Multiple guests in a single host: interactions across symbiotic and phytopathogenic bacteria in phloem-feeding vectors – a review

Published version:

DOI:10.1111/eea.12766

Multiple guests in a single host: interactions across symbiotic and phytopathogenic bacteria in phloem-feeding vectors

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Running title: Bacterial interactions in phloem phytopathogen vectors

Abstract

Phloem-limited bacteria are a major threat for worldwide agriculture due to the heavy economic losses caused to many high-value crops. These disease agents, namely phytoplasmas, spiroplasmas, liberibacters and *Arsenophonus*-like bacteria, are transmitted from plant to plant by phloem-feeding Hemiptera vectors. The associations established among pathogens and vectors often derive from co-evolution, and hence could result in a complex network of interactions involving also the whole microbial community harboured by the insect host. Interactions among bacteria may be beneficial, competitive, or detrimental for the involved microorganisms, and can dramatically affect the insect vector competence and consequently the spread of diseases. Interferences are observed among different pathogen strains competing to invade the same vector specimen, causing selective acquisition or transmission. Bacterial symbionts are another pivotal element for interactions existing between vectors and phytopatogens, because of their central roles for insect life cycle. Some symbionts, either obligate or facultative, were shown to have antagonistic effects on the colonization by plant pathogens, by producing or stimulating the insect production of antimicrobial substances, or competing for host infection. In other cases, evidences of mutual exclusion between symbiont and pathogen suggested possible detrimental influence on phytopathogens displayed by symbiotic bacteria; conversely examples of microbes enhancing pathogen load are available as well. Whether and how bacterial exchanges occurring in vectors affect the relationships between insects, plants and phytopathogens are still incompletely characterized issues, leaving room for many open questions concerning the significance of some traits of these multitrophic interactions. However, such complex interplays may have a serious impact on pathogen spread and control, having the potential to drive new strategies for the containment of important diseases.

Keywords: phytoplasma, liberibacter, spiroplasma, *Arsenophonus*, symbiotic bacteria, antagonism, competition

Introduction

Phloem-limited bacterial phytopathogens, which are among the most devastating agricultural threats globally due to their wide host range and symptom severity, strictly rely on insect vectors to be spread from plant to plant. These pathogenic bacteria are walled Proteobacteria (α- and γ- subclades), and wall-less Mollicutes. The first group encompasses the α-proteobacterial '*Candidatus* Liberibacter spp.', including important pathogens of citrus and vegetable crops (Haapalainen, 2014), and two *Arsenophonus*-related γ-Proteobacteria, namely '*Ca.* Phlomobacter fragariae' and '*Ca.* Arsenophonus phytopathogenicus' (Bressan, 2014). Plant pathogenic Mollicutes embrace the genera '*Ca*. Phytoplasma' and *Spiroplasma*.

All vectors of plant pathogenic bacteria residing in the phloem are Hemiptera belonging to the suborders Auchenorrhyncha (with the families Cixiidae, Dictyopharidae and Flatidae in the Fulgoromorpha infraorder and Cicadellidae in the Cicadomorpha infraorder) and Sternorrhyncha (superfamily Psylloidaea). Vectors are able to ingest bacteria by feeding in the phloem with their piecing-sucking mouthparts. Liberibacters are transmitted by psyllids, and *Arsenophonus*-like bacteria are vectored by planthoppers in the family Cixiidae. On the other hand, phytoplasmas are transmitted by leafhoppers (family Cicadellidae), planthoppers (superfamily Fulgoroidea), and psyllids (superfamily Psylloidea); while spiroplasmas are vectored by leafhoppers only (Gasparich, 2010).

The interactions between plant pathogens and their vectors are not limited to a carrier-carried relation: different species or strains of a plant pathogen have divergent behaviours in different insect hosts. Moreover, phytopathogenic bacteria are included in a complex network of interactions occurring in vectors, being actual members of the multifaceted insect microbiomes, which have a significant influence on the biology of the hosts. Members of the Hemiptera, including all of the vectors of phloem-limited bacterial plant pathogens, rely on bacterial symbionts for supplying nutrients lacking in their unbalanced diet (Baumann, 2005). The nutritional provisioning operated by obligate symbionts has been a crucial condition for insect persistence and diversification on a limited food niche such as plant phloem (Skidmore and Hansen, 2017), then affecting the host range of vectors. High polyphagy deriving from mutualistic associations may in turn influence the chance of different plants to be infected by a plant pathogen. Moreover, facultative symbionts are commonly found in many vectors, showing protective functions, or being capable to manipulate the host's reproduction (Zchori-Fein & Bourtzis, 2011). In addition, different species or strains of plant pathogens may be hosted by the same individual vector (Table 1), possibly being transferred together to the host plant (Bosco & D'Amelio, 2010). Such multipartite interactions most commonly result in microbial synergies or interference, with potential implications for bacterial transmission as well (Bosco & D'Amelio, 2010; Saldaña et al., 2017). This review summarizes the available knowledge concerning microbial exchanges occurring in the vectors of phloem bacterial pathogens, with special regard to the consequences on their transmission. Disease management could take advantage of these interactions to develop microbe-based control strategies (Crotti et al., 2012) (Figure 1). Indeed, despite their capability to easily adapt to, and grow in, different hosts such as plants and insects, currently these phloem-restricted bacteria cannot be cultured or are difficultly cultivated in cell-free media –with few exceptions such as spiroplasmas and a single liberibacter species– (Perilla-Herao &Casteel, 2016), thus limiting experimentations aimed to identify new control strategies. Control is generally based on the use of healthy plant propagation material, elimination of symptomatic plants, and control of insect populations spreading the disease. Unravelling the interactions established between phytopathogens and insect symbionts could offer an interesting tool to impair the transmission of phloem-limited plant pathogens in a sustainable perspective.

Phloem-limited bacterial plant pathogens

Liberibacters

Transmitted by psyllids, '*Ca.* Liberibacter' pathogens include primarily obligate parasites of plants and insects, responsible for several plant diseases, among which huanglongbing (HLB) in citrus trees and zebra chip (ZC) in potatoes are the most severe ones in terms of crop damage and economic losses (Gottwald et al., 2007; Haapalainen, 2014). Three species of '*Ca*. Liberibacter' have been indicated as the causal agents of citrus HLB, previously known as citrus greening, i.e. '*Ca*. L. asiaticus' (CLas), '*Ca*. L. africanus' (CLaf), and '*Ca*. L. americanus' (CLam), the names of which have been derived from the continents where these bacteria have been originally found and are mainly distributed (Haapalainen, 2014). While CLaf is transmitted by the African citrus psyllid *Trioza erytreae* Del Guercio (McClean & Oberholzer, 1965), CLas and CLam are mainly vectored by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Capoor et al, 1967; Teixeira et al., 2005). *D. citri*, native to southeastern Asia, has been recently diffused in America probably in consequence of international commerce (Halbert & Núñez, 2004; Bayles et al., 2017). Despite similar symptoms are recorded after infection by each of the three HLB-causing species, CLas is the most destructive one, inducing devastating epidemics in several countries (Haapalainen, 2014). On the other hand, ZC in potatoes and other diseases in vegetable crops are caused by '*Ca*. L. solanacearum' (CLso), which has been initially indicated with the name '*Ca*. L. psyllaurous' (Liefting et al., 2009). Geographically distinct CLso haplotypes are known, whose differential distribution results in the association with separate plant and insect host species. While in North America and Oceania this pathogen is vectored by the potato/tomato psyllid *Bactericera cockerelli* Šulc, causing severe damage in potato and tomato crops, in Europe -where it is transmitted by psyllids of the species *Trioza apicalis* Förster and *Bactericera trigonica* Hodkinson- it is associated with diseases of the Apiaceae family plants, such as carrot and celery.

In the last years, other liberibacter species have been identified, i.e. '*Ca.* L. europeaus' (CLeu) and *Liberibacter crescens*, but differently from the aforementioned species these latter are not reported as phytopathogens, rather showing an endophytic behaviour (Raddadi et al., 2011; Leonard et al., 2012). Interestingly, *L. crescens*, found in mountain papaya in Puerto Rico, can be grown in axenic cultures, making it an optimal candidate to study liberibacters' biology (Leonard et al., 2012; Fagen et al., 2014a,b). On the other hand, CLeu, reported as an endophyte of pear, apple, blackthorn and hawthorn, transmitted by *Cacopsylla* spp. (Raddadi et al., 2011; Camerota et al., 2012), has been recently indicated as a pathogen in Scotch broom (*Cytisus scoparius*) in New Zealand (Thompson et al., 2013). Recently, other two new candidate liberibacter species were recently reported: the '*Ca.* Liberibacter caribbeanus' (CLca) detected in *Citrus sinensis* (L.) Osbeck and in the citrus psyllid *D. citri* from Colombia (Keremane et al., 2015) and the '*Ca.* Liberibacter brunswickensis' (CLbr) detected in the native Australian eggplant psyllid, *Acizzia solanicola* Kent & Taylor (Morris et al., 2017). Neither these new species were associated with plant disease but a co-evolution with psyllids as secondary symbionts is inferred (Morris et al., 2017).

Arsenophonus-like bacteria

Arsenophonus genus includes not only plant pathogens, but also insect parasites and symbionts (Bressan, 2014). For instance, in a survey performed on 136 arthropod species it has been found that *Arsenophonus* bacteria are associated with 5% of the tested hosts (Duron et al., 2008), where they can establish complex interactions with beneficial or parasitic features (Wilkes et al., 2011). Conversely, two species are cause of disease to strawberry and sugar beet plants (Danet et al., 2003; Bressan et al., 2008). The first pathogenic agent was discovered at the end of last century in France on strawberries affected by marginal chlorosis. Because at that time very little was known about this genus, the pathogen was considered as a separate species that was named '*Ca.* Phlomobacter fragariae' (Zreik et al., 1998); nonetheless the increase of sequence data availability led to propose it to be an *Arsenophonus* (Bressan, 2014). The other plant pathogenic *Arsenophonus* is '*Ca.* Arsenophonus phytopathogenicus' which infects sugar beet, causing a disease defined as "basses richesses" syndrome, because diseased plants show decreased sugar content (Richard-Molard et al., 1995). The insect vectors of pathogens in the *Arsenophonus* group are cixiids: '*Ca.* Phlomobacter fragariae' is vectored by *Cixius wagneri* (China) (Danet et al., 2003), whereas '*Ca.* Arsenophonus phytopathogenicus' is transmitted by *Pentastiridius leporinus* (L.) (Gatineau et al., 2002). These two pathogens are phylogenetically distinct, and can differentially interact with plants and insects in different contexts. '*Ca*. Arsenophonus phytopathogenicus' was observed in Italy to be related to a strawberry marginal chlorosis disease (Terlizzi et al., 2007); likewise it was detected in *C. wagneri*, which was able to inoculate it to sugar beet plants, whereas strawberries were not infected (Bressan et al., 2008). Moreover, the epidemiology of this group of diseases is complicated by the fact that they can be induced also by phytoplasmas transmitted by *Hyalesthes obsoletus* Signoret (Gatineau et al., 2002, Danet et al., 2003). Even being plant pathogens, there is evidence that many traits of *Arsenophonus*-like bacteria are characteristic of an insect symbiont lifestyle, such as reproductive tissue colonization and vertical transmission, absence of entomopathogenic activity, high infection rate and a life cycle prevalently related to insect hosts (Bressan, 2009b; 2014). Thus, these bacteria could easily initiate new associations with additional cixiid species. The complexity of their associations with insects and plants, jointly to cixiids' capability to easily adapt to new environments and host plants, could effectively explain the increasing appearance of emerging *Arsenophonus*-related diseases.

Phytoplasmas

Phytoplasmas are known to be responsible for diseases in over a thousand of economically important crops globally distributed (Marcone, 2014): typical symptoms include yellowing, witches' broom, virescence, phyllody, bolting, reddening of leaves and stems, decline and stunting of plants (Hogenhout et al., 2008). To date, all known phytoplasmas are reported to be pathogenic for at least one plant, even though asymptomatic hosts may be recruited. Phytoplasma taxonomy has been hampered by their recalcitrance to be cultured in vitro; therefore these bacteria are partially classified in the provisional genus '*Ca.* Phytoplasma' based on sequence analysis; up to now 42 '*Ca.* Phytoplasma' species have been reported (Zhao & Davis, 2016). A more exhaustive categorization defines phylogenetic clusters (16SrI-XXXIII groups, each one divided in many subgroups) according to 16S rRNA gene sequence (Lee et al., 1993, 1998b; Zhao & Davis, 2016).

Since most phytoplasmas are capable to cause symptoms to a number of plants belonging to different families, such phytopathogens are regarded as some of the most troubling disease agents in these areas. Moreover, some phytoplasmas are successfully transmitted by polyphagous vectors, furtherly incrementing their chance to infect a huge number of plants. For example, Aster Yellows phytoplasmas (16SrI) are vectored by many polyphagous leafhoppers to several plants (Weintraub & Beanland, 2006), including different flowers, vegetables, or grapevine. The broad range of wild and cultivated plants that are affected by these pathogens can be explained by the polyphagy recorded for most of vectors, along with the great diversity of phytoplasma subclades within this group (Hogenhout et al., 2008).

Considering vector-phytoplasma interplays, many specific interactions are acknowledged between different phytoplasma phylogenetic groups and distinct taxa of vectors. As an example, only leafhoppers in the family Cicadellidae have been reported to transmit phytoplasmas of the 16SrI group (Alma et al., 2015). On the other hand, many phytoplasmas are indistinctively vectored by distant insects. For instance, phytoplasmas of the phylogenetic groups 16SrV and 16SrXII may be vectored by members of either Fulgoromorpha and Cicadomorpha, and 16SX phytoplasma can be transmitted both by Auchenorrhyncha and Sternorrhyncha (Alma et al., 2015). However, a single family with major vector importance can be generally recognized even for pathogens transmitted by distinct taxa: in the case of 16SrV phytoplasmas, most of vectors belong to Cicadellidae, 16SrXII phytoplasmas are mainly transmitted by cixiids, and the major vectors 16SrX phytoplasmas are members of Psyllidae.

Spiroplasmas

Spiroplasmas are regarded as an extremely harmful group for global agriculture, even though only few species have been accounted as phytopathogens, i.e. *Spiroplasma citri* in citrus, *S. kunkelii* in maize and *S. phoeniceum* in aster (Gasparich, 2010). All plant pathogenic spiroplasmas are phylogenetically related, being included in the same taxonomic lineage, namely the Citri clade

(Garsparich, 2010). Despite spiroplasmas and phytoplasmas establish similar pathogenic relationships with host plants, inducing analogous symptoms, major biological differences are evident between these genera. Distinctions include the bacterial shape, as spiroplasmas are characterized by the helical morphology and phytoplasmas are pleomorphic, and cultivation suitability, as spiroplasmas can be cultured in nutrient-rich media while phytoplasmas are recalcitrant to cultivation (Gasparich, 2010).

S. citri is mainly related to heavy economic losses to citrus productions; however this pathogen, as well as its vectors, may be found on many different host plants. Namely, *S. citri* is the agent of citrus stubborn, brittle root disease of horseradish, sesame yellowing, and carrot purple leaf (Zarei et al., 2017); it is transmitted by the leafhoppers *Circulifer haematoceps* (Mulsant & Rey) in the Mediterranean basin and *Circulifer tenellus* (Baker) in North America (Renaudin, 2006). The main areas affected by *S. citri*-related diseases are the Mediterranean countries of Europe, North Africa, and western Asia, as well as the Nearctic region, whereas the pathogen is absent in South America.

S. kunkelii is an important pathogen of maize crops, even though its distribution is restricted to the Americas. Its natural vector is the cicadellid *Dalbulus maidis* (Delong & Wolcott), which is a specialist of the genus *Zea* present in the Nearctic and Neotropical areas. *D. maidis* is co-evolved with maize, where it can be among the most prevalent leafhoppers (Palomera et al., 2012).

The third plant pathogenic spiroplasma species is *S. phoenicium*, which was retrieved from periwinkle plants affected by yellows in Syria. This pathogen is experimentally transmitted by the leafhopper *Macrosteles fascifrons* (Stål); however, at present no information is available concerning the natural vectors of *S. phoeniceum* in the infested area (Saillard et al., 1987).

Bacterial phytopathogen-vector relations

In the vectors, the phloem-restricted pathogens are transmitted in a persistent manner: once ingested by trophic activity on infected plants, bacterial cells multiply in the insect midgut, cross the epithelium, replicate in the hemolymph and, ultimately, infect the salivary glands to be further injected in the new host plant (Figure 1; Gasparich, 2010; Bressan, 2014; Haapalainen, 2014). This process implies complex interplays, spanning from beneficial to adverse. A benign role was suggested for CLas in *D. citri* (Duan et al., 2009; Mann et al., 2011), although an increased susceptibility to selected insecticides was observed in infected psyllids, resulting in fitness decrement (Mann et al., 2011). Similarly, a negative density-dependent effect of CLso infection on the fecundity of *B. cockerelli* was reported by (Nachappa et al., 2014), whereas no significant detrimental effects on the biology of infected individuals occurs according to Thinakaran et al (2015). Effects of vector manipulation by a phytopathogen have been observed also at the hemolymph level, as in CLas-infected *D. citri* showing changes in proteins related to energy metabolism, immunity, and lipid transport (Kruse et al., 2018). Differential effects have been reported for insect-phytoplasma associations: for example, shorter survival and a lower egg production were observed in individuals of *Scaphoideus titanus* Ball infected by 16SrV phytoplasmas (Bressan et al., 2005a), whereas a positive influence have been recorded for 16SrI phytoplasmas in *Macrosteles quadrilineatus* DeLong & Caldwell (Beanland et al., 2000).

The molecular mechanisms regulating plant pathogens retention, multiplication and spread in the body of some species, and not in others, are still poorly understood. The biological adaptation of vectors to harbour plant pathogens suggest a co-evolution between insects and bacteria; however, these interactions have polyphyletic traits, indicating multiple independent evolution events (Orlovskis et al., 2015). The evolution of pathogen transmission shares some traits with insect symbiosis, as most of plant pathogens are phylogenetically related to many symbiotic bacteria of Hemiptera, and similarly to endosymbionts they have reduced genomes, reflecting the adaptation to obligate associations (Bendix & Lewis, 2018). Indeed, a major consequence of a host-dependant life style is an extreme gene loss, due to the lack of a selection process capable to maintain superfluous genes in the rich environment provided by the insect body (Latorre & Manzano-Marín, 2017). In most cases, the associations between plant pathogens and their vectors are believed to be originated from bacterial internalization and successful survival in insects feeding transiently in infected plants (plant-first model). Conversely, some phytopathogens, especially those in the Enterobacteriaceae family, may have been initially insect commensals (i.e. non-harmful associates) that have evolved as plant pathogens following repeated inoculations in the phloem by their insect hosts (insect-first model) (Bové & Garnier, 2002; Nadarasah & Stavrinides, 2011).

Traits affecting vector suitability and specificity are thought to be related to difference in insect physiology, immunity, and behaviour, as well as to their geographical and seasonal distribution (Perilla-Hernao & Casteel, 2016). For instance, divergent plant host-dependant feeding behaviours have been suggested to play an important role in differential transmission competence observed in the leafhopper phytoplasma vectors *Euscelidius variegatus* (Kirschbaum) and *Empoasca decipiens* Paoli (Galetto et al., 2011). Moreover, the vector immune system may limit pathogen invasion. In *D. citri*, CLas acquisition by adult specimens was proven to be significantly less efficient than by nymphs due to differential immune responses, like melanization and apoptosis of gut cells (Kruse et al., 2017). Similarly, immune response may be the cause of limited phytoplasma cell number found in non-transmitting individuals of different vector species after experimental exposure to the pathogens (Galetto et al., 2009). A crucial phase of the transmission process is the protein interaction between pathogen cells and those of the host, regulating pathogen crossing of gut and salivary glands epithelia. The main strategy for bacterial internalization reported for plant pathogenic agents is endo-exocytosis (Kwon et al., 1999; Hogenhout et al., 2008; Cicero et al., 2016), mediated by different membrane proteins (Labroussaa et al., 2010, 2011; Béven et al., 2012; Duret et al., 2014; Konnerth et al., 2016; Arricau-Bouvery et al., 2018). The absence of specific adhesion machinery to host cells seriously weakens the vector competence (Weintraub & Beanland, 2006). For example, *S. citri* strains lacking adhesion-related proteins are not transmissible by insects (Kruse et al., 2017).

The transmission of a plant pathogen by vectors is affected also by the fact that different species or strains of a plant pathogen have divergent behaviours in different insect hosts. This is especially observed for those phytopathogens that most probably derive from insect symbionts, such as *Arsenophonus* bacteria and spiroplasmas. Both the genera *Arsenophonus* and *Spiroplasma* encompass inter- and intracellular symbiotic bacteria displaying a diversity of roles, from mutualism to reproductive manipulation, or may even be entomopathogenic (Gasparich, 2010, Bressan, 2014). In '*Ca*. A. phytopathogenicus' and '*Ca*. P. fragariae', it has been shown that the exploitation of plants resulted from independent evolutionary events from a common endosymbiotic ancestor (Bressan, 2014). This evidence, along with the observation of typical symbiotic traits in insects, like high prevalence and maternal transmission, suggests their transition from endosymbiotic to plant pathogenic life style (Bressan, 2014). Besides, some species belonging to other phytopathogen groups could actually derive from insect commensals. For example, phylogenetic studies demonstrated a match between the affinity level of liberibacter species restricted to different continents and the geographical distribution of psyllid hosts. This supported the hypothesis of a co-evolution between CLbr, behaving as an insect secondary symbiont, and its host *A. solanicola* (Morris et al., 2017). On the other hand, co-evolved associations involving a plant pathogen and an insect vector may lead to mitigate possible harmful effects exhibited on the host fitness (Purcell, 1982). The growing number of observed transitions from insect endosymbiosis to plant pathogeny and vice versa is certainly indicative of the possibility that new bacterial species, currently believed to be horizontally transmitted insect commensals or mutualists, will become emerging plant pathogens in the future.

The study of phytopathogen-vector interactions has a remarkable pertinence from a disease containment perspective, because differential molecular targets for control could be derived from distinct associations involving co-evolution, mutualism or insect injury. For example, the enhancement of insect immunity could be a specific control objective in case of pathogen-vector interactions where the bacterium is definitely recognized and attacked by immune cells due to nonbeneficial interchange (Weiss & Aksoy, 2011). In contrast, some phytopathogens that are anciently related to and co-evolved with their insect hosts are able to escape the immune response. For example, *S. citri* has been reported to evade phagocytosis and limit phenoloxidase activity in its vector *C. haematoceps* (Eliautout et al., 2016). In those cases, control approaches based on immune augmentation may be insufficient.

Multiple pathogen infections and competition

The interaction among pathogens, plants and vectors can be extremely complex. Mixed infections by different bacterial pathogens can quite commonly be observed in the phloem of the same plant. The simultaneous occurrence of multiple pathogens in the same plant is rather frequent in herbaceous plants and trees belonging to many families; either related and phylogenetically distant pathogenic agents may co-exist (Križanac et al., 2010; Nicolaisen et al., 2011; Arratia-Castro et al., 2016; Satta et al., 2016; Swisher et al., 2018). Moreover, a single insect can feed on several plants, or even different plant species, during its life cycle, possibly being exposed to mixed pathogen infections. As a consequence, insect vectors may acquire many pathogen species or strains during the same feeding event, or by feeding sequentially on host plants infected by different bacteria (Križanac et al., 2010; Raddadi et al., 2011; Swisher et al., 2018) (Table 1). However, in some cases, the co-occurrence of multiple pathogens in the same insect's body is inhibited by interferential interactions such as selective acquisition or transmission of a single microbe (Bosco & D'Amelio, 2010). For example, in the leafhopper *Dalbulus maidis* (Delong & Wolcott), which is the natural vector of maize bushy stunt phytoplasma (MBSP) and corn stunt spiroplasma (CSS), competition for transmission was reported after co-occurrence during a long-term latency period (de Oliveira et al., 2007). This competition resulted in suppression of prolonged transmission of MBSP after acquisition of CSS, as the latter is thought to have faster rates of multiplication and spread, hence being more competitive during the latency period required for successful transmission. Similar results were obtained with the cicadellid *M. quadrilineatus*, vector of several strains of Aster Yellows Phytoplasma. Leafhoppers exposed to sequential acquisition of different phytoplasma strains most frequently transmitted the first provided isolate exclusively (Freitag, 1967). These evidences suggest competitive colonization of the insect's body, where the first strain starting multiplication and reaching the salivary glands is more competitive and hence preferentially transmitted (Bosco & D'Amelio, 2010). The same competitive colonization process was proposed for *Osbornellus horvathi* Matsumura, since '*Ca*. P. asteris' and '*Ca*. P. phoenicium' double-infected adult leafhoppers were able to transmit the former, but not the latter, to different plants in experimental conditions (Rizza et al., 2016). Considering *Arsenophonus*-related plant pathogens, no specific transmission trial from double-infected sources has been reported yet; however there are evidences that separated populations of *Cixius wagneri* (China), the only known vector of both pathogens, exclusively transmit '*Ca*. A. phytopathogenicus' or '*Ca*. P. fragariae' but do not carry the two bacteria together (Bressan et al., 2008). Many factors must be taken into account to explain exclusive pathogen acquisition by *C. wagneri*, including vector ecology and population dynamics, which could lead to limited chance for the same individual to be exposed to both pathogens; however the competition between '*Ca*. A. phytopathogenicus' and '*Ca*. P. fragariae' for insect colonization cannot be ruled out.

The competition between two bacterial pathogens in the vectors has been better dissected by Rashidi et al. (2014), by using the leafhopper *E. variegatus* and two unrelated phytoplasmas, namely Chrysantheum Yellows phytoplasma (CYP) and Flavescence Dorée Phytoplasma (FDP), experimentally transmitted to broad bean plants. The authors found that insects sequentially exposed to acquisition of CYP and FDP showed unilateral interference, with the suppression of FDP transmission regardless of the feeding order. On the other hand, the acquisition of each pathogen was not affected by the presence of the other one, suggesting no competition at the earlier infection stages. The barrier where competition takes place was rather identified in salivary glands, which were more rapidly invaded by CYP due to its capability to multiply faster than FDP, even though the latter bloomed to higher concentrations. The higher speed in reaching salivary glands displayed by CYP was suggested to be related to: i) long co-evolution with the insect host and consequent mitigated immune response, and ii) broad phytoplasma host range supporting the evolution of traits that promote acceptability by a broad vector range (Rashidi et al., 2014). Transcriptomic analysis of infected leafhoppers with single phytoplasma strains demonstrated the activation of insect immune response (by activation of Kazal type 1 serine protease inhibitor and melanisation pathway) after infection by FDP, which reduces the host fitness and is then perceived as a potential pathogen (Galetto et al., 2018). Instead, the most competitive CYP increased energy metabolism, providing molecular confirmation for different competition levels.

The knowledge on competition between co-occurring pathogen strains in the same host, although being still limited, could support the study of pathogen transmission. Indeed, the observation and characterization of competition events may contribute to unravel meaningful details of the processes determining insect invasion and spread of phytopathogens, possibly identifying weaknesses of single associations and revealing new control targets. Moreover, competitive transmission of different plant pathogens may seriously alter disease epidemiology in the field.

Symbiont-pathogen interactions

The Auchenorrhyncha and Sternorrhyncha, including the vectors of plant pathogenic bacteria, harbour both obligate and facultative endosymbionts which play important roles in supplying nutrients and providing the host with other fitness benefits (Baumann, 2005; Morrow et al., 2017). The main obligate (primary) symbiont are '*Ca*. Sulcia muelleri' in Auchenorrhyncha, and '*Ca*. Carsonella ruddii' in psyllids. Moreover, *Sulcia* requires complementary (co-primary) symbiotic bacteria to integrate its nutrient supply to the insect (McCutcheon & Moran, 2010). Similarly, psyllids harbour secondary symbionts, such as *Sodalis* or *Arsenophonus* bacteria, with nutritional roles (Morrow et al., 2017). In addition, the function of some symbionts of hemipterans vectors is still unrecognized. For example, many bacteria generally known as reproductive manipulators, such as *Wolbachia*, *Cardinium*, *Rickettsia* and *Arsenophonus*, have been found in several vector species;

however their role has not been characterized yet (Marzorati et al., 2006; Gonella et al., 2011; Jing et al., 2014; Morrow et al., 2017; Iasur-Kruh et al., 2017). Moreover, some insect beneficial microorganisms (e.g. *Rickettsia* and *Cardinium*), capable to colonize the salivary glands, may be transferred from insect to plant and vice versa, possibly establishing endophytic relationships as well (Caspi-Fluger & Zchori-Fein, 2010; Gonella et al., 2015; Iasur-Kruh et al., 2017). Despite the emerging recognized need to study microbial communities affiliated to non-model insects (Prosdocimi et al., 2015), which recently led to a growing number of evidences of co-existence of plant pathogens and other microbes in the insect vectors, few studies directly investigated their interactions (Table 2). Symbiont-pathogen exchanges were firstly studied in psyllids, and more specifically in the CLas vector *D. citri*. This psyllid harbours three main endosymbionts: a species of *Wolbachia*, the γ-Proteobacterium '*Ca.* Carsonella ruddii', an endosymbiont which may provide nutritional benefits to its host (Thao et al., 2000), and '*Ca.* Profftella armatura', a β-Proteobacterium with defensive function (Nakabachi et al. 2013). Fagen et al. (2012) firstly observed a negative correlation between CLas infection rate with the relative abundance, within the microbial community, of *Profftella*. Based on its genome sequence, *Profftella* was predicted to produce defensive toxins, i.e. diaphorin and diaphorin-related polyketides. CLas-infected [CLas(+)] insects were found to have dramatically elevated levels of two proteins involved in polyketide biosynthesis. In contrast, the protein responsible for initiating diaphorin biosynthesis is down-regulated in CLas(+) *D. citri* (Ramsey et al., 2015). Moreover, Ramsey et al. (2015) observed that the ratio between levels of diaphorin and the related polyketide is significantly increased in CLas (+) compared to CLas uninfected [CLas(-)] *D. citri*, suggesting changes in *Profftella* polyketide metabolism in response to the presence of the pathogen or in direct or indirect response to changes induced by the pathogen in infected plants. The up-regulation of the polyketide synthase (PKS) gene expression in CLas(+) *D. citri* may be a specific response of *Profftella* to the presence of CLas, as part of an infection response that may be mediated by *D. citri* (Ramsey et al., 2015). Such an interactive response may involve

Carsonella as well, which could provide the host with essential amino acids required for polyketide production (Ramsey et al., 2015).

Besides psyllid-liberibacter interactions, further evidences of antagonistic relationships between symbiotic bacteria and plant pathogens are reported for some Auchenorrhyncha vectors of phytoplasmas. A bacterium in the Xanthomonadaceae, provisionally named *Dyella*-like bacterium (DLB) (Iasur-Kruh et al., 2017), was isolated from the planthopper *H. obsoletus*, and showed anti-phytoplasmal activity in inoculated plants (Iasur-Kruh et al., 2018). Indeed, despite being isolated from an insect source, DLB showed endophytic traits: it was consistently found in the wild bush *Vitex agnus-castus* L., and it was able to long-term colonize the phloem of different plant species, including many hosts of phytoplasmas and liberibacters (Lidor et al., 2018). Once established in grapevines infected by phytoplasmas, DLB reduced disease symptoms (Iasur-Kruh et al., 2018). Based on DLB genome analysis, the authors suggested that such a drop of symptoms is related to inhibition of pathogens, rather than competition or production of substances stimulating plant growth or defense (Lahav et al., 2016; Iasur-Kruh et al., 2018). Moreover, DLB was demonstrated to inhibit the growth of the cultivable model Mollicute *Spiroplasma melliferum* (Iasur Kruh et al., 2017).

Acetic acid bacteria in the genus *Asaia* are widespread in insects, including leafhoppers transmitting phytoplasmas, and they were proposed to interact with insect vectors, possibly altering their spread (Crotti et al., 2009). Strains with different phenotypes previously isolated from mosquitoes were orally supplied to the experimental vector of FDP *E. variegatus*, which was successfully colonized. One *Asaia* strain producing an air-liquid interface biofilm, after establishing in *E. variegatus,* reduced its acquisition of FDP from broad beans in experimental conditions (Gonella et al., 2018). These authors suggested that the strain of *Asaia* could affect the capability of the phytoplasma to cross the gut epithelia for reaching salivary glands, even though the mechanisms regulating this interference remain to be elucidated. However, such an alteration was imperfect and, when the pathogen succeeded in colonizing the insect, transmission rates to broad beans were similar to those recorded for control leafhoppers unexposed to *Asaia* (Gonella et al., 2018).

Additional interplays between symbiotic bacteria and plant pathogens have been suggested by multiple prevalence studies, as in some cases positive correlation or mutual exclusion could be detected between symbiotic and phytopathogenic bacteria. For example, the obligate symbiont *Nasuia*, largely widespread in the family Cicadellidae, is present in most of leafhopper species transmitting phytoplasmas, while non-vector species were shown to lack it (Wangkeeree et al., 2012). It has been suggested that *Nasuia* could be required for successful transmission. Likewise, in the planthopper FDP vector, *Dictyophara europaea* L., a negative correlation between infections by phytoplasma and *Wolbachia* was reported, suggesting that the *Wolbachia* strain infecting *D. europaea* displays antagonistic activities against the pathogen, or alternatively competes for insect colonization (Krstić et al., 2018). On the other hand, in *D. citri* an increase in the ubiquitous *Wolbachia* titre was reported with CLas infection (Fagen et al., 2012), indicating a more complicated interplay mechanism with strain-specific variability. Direct interaction has been documented between *Wolbachia* and CLas, as the first suppress the holing lytic promoter in a CLas-infecting phage in *D. citri* (Jain et al., 2017).

The studies regarding synergies and interferences between symbiotic agents and plant pathogens offer significant cues for disease treatment; moreover, further work is still required to describe new interactive associations. Future work concerning such interplays should be aimed not only to identify direct anti-pathogen activity expressed by symbionts, but also to alter the mutualistic exchange recorded among vectors, symbionts and phytopathogens, and to influence insect ecology (e.g. by driving plant choice and governing interactions with stresses).

Conclusions and open issues

The interactive roles of phytopathogenic and symbiotic bacteria in insects certainly represent an emerging topic for researchers focusing on the transmission process of disease agents. A multi-actor picture, involving insects, plants, and microbes, is resulting as the condition where the transmission of plant pathogens arises. Consequently, the bacterial interactions occurring in insects affect the life cycle of the host as well. First, considering the reported evolutive bilateral transition of the role of many disease agents in their vectors from symbiotic to phytopathogenic life style, the effects of these bacteria are a key issue for the study of insect-microbe relationships; however they are still mostly unknown. Such effects may also result in the uneven competitive behaviours described for both closely and distantly related pathogens. Many questions arise from this hypothesis. How is insect immunity involved in differential growth rates of plant pathogens? What are the traits of vector-pathogen interaction originating possible diversity in host responses? Are these bacteria at different steps of transition from symbiont to pathogen or vice versa (e.g. the most competitive pathogens supply the host with fitness advantages)? Most of these questions were addressed by Galetto et al. (2018) using the *E. variegatus*-CYP-FDP model, but more work is needed to expand the analysis of competitiveness conditions to different pathogens and vectors. Moreover, it is still unclear whether non-competitive or beneficial interactions take place among pathogens in insects where multiple infections are observed. Finally, how the plants are implicated in these interactions? Many examples are available on the effects displayed by phytopathogens on the plant processes in favour of insects, such as the promotion of insect attraction to infected hosts, allowing the pathogen spreading (Orlovskis et al., 2015). However, whether pathogens that are capable to modulate their attractiveness could display enhanced competitiveness against horizontally transmitted microbes (including other phytopathogens) is poorly understood. Deep surveys of molecular and cellular machineries of insect-phytopathogen-host plant relations could provide the answers to these issues.

Additional open questions involve the role of bacterial symbionts in plant pathogen competition and spread. Only few examples of interactions between symbionts and pathogens have been described, in spite of the high number of symbiotic bacteria depicted in most of vectors: direct evidences of an interference with the transmission process in the insect or with symptom development in the plant have been provided only for phytoplasmas (Gonella et al., 2018; Iasur-Kruh et al., 2018).

Furthermore, the mechanisms regulating beneficial or hostile exchanges have been only rarely elucidated, and some bacterial pathogens were shown to exhibit mutualistic effects on their vectors, while other caused fitness costs (Hogenhout et al., 2008; Tamborindeguy et al., 2017). An open field for future research is the awareness of whether harmful or beneficial roles are in some way the result of interactions with bacterial symbionts co-inhabiting the same host. A similar evidence of indirect effect on the insect fitness as a consequence of symbiont suppression was observed in virus-transmitting aphids. In the soybean aphid *Aphis glycines* Matsumura, a drop in the concentration of the endosymbiotic *Buchnera* was observed in insects exposed to the beetle-transmitted bean pod mottle virus, resulting in reduced aphid fecundity (Cassone et al., 2015).

Finally, a still unexplored field for vectors of phloem-limited pathogen is the manipulation of symbiotic microbes to drive their interaction with plant pathogens toward antagonistic activities, by means of paratransgenesis. A similar approach was proposed for example for a xylem-restricted pathogenic agent, i.e. the *Xylella fastidiosa* strain causing Pierce disease to grapevine. A bacterium reported as an insect symbiont and an endophyte, *Alcaligenes xylodoxidans denitrificans*, was proposed as a candidate agent to be genetically transformed to display anti-*Xylella* molecules (Bextine et al., 2004).

Along with being of certain interest to elucidate biological mechanisms regulating insect-bacteria relationships, the gain of knowledge concerning microbial interactions occurring in insect vectors have important implications for disease epidemiology and control. From the epidemiological point of view, the competition among plant pathogens alters the rates of transmission by vectors, and possibly influences their fitness as well, with a final impact of the spread of diseases on different plants. From the point of view of disease control, the study of microbial interactions in the vectors could provide valuable tools to manage crop infections by altering vector competence via symbiotic control approaches (Alma et al., 2010). Possible strategies include the identification of detrimental effects played by symbionts on plant pathogens in the insect, or the selection of new molecular targets to interrupt beneficial interplays among bacteria.

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Table legends

Table 1 Multiple bacterial infections in the vectors of phloem-limited pathogens. Only reports showing mixed infections in the same host individual, involving distinct plant disease agents or symbiotic bacteria with phytopathogens, are listed.

Table 2 Symbiont-pathogen interactions reported in the vectors of phloem-limited plant pathogenic bacteria.

Figure legends

Figure 1 Insect symbionts could be useful for controlling the transmission of phloem-limited plant pathogens. Phloem-restricted plant pathogens are indicated as red, purple or violet dots, while microbial symbionts are depicted with other different colours. Microbe movements are indicated with red arrows. Symbiont-mediated control mechanisms of pathogen transmission are listed on the right and corresponding numbers are depicted in gut (in green circles), hemolymph (in orange circles) and salivary glands (inset, in blue circles).

TABLES

Vector taxonomic position	Vector family	Vector species	Phytopathogen multiple infection	Symbiont - phytopathogen multiple infection	Reference
Auchenorrhyncha - Fulgoromorpha	Cixiidae	*Hyalesthes obsoletus* Signoret		*'Ca.* Sulcia muelleri' + *Wolbachia* + '*Ca.* Vidania fulgoroidaeae' + '*Ca.* Purcelliella pentastirinorum' + 16SrXII phytoplasma	Gonella et al., 2011
		Pentastiridius leporinus L.		*'Ca.* Sulcia muelleri' + '*Ca.* Purcelliella pentastirinorum' + *Wolbachia* + '*Ca.* Arsenophonus phytopathogenicus'	Bressan et al., 2009a
Auchenorrhyncha-Cicadomorpha	Cicadellidae	*Amplicephalus curtulus* Linnavuori & DeLong	Phytoplasmas, groups: 16SrI + 16SrXII		Longone et al., 2011
		Amplicephalus funzaensis Linnavuori	Phytoplasmas, groups: 16SrI + 16SrVII		Perilla-Henao et al., 2016
		Circulifer tenellus (Baker)	16SrVI phytoplasma + *S. citri*		Lee et al., 1998a Swisher et al., 2018
		Euscelidius variegatus Kirshbaum	Phytoplasmas, groups: 16SrI + 16SrV	bacterium of *E. variegatus* (BEV) + 16SrI phytoplasma *Asaia* + 16SrV phytoplasma	Rashidi et al., 2014 Galetto et al., 2009 Gonella et al., 2018
		Euscelis incisus (Kirschbaum)	Phytoplasmas, groups: 16SrI + 16SrIII + 'Ca. Phytoplasma pruni'		Orságová et al., 2011
		Euscelis lineolatus Brulle	Phytoplasmas, groups: 16SrII+16SrXII		Landi et al., 2013
		Exitianus atratus Linnavuori	Phytoplasmas, groups: 16SrI + 16SrVII		Perilla-Henao et al., 2016
		Graminella nigrifrons (Forbes)	Phytoplasmas, groups: 16SrI + 16SrVII 16SrI + 16SrX		Arocha-Rosete et al., 2011
		Macrosteles sexnotatus (Fallén)		*'Ca.* Sulcia muelleri' + *Nasuia* + 16SrI phytoplasma	Ishii et al., 2013
		Macrosteles striifrons Anufriev		*'Ca.* Sulcia muelleri' + '*Ca.* Nasuia deltocephalinicola' + 16SrI phytoplasma	Ishii et al., 2013
		Matsumuratettix hiroglyphicus (Matsumura)		Bacterium associated with *M. hiroglyphicus* (BAMH) (*Nasuia*) + *'Ca.* Sulcia muelleri' + 16SrXI phytoplasma	Wangkeeree et al., 2012
		Osbornellus horvathi Matsumura	'*Ca.* Phytoplasma asteris' + '*Ca.* Phytoplasma phoenicium'		Rizza et al., 2016

		Paratanus exitiosus (Beamer)	Phytoplasmas, groups: 16SrII + 16SrVII + 16SrXII		Longone et al., 2011
		Recilia dorsalis Motschulsky		BAMH + *'Ca.* Sulcia muelleri' +16SrXI phytoplasma	Wangkeeree et al., 2012
		Recilia sp. nr. *vetus*		BAMH + *'Ca.* Sulcia muelleri' +16SrXI phytoplasma	Wangkeeree et al., 2012
		Scaphoideus titanus Ball		'*Ca*. Cardinium hertigii' + 16SrV phytoplasma	Marzorati et al., 2006
Sternorrhyncha	Psyllidae	*Cacopsylla chinensis* (Yang & Li)	Phytoplasmas, groups: 16SrII + 16SrX		Liu et al., 2011
		*Cacopsylla melanoneura (*Förster*)*		CLeu + '*Ca.* Phytoplasma mali'	Camerota et al., 2012
		Cacopsylla pyri L.	Phytoplasmas, groups: 16SrI + 16SrXII 16SrX + 16SrXII 16SrI + 16SrX	CLeu + '*Ca*. Carsonella ruddii' + *Arsenophonus* + *Ralstonia* + '*Ca*. Phytoplasma pyri'	Križanac et al., 2010 Raddadi et al., 2011 Camerota et al., 2012
		Cacopsylla pyricola Förster		*Arsenophonus* + '*Ca*. Phytoplasma pyri'	Cooper et al., 2017
		Diaphorina citri Kuwayama		'*Ca*. Carsonella ruddii' + '*Ca*. Profftella aramtura' + *Wolbachia* + CLas *Ralstonia* + CLas	Kruse et al., 2017 Ramsey et al., 2017 Kolora et al., 2015
	Triozidae	*Bactericera cockerelli* (Sulc)		*Erwinia* sp. + *Wolbachia* + *Staphylococcus* sp. + *Enterococcus* sp. + CLso	Kolora et al., 2015
		Bactericera trigonica Hodkinson	CLso + phytoplasmas, (group 16SrVI+ 16SrI)		Swisher et al., 2018

Table 2 Symbiont-pathogen interactions reported in the vectors of phloem-limited plant pathogenic bacteria.

Insect	Phytopathogen	Symbiont	Interaction	Reference
Diaphorina citri	CLas	'*Ca.* Profftella armatura'	Upregulation of genes involved in biosynthesis of diaphorin polyketide.	Ramsey et al., 2015
		Wolbachia	Positive correlation	Fagen et al., 2012
Hyalethes obsoletus	16SrXII phytoplasma	*Dyella*-like bacterium (DLB)	Reduction of phytoplasma-related symptoms in grapevine	Iasur-Kruh et al., 2018
Euscelidius variegatus	16SrV phytoplasma	*Asaia* sp.	Reduced phytoplasma acquisition in Asaia-infected individuals	Gonella et al., 2018
Matsumuratettix hiroglyphicus *Recilia dorsalis* *Recilia* sp. nr. vetus	phytoplasmas	Bacterium associated with *M. hiroglyphicus* (BAMH) (*Nasuia*)	BAHM suggested to be required for successful phytoplasma transmission	Wangkeeree et al., 2012
Dyctiophara europaea	16SrV phytoplasma	*Wolbachia*	Mutual exclusion	Krstić et al., 2018