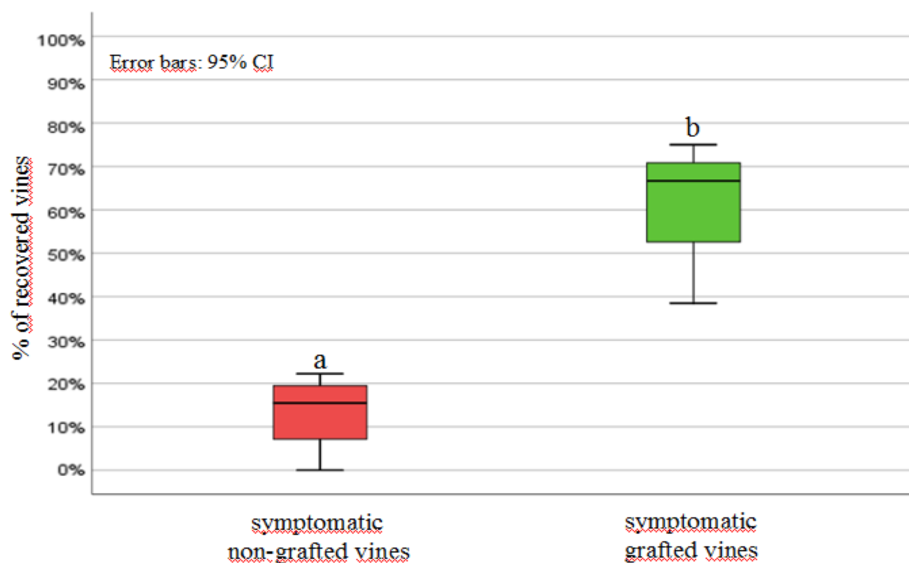


Figure 25 Recovery rate in symptomatic grafted and non-grafted vines.

5- Discussion

Bois noir is one of the grapevine yellows disease that is causing considerable losses to vineyards in Europe and the Mediterranean areas. Its epidemiology is very complex and further investigations are needed to develop sustainable control strategy. To date, the management of the spread of the disease is based almost exclusively on preventive measures, which imply mainly the reduction of inoculation sources (Mori *et al*., 2015). In the last years, the interest in grapevine yellows control strategies acting by promoting the spontaneous recovery of infected grapevines, leading to the remission of symptoms along with the elimination of the pathogen (at least from the canopy) is increasing. Several studies reported that agronomic interventions like pruning of infected vines, partial uprooting followed by replanting, and treatment with resistance inducers can stimulate recovery and can be considered as a possible containment practices (Romanazzi and Murolo, 2008; Belli et al., 2010; Romanazzi et al., 2013).

The use of recovered plant materials tends to have a promising potential as a preventive and curative control measure. Considering the curative effect, our results confirmed that the percentage of symptomatic and CaPsol-infected vines decreased in statistically significant manner in grafted plants compared to non-grafted ones. Regarding the preventive effect, our results indicated that the grafting of recovered materials reduced, but not in statistically significant mode, the rate of new CaPsol-infections. In order to determine the induction of recovery, symptoms and infection by CaPsol in grafted and non-grafted plants were monitored for three consecutive years, as established in previous study (Osler et al., 1993). Obtained results showed that the percentage of recovered grapevine plants was significantly higher (60.86%) in symptomatic grafted vines compared to symptomatic non-grafted vines. Such results give a strong evidence that the use of grafting with recovered material can decrease the incidence of the disease and therefore represents a valid control strategy against BN. However, a cost benefit analysis is necessary to evaluate the profitability of grafting symptomatic plants before to suggest this technique in the integrated BN management.

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Chapter. 7: General conclusion

Conclusion

In the last 50 years since the beginning of disease onset till the catastrophic diffusion worldwide, studies were mostly focusing on the disease epidemiology. The heavy investigations made by phytoplasmologists worldwide led to the identification of the main pillars involved in the disease cycle including '*Candidatus* Phytoplasma solani', the associated agent of Bois noir (BN), and its insect vectors(s). During the PhD research work, we targeted the previous mentioned disease pillars for disease diffusion containment within organically cultivated vineyards. Firstly, insect vectors were on the top priority since they are the main disease diffusers. Within the research focused on insect vectors, the main attention was given to the control of the main insect vector *H. obsoletus* through the use of different strategies including agricultural practices, biological control and disease-insect vector(s) related ecological studies. Regarding the agricultural practices, the use of chaste tree (*Vitex agnus-castus*) as a trap plant for *H. obsoletus* was considered. Chaste tree showed to be a preferred host of *H. obsoletus*, which can complete its life cycle on this plant. However, chaste tree cannot be used as a trap plant since adults of *H. obsoletus* can transmit CaPsol to it. Further investigation will be required to find other preferred host plants that can be used as a trap plants to reduce *H. obsoletus* population within the vineyard. In terms of biological control, entomopathogenic nematodes and fungi showed a promising efficacy against *H. obsoletus* nymphs and adults under laboratory and semi-field conditions. More studies should be carried out on the application of these arsenals in the field condition taking in consideration the environmental factors that boost their presence and enhance their efficacy. The puzzling increment in disease incidence with absence of the main insect vector *H. obsoletus* led to the survey of the Auchenorrhyncha community in vineyards for studying the possible involvement of other insect vectors. Eight alternative insect vectors (*Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Philaenus spumarius*, and *Psammotettix alienus/confinis*) transmitting CaPsol to grapevine were found. These novel findings highlight that BN epidemiology in vineyard agro-ecosystems is more complex than previously known, opening up new perspectives in the disease management. In addition to the complexity of BN epidemiology, alternative control measures have to be developed rather than the direct control strategies of insect vectors. In this context, bacterial microbiota was characterized in *H. obsoletus*, the newly identified alternative insect vectors, and *Cicadella viridis* (non-vector CaPsol-host) to investigate if phytoplasma presence can shape the microbiota, with special

attention for bacteria known as essential for insect survival, parasites, or phytoplasma antagonists. The mutualistic endosymbiont *Sulcia* was found as the prevalent member of the microbiota in all insect individuals non-infected by the phytoplasma. The non-vector *C. viridis* carries unique bacterial signatures (i.e, *Sodalis*, *Cronobacter*) distinguishing its microbiota from that of vector insects, including its fellow xylem-feeder *P. spumarius*. Beta-diversity analysis revealed that the xylem-feeding behavior of *C. viridis* and *P. spumarius* gave a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. Anyway, the aforementioned unicity of *C. viridis* reinforces the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects. Analyses highlighted that, in North Italy, phytoplasma infection (not related to its abundance) is associated with major change due to an increase of diversity in the microbiota structure exclusively in *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, the only species, among the analyzed ones, belonging to the subfamily Deltocephalinae. Considering the specific bacterial genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), obtained data showed that '*Ca*. P. solani' may have an adverse effect on the presence of *Sulcia* as well as *Wolbachia*, while *Dyella* was not found. Further studies are necessary to elucidate whether observed differences (reduction of *Sulcia* and *Wolbachia and* increase of bacterial diversity) in phytoplasma infected insects are associated with fitness increase or decrease. The results of this study indicate an interesting perspective regarding the microbial signatures that could be relevant to determine whether an insect species can be a vector or not, opening up new avenues for developing microbial resource management (MRM)-based approaches to contain BN spreading. The use of cleft grating of BN recovered grapevine materials tended to have a preventive and curative rule that is comparable with the results obtained by partial up-rooting or replanting. More studied must be carried out on the physiological changes accompanied in the enhancement of the recovery phenomena when recovered materials are used as a grafting source. Results obtained in the present PhD thesis provided new insights in BN epidemiology and opened new avenues for developing integrated sustainable strategies for BN management.