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Vector mediated transmission of *Candidatus Liberibacter solanacearum* and
epidemiological consequences

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**VECTOR MEDIATED TRANSMISSION OF *CANDIDATUS LIBERIBACTER*
SOLANACEARUM AND EPIDEMIOLOGICAL CONSEQUENCES.**

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ACRONYMS AND ABBREVIATIONS

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

CLas: *Candidatus Liberibacter asiaticus*

°C: Centigrade degrees

cm: Centimeters

cv: Cultivar

D: Dark

d: Days

df: degree freedom

EMF: Electromotive force

EPG: Electrical Penetration Graph

g: Grams

h: Hours

Hz: Hertz

IAP: Inoculation access period

L: Light

l: Liters

ln: Natural logarithm

LSD: Least significant differences

Lso: *Candidatus Liberibacter solanacearum*

min: Minutes

mm: Milimeters

ng: Nanogram

NPI: Number of probes per insect

N: P: K: nitrogen, phosphorous, potassium

NWEI: Number of waveform events per insect

P: *P* value

pd: potential drop, stylet intracellular puncture.

PCR: Polymerase chain reaction

PPW: Proportion of insects who produced a waveform

Pseudo-pd: Pseudo potential drop

R: Resistance

RH: Relative humidity

s: Seconds

sqrt: Square root

t: t-student

TWD: Total waveform duration

U= Mann Whitney test

μl: Microliters

μm: Micrometers

V: Voltage

WDI: Waveform duration per insect

WDE: Waveform duration per event

WDEI: Mean waveform duration per event per insect

RESUMEN

Candidatus Liberibacter solanacearum (Lso) es una bacteria fitopatógena restringida al floema que causa grandes pérdidas económicas en diferentes cultivos a nivel mundial. Esta bacteria es transmitida de manera circulatoria propagativa por diferentes especies de Psílidos (Hemiptera : Psylloidea). En Europa, Lso causa importantes pérdidas en cultivos de Apiáceas y también representa una potencial amenaza para la industria de la patata. El objetivo principal de este trabajo ha sido estudiar la transmisión de Lso mediada por psílidos y sus consecuencias epidemiológicas.

El primer objetivo fue estudiar el comportamiento alimenticio de *B. trigonica* el principal vector de Lso en el Mediterráneo y evaluar el efecto de Lso sobre dicho comportamiento. Para esto se caracterizaron las ondas de gráficos de penetración eléctrica (EPG) producidas por este insecto en plantas de zanahoria. Una vez caracterizadas, las ondas descritas fueron correlacionadas con la posición de la vaina salivar producida por el insecto durante cada onda y con la inoculación de Lso. Se identificaron 7 ondas (C1, C2, Pseudo-pd, G, D, E1 y E2) y se encontraron las siguientes correlaciones directas con la posición de las vainas salivares en diferentes tejidos: ondas C1, C2 y pseudo-pd en mesófilo, onda G en xilema, ondas D, E1 y E2 en floema. Se identificó y describió una onda no descrita previamente en psílidos “pseudo-potential drop” y se discutió su significado biológico. Este trabajo también demuestra que periodos cortos de E1 están relacionados con altos porcentajes de inoculación de Lso. Finalmente, se observó que aunque Lso afecta de manera directa el comportamiento alimenticio de *B. trigonica* los cambios producidos no afectan a los parámetros relacionados con su transmisión.

También se estudió cómo el sexo del vector afecta el comportamiento alimenticio y cómo esto afecta la transmisión de Lso. Por medio de la técnica de EPG, se monitorizó el

comportamiento alimenticio de machos y hembras de *B. trigonica* en plantas de zanahoria y posteriormente se correlacionó con la transmisión de Lso. Para determinar la transmisión de machos y hembras se realizaron ensayos con un psílido por planta y ocho psílicos por planta. Por último se cuantificó la cantidad de Lso transmitida y los síntomas producidos cuando Lso fue transmitido por machos o por hembras. Este trabajo demostró diferencias en el comportamiento alimenticio de los diferentes sexos se correlacionan con diferencias en la cantidad de Lso transmitida. Las hembras probaron e ingirieron más savia del floema que los machos lo cual se correlaciono con mayores cantidades de Lso inoculadas por hembras. A pesar de las diferencias en la cantidad de Lso inoculada por machos o hembras, la tasa de transmisión de Lso no se vio afectada por el sexo del vector y tanto machos como hembras transmitieron eficientemente la bacteria. Nuestro estudio también demostró que cuando Lso es transmitido por grupos de hembras los síntomas producidos en planta fueron más severos que los causados por Lso cuando es transmitida por grupos de machos. Estos resultados sugieren una alta propensión vectorial de *B. trigonica* y demuestran que el sexo del vector puede afectar la transmisión de Lso.

Debido a que *B. trigonica* y *B. tremblayi* se encuentran ampliamente distribuidos en España y causan pérdidas económicas importantes se evaluó la transmisión de Lso por *B. trigonica* y *B. tremblayi* en cultivos de apio, zanahoria y patata en ensayos con y sin elección, así como su preferencia por los diferentes cultivos. Además, se monitorizó el comportamiento alimenticio de ambas especies en los diferentes cultivos mediante la técnica de gráficos de penetración eléctrica (EPG). *Bactericera trigonica* fue incapaz de colonizar plantas de patata y tampoco pudo transmitir Lso a este cultivo. Además *B. trigonica* mostró una clara preferencia a aterrizar, ovipositar y alimentarse en plantas de zanahoria y apio lo cual se correlaciono con una alta tasa de transmisión en ambas especies de plantas. Por otro lado, a pesar que *B. tremblayi* fue capaz de adquirir Lso de zanahorias infectadas fue incapaz de

transmitirlo a plantas de zanahoria sanas. Los resultados demuestran que *B. trigonica* es un vector eficaz de Lso en zanahoria y apio pero no representa un riesgo importante para la industria de la patata, a la vez que no hay evidencia suficiente para considerar a *B. tremblayi* como vector de Lso.

Por último, la densidad poblacional de las especies de psílicos asociadas a cultivos comerciales de patata y zanahoria fue estimada en 2015 y 2016 utilizando diferentes tipos de muestreo, (muestreo con manga entomológica, trampas de agua e inspección visual). Se colectaron psílicos en tres de las zonas más importantes de España en cuanto a producción de zanahoria, Gomezserracín (Segovia), Íscar (Valladolid) y Villena (Alicante). Además se muestrearon psílicos en cultivos de patata en las zonas de Gomezserracín (Segovia) y Aldearrubia (Salamanca). Los muestreos mostraron que *B. trigonica* fue el psílido más abundante en todos los cultivos de zanahoria seguido por *B. nigricornis*, especie que se encontró en bajos números poblacionales. Por otro lado, *B. nigricornis* fue la única especie asociada a todos los campos de patata en 2015 y 2016. Siendo a su vez la especie más abundante en patata y la única que tiene la capacidad de completar su ciclo de vida en este cultivo. Estos datos sugieren que *B. trigonica* es capaz de colonizar zanahoria pero no patata y que *B. nigricornis* puede colonizar tanto zanahoria como patata.

Esta Tesis generó nueva información del comportamiento alimenticio, la capacidad de transmisión y la dinámica poblacional de psílicos en cultivos de Apiáceas y de patata. Esta información es fundamental para desarrollar estrategias de control a largo plazo.

SUMMARY

Candidatus Liberibacter solanaceum (Lso) is an emerging pathogenic plant bacterium causing major economic losses in vegetable crops worldwide. Lso is transmitted in a circulative-propagative manner by different psyllid species (Hemiptera : Psylloidea). In Europe Lso causes economically important losses in carrot (*Daucus carota* L.) and celery (*Apium graveolens* L.) crops and has become a potential threat to the potato (*Solanum tuberosum* L.) industry. This Thesis focuses on studying the vector transmission of Lso by psyllids and its epidemiological implications.

The first objective of this Thesis was to study the feeding behavior of the main Lso vector *Bactericera trigonica* Hodkinson in the Mediterranean region, by characterizing its feeding behavior on carrot seedlings by means of the Electrical Penetration Graph (EPG) technique. Waveforms identified were correlated with stylet tracks and salivary sheath termini on plant tissues as well as with Lso inoculation. Also, the effect of Lso in the feeding behaviour of *B. trigonica* was tested by EPGs. Seven different waveforms (C1, C2, pseudo-potential drop, G, D, E1 and E2) were identified and correlated with salivary sheath termini in mesophyll (C1, C2 and Pseudo-potential drop), xylem (G) and phloem tissues (D, E1 and E2). “Pseudo-potential drop” is a novel waveform pattern never previously described for psyllids. Here, the first and full description of this waveform is provided and its biological meaning discussed. This study showed that *B. trigonica* is a highly efficient vector of Lso and that there is a direct correlation between waveform E1 and the inoculation of Lso. Additionally, I found that Lso directly affects the feeding behaviour of *B. trigonica*, but has no influence in its own transmission (the pathogen does not enhance or impair transmission).

The second objective of this study focused on how vector sex affected the feeding behaviour and consequently the transmission of Lso. By using EPGs the feeding behaviour of males and

females of *B. trigonica* on carrots was monitored and correlated with Lso transmission rate by single or groups of males and females of *B. trigonica*. In addition, the titre of Lso transmitted by males or females as well as the symptoms expression produced by Lso on carrots was quantified. The sex of the vector affected the feeding behaviour and Lso titre. Females probed and ingested more from the phloem tissues than males and this was correlated with more Lso titter inoculated by females than by males. Despite Lso titter differences, transmission rate was not affected by sex and both males and females transmitted Lso very efficiently. This study also showed that groups of females more severe symptoms than groups of males, however this difference was not evident when comparing symptoms produced by single males or single females. These results show a high vector efficiency of *B. trigonica* and highlight the importance of vector sex in the transmission of Lso.

Third, the risk of transmission of Lso from carrots to other economically important crops was assessed. In the Mediterranean region, *B. trigonica* is the only known vector of Lso, but the leek-onion psyllid *Bactericera tremblayi* Wagner is another widespread psyllid and potential vector of Lso. Thus, the settling, oviposition and feeding behaviour of *B. trigonica* and *B. tremblayi* in potato, carrot, celery and leek were studied and the likely implications for the spread of Lso were determined. *Bactericera trigonica* was unable to transmit Lso efficiently to potato nor to colonize potato. Additionally, *B. trigonica* showed a clear preference to ingest sap from the phloem, settle and oviposit on carrot and celery. Consequently, Lso was efficiently transmitted to both carrot and celery. On the other hand *B. tremblayi* preferred leek over carrot and potato, with potato being the less preferred host. Additionally *B. tremblayi* was able to ingest from the phloem of carrot and consequently could acquire Lso but failed to transmit the bacterium from carrot to carrot. Results showed that despite *B. trigonica* can transmit Lso to potato with very low efficiency it is not a threat to potato crops. Furthermore, *B. tremblayi* is not a likely vector of Lso.

Finally, population densities of psyllid associated to commercial carrot and potato fields were estimated in 2015 and 2016 by three different sampling methods (sweep net, green tile traps and visual inspection of eggs and nymphs). Commercial plots were surveyed in two of the most important carrot producing regions in Spain: Castilla-Leon and Comunidad Valenciana, more precisely in Gomezserracín (Segovia), Ìscar (Valladolid) and Villena (Alicante). In addition, psyllid populations in two commercial potato plots Aldearrubia (Salamanca) and Gomezserrcìn (Segovia) were monitored. *B. trigonica* was the most abundant psyllid in all carrot fields followed by *B. nigricornis*. On the other hand, *B. nigricornis* was the most abundant psyllid species associated with potato fields in both 2015 and 2016 and was the only psyllid species able to complete its life cycle in potato plants. *B. trigonica* was associated with one potato plot that was planted next to a carrot infested crop. Data suggests that *B. trigonica* can colonize carrot but not potato and that *B. nigricornis* is able to colonize both carrot and potato.

This Thesis provides new information on the feeding behaviour, the transmission ability and the population dynamics of psyllids on Apiaceae and potato crops. Such information is critical for understanding the epidemiology of Lso and for developing effective disease management strategies.

CHAPTER I. Introduction

Vegetable production in Spain.

Vegetable crop production is a key sector in Spanish agriculture and in 2015 it accounted for 16,7 % of the vegetable output of the European Union (Eurostat, 2017a). Overall Spain's agricultural output in 2014 amounted to 46.2 billion Euros and provided employment for approximately 824,00 people (Ministry of Agriculture, Food and Environmental Affairs, 2017). Among horticultural crops produced in Spain tomato, carrot and onions were the most valuable crops for the European Union market in 2015 and the vast majority of these products are destined for exportation. In 2015, Spain was the second largest tomato producer in the European Union with a production of 4,838,000 tonnes (Eurostat, 2017b). For carrot Spanish production in 2016 was 401,00 tonnes and the most important growing zones were the communities of Andalucía followed by Castilla y Leon (Statista, 2017a) (MAGRAMA, 2017). On the other hand, Spain is the third largest onion producer in Europe with a production of 1.2 million tonnes (Fayos et al., 2015). The most important onion and leek producing zones in Spain are Cataluña, Valencia and Castilla y Leon (MAGRAMA, 2017).

Other important agricultural crops for the internal agricultural market in Spain are potato and different Apiaceae plants such as celery, parsley and parsnip. Spain produced up to 2,285 tonnes of potatoes in 2015 and its production is ranked 7th in the top ten European ranking of agricultural products (Eurostat, 2017c). Celery production amounted to 98,700 tonnes in 2016 (Statista, 2017b). Several factors threaten the yield and thus the economic benefits associated with these crops of which pests and diseases are one of the most important factors associated with crop losses. Recently, a group of hemipteran insects known as psyllids have become important pests of carrot, celery, potato leek and onion crops in Europe (Munyanza

et al., 2010a; Alfaro-Fernández et al., 2012a,b; Ouvrard and Burckhardt, 2012). This group of insects cause direct damage to the plants and also transmit pathogens. Among the transmissible pathogens, the most economically important is the phloem-restricted bacterium *Candidatus Liberibacter solanacearum* (Lso). Currently, psyllids as well as Lso are well established in different European countries and are spreading to new areas (Munyanze et al., 2010a; Alfaro-Fernández et al., 2012a; Ouvrard and Burckhardt, 2012; Loiseau et al., 2014; Munyanze et al., 2015). Therefore, information about how psyllids transmits Lso is important to understanding the epidemiology of this pathogen and to establish effective management practices.

Psyllids as agricultural pests.

Psyllids (Hemiptera: Psylloidea), commonly called jumping plant lice, are a group of piercing sucking insects that feeds on plant sap (Hodkinson, 2009). This group is the sister taxon of whiteflies, aphids, and coccids and together make up the Sternorrhyncha group, a monophyletic group within the insect order Hemiptera (Campbell et al., 1994). Psyllids constitute a morphologically well-defined group with up to 3,800 described species distributed worldwide (Burckhardt et al., 2014; Ouvrard et al., 2015). The psyllid life cycle comprises: the egg stage, five nymphal stages and sexually reproducing adults (Hodkinson, 1974). Parthenogenetic reproduction is possible but rare and it has been documented only in few psyllid species (Hodkinson, 2009; Nokkala et al., 2013). In the sexual reproduction phase, after mating and egg maturation, females oviposit in the edges or in the underside of the host plant leaves. The eggs possess a characteristic basal pedicel that is inserted in to the host plant tissue through which is believed an interchange of water with the plant occurs to avoid desiccation of the egg (Hodkinson, 1974). After egg hatching, psyllids must pass

through five nymphal or immature stages before becoming adults. The body size of adult psyllids is in the range of 1 to 4 mm and its general appearance resembles miniature cicadas (Hodkinson, 1974, 1988; Santana and Burckhardt, 2007). Adults are four winged and the dispersal form of the insect. Adults are also characterized by a strong pair of hind legs well adapted for jumping (Hodkinson, 1974). Males and females can be clearly distinguished by their genitalia; in field conditions both sexes usually occur in similar proportions and can disperse long distances facilitated by air currents (Hodkinson, 1974, 2009).

Typically, the psyllid life cycle could takes around 30 days to be completed (Brown, 2016). However, environmental conditions such as temperature play an important role, determining the length of its life cycle and the number of generations per year (voltinism) (Hodkinson, 2009). A few number of psyllid species distributed in cold regions have been reported as uni or bivoltine (one or two generations per year) but several psyllid species distributed in temperate, tropical and subtropical regions are multivoltine (multiple generations per year) (Hodkinson, 1974, 2009). Multivoltinism and the high psyllid reproduction rate shown by different psyllid species are linked to their capacity to cause epidemics on different crop plants.

Physical damage to crop plants occurs when psyllids suck nutrients from the vascular tissues of the plant while using its needle-like mouthparts (stylets). Furthermore, direct psyllid feeding could also produce symptoms such as leaf yellowing, leaf distortion and root reduction (Nissinen et al., 2007, 2012). However, the most important damage associated with psyllids is the indirect damage produced by the transmission of plant pathogens. Psyllids transmit several phloem restricted bacteria such as phytoplasmas and different species of the genus *Candidatus Liberibacter* (Bové and Garnier, 2003; Munyaneza, 2010; Trivedi et al., 2016). Among the *Candidatus Liberibacter* species, found in Europe, *Candidatus Liberibacter solanacearum* (Lso) is considered one of the most important ones due to the

serious losses it cause to the carrot and celery industries (Munyaneza et al., 2010a; Bertolini et al., 2015). In Europe, two carrot psyllid species have been reported as vectors of Lso, *Bactericera trigonica* Hodkinson and *Trioza apicalis* Foester (Munyaneza et al., 2010a,b; Alfaro-Fernández et al., 2012b;). Recent research has shown that other psyllid species such as *B. tremblayi* Wagner, and *B. nigricornis* Foerster, could be found on carrot crops and also test positive for Lso using real-time PCR (Teresani et al., 2015). These findings suggest that these two psyllid species might be potential vectors of Lso in Europe although this requires to be confirmed.

In the Mediterranean region *T. apicalis* has not been found and the only known vector of Lso in Apiaceae crops is *B. trigonica*. This species together with the closely related species *B. tremblayi* and *B. nigricornis* form the “*Bactericera nigricornis* Föster complex” (Hodkinson, 1981). This group belongs to the family Triozidae and is characterized by the absence of surface spinules in the forewing, small genal cones and a narrow third antennal segment (Hodkinson, 1981). Psyllid species of this complex show short terminalia in females and males are characterized by the posterior extension of the proctiger. Differentiation between these three psyllid species is difficult and mainly relies on the appearance of the genal cones and the shape of different structures of the male genitalia such as parameres, aedeagus and proctiger. Feeding behavior and host range of these three psyllid species also varies, but unfortunately these two characteristics are poorly studied and the literature is full of reports of incidental findings on different plant species. Thus, from those studies it is difficult to differentiate which are the host plants on which the psyllids can multiply and grow and those which are non-hosts (i.e. rest plants or temporary food plants) (Burckhardt et al., 2014).

To date *B. trigonica* has been found in different countries in the Mediterranean region mainly associated with carrot and celery crops, but low numbers of adults have been also collected from potato fields (Alfaro-Fernández et al., 2012b; Teresani et al., 2014, 2015). Additionally,

B. tremblayi has been associated to leek and onion crops in Spain, France, Serbia and Iran where it causes severe economic damage (Kazemi and Jafarloo, 2008; Ouvrard and Burckhardt, 2012). *Bactericera tremblayi* has been also collected in carrot fields in Spain (Teresani et al., 2015), but is still uncertain if this psyllid is able to reproduce on this crop. Finally, *B. nigricornis* has been described as pest of potato in Iran, where it is associated with leaf yellowing and yield reduction (Fathi, 2011). Other reports suggest that this psyllid species is able to feed and reproduce on carrots and plants in the family *Brassicaceae* (Ouvrard, 2017). According to this, *B. nigricornis* shows polyphagous habits, however detailed information about its feeding habits and host range is missing.

Geographical distribution of *B. trigonica*, *B. tremblayi* and *B. nigricornis* commonly overlaps and host plants of the three species are grown in the same production zones (Ouvrard, 2017). Although, only *B. trigonica* has been demonstrated as a Lso vector, there is a potential threat of Lso cross transmission from carrots to other susceptible crops such as potato. To fully understand the epidemiology of Lso, information on vector feeding habits as well as how vectors transmit the bacterium is necessary. This work aims to fill this gap in knowledge, studying the transmission of Lso mediated by *B. trigonica* and identifying the risk of Lso transmission mediated by other psyllids belonging to the “*Bactericera nigricornis* Föster complex”.

The pathogen *Candidatus Liberibacter solanacearum*.

Candidatus Liberibacter solanacearum (Lso) is a Gram-negative, unculturable and phloem restricted plant pathogenic bacterium (Hansen et al., 2008; Liefting et al., 2009). This bacterium belongs to the *Rhizobiaceae* family and it is characterized by a rod-shaped morphology of approximately 0.2 µm wide and 4 µm long (Liefting et al., 2009; Secor et al.,

2009). Lso is an intracellular bacterium that infects and replicates in the sieve elements of plants as well as in different organs and tissues of its psyllid vectors (Brown, 2016; Haapalainen, 2014; Perilla-Henao and Casteel, 2016). This bacterium was first identified in 2008 in tomato and pepper in New Zealand and latter was associated with disease in potatoes in North America (Hansen et al., 2008; Liefting et al., 2008, 2009; Secor et al., 2009; Crosslin et al., 2010). This bacterium has also been found to infect other cultivated and wild solanaceous plants such as tobacco (*Nicotiana tabacum* L), capegooseberry (*Physallis peruviana* L), tamarillo (*Solanum betaceum*) nightshade (*Solanum spp.*) and ground cherry (*Physallis spp.*) in North and Central America as well as in New Zealand (Liefting et al., 2009).

Lso is especially important in potato where it causes a disease called “Zebra chip”. The ZC disease is characterized by collapsed stolons, aerial tubers, upward rolling of leaflets, purple or yellow discoloration, leaf scorch, aerial tubers and early senescence; also dark and striped pattern clearly visible in raw tubers and fried chips (Munyaneza, 2012). As a consequence, it causes yield and quality reduction leading to substantial economic losses. For example, in New Zealand annual potato losses have been estimated at 30 million Euro and in the state of Texas, USA this disease has caused losses up to 33 million dollars in the state of Texas alone (Soliman et al., 2013).

Interestingly, Lso also affects plants from the family Apiaceae such as celery and carrot in Europe, Africa and the Middle East (Alfaro-Fernández et al., 2012a,b; Munyaneza et al., 2010a, 2015; Loiseau et al., 2014; Tahzima et al., 2014; Teresani et al., 2014). Symptoms of Lso in carrot and celery include abnormal proliferation of shoots, stem curling as well as purple or yellow discoloration and root size reduction. In recent years, Lso has caused severe economic losses to the fresh carrot market in Norway, Finland, Germany and Spain. These losses can lead to important decrease in marketable yield (Munyaneza et al., 2010a; Laska,

2011; Teresani et al., 2014; Bertolini et al., 2015). Five haplotypes of Lso have been described so far. These haplotypes are genetic variants based on differences in single nucleotide polymorphism (SNPs) of the intergenic spacer region of the 16S, 16S/23S and 50S RNA genes (Nelson et al., 2011, 2012, 2013; Teresani et al., 2014). Lso haplotypes are distributed in different geographical regions and have been designated as A, B, C, D, E, according to the order they have been described. Haplotype A has been detected in solanaceous crops in the United States of America, Mexico, Honduras, Guatemala and New Zealand. Haplotype B overlaps with haplotype A in Solanaceae in the United States of America and Mexico (Nelson et al., 2011). Haplotypes C, D, E have been associated to Apiaceae crops in Europe (Nelson et al., 2012). In North Europe and Central Europe (Norway, Finland, Sweden, Germany) haplotype C has been described in carrot (EPPO, 2013) whereas in Southern Europe (Spain, France), Northern Africa (Morocco) haplotypes D and E have been found in carrot and celery.

Vector transmission of *Candidatus Liberibacter solanacearum*.

Lso can be transmitted by infected carrot seed and infected potato tubers (Pitman et al., 2013; Bertolini et al., 2015). However, the main pathway for transmission is psyllid vectors. Recent studies have shown that various species of psyllids with different geographical distributions could transmit Lso to different hosts plants (Alfaro-Fernández et al., 2012b; Munyaneza et al., 2007; Munyaneza, 2010; Munyaneza et al., 2010a). In North, Central America and New Zealand Lso haplotypes A and B are transmitted to Solanaceae plants by the potato/tomato psyllid *Bactericera cockerelli* Šulc. In Europe and Africa Lso is transmitted to carrots by two different psyllids species. *Trioza apicalis* transmits the Lso haplotype C to carrots in Northern and central Europe whereas *B. trigonica* transmit the haplotypes D and E to carrot

and celery in the Mediterranean region. The host range of Lso vectors may explain the different host range shown by this bacterium in different continents because psyllid species that affect carrots do not overlap with the psyllid species that affects potato (Haapalainen, 2014; Munyaneza et al., 2016). However, in Europe the plant host range and feeding habits of psyllids are poorly documented.

Psyllid feeding behaviour plays a key role in the transmission of Lso, because the bacterium spreads from plant to plant facilitated by the psyllid feeding. The Lso transmission is a complex process and it includes various steps. First, the psyllid vector acquires the bacterium from the phloem sieve elements of an infected plant during feeding (Mustafa et al., 2015; Sandanayaka et al., 2014). Once Lso reaches the psyllid digestive tract, the bacteria must pass through the midgut epithelium in order to reach the haemocoel and circulate in the haemolymph (Cicero et al., 2016; Fisher et al., 2014). From the haemolymph, Lso spreads to different tissues and organs where it accumulates and replicates to finally reach the salivary glands (Cicero et al., 2016b). When it colonizes the salivary glands, Lso is persistently transmitted with the saliva while the insect is feeding on the phloem of a susceptible host. This entire process from acquisition to inoculation is called the “latency period” and takes approximately two weeks to complete (Sengoda et al., 2014). This mechanism of transmission is known as circulative–propagative because it requires pathogen to circulate in the psyllid haemolymph as well as to multiply in different organs.

The ability of Lso to survive and replicate in different vectors organs suggests a high degree of specialization (Perilla-Henao and Casteel, 2016). Lso is not only a plant pathogen but is also considered to be an endosymbiont of its psyllid hosts that and can be transmitted transovarially (Casteel et al., 2012; Hansen et al., 2008). In transovarial (or vertical) transmission, a symbiont or pathogen is transmitted from the mother to the offspring. Vertical transmission rates of Lso in *B. cockerelli* might be up to 87% (Casteel et al., 2012). When

this vertical transmission occurs, nymphs resulting from infected eggs and newly emerged adults could efficiently transmit Lso to susceptible plants. This increases the vector propensity and makes Lso difficult to control in field conditions.

Feeding behaviour monitoring and how to study the transmission of *Candidatus Liberibacter solanacearum*.

The transmission mechanism of Lso is entirely dependent on the feeding behaviour of its vectors. Thus, understanding the psyllid stylet penetration process in susceptible host plants is fundamental to understanding the transmission of Lso. Psyllid stylet penetration starts with a “pathway phase” through the ground tissues of the plant until the stylet, reaches the vascular bundle (Bonani et al., 2010). In this pathway phase, stylet tip penetrates deep into the plant tissue while the psyllid produces a salivary sheath that guides the stylet movement. This entire process is intercellular and might take from minutes to hours. The pathway phase continues until the stylet reaches phloem or xylem tissues. Once in the vascular tissues, psyllids could ingest sap from xylem or phloem tissues (Bonani et al., 2010; Civolani et al., 2011; Pearson et al., 2014; George et al., 2017;). Nevertheless, psyllids are commonly referred to as phloem feeders because they spend more time in the phloem and also because nutrients from this tissue are essential to complete its life cycle (Bonani et al., 2010; Hodkinson, 1974).

As reported from other Hemipterans, it is believed that as soon as psyllid stylet penetrates into a phloem sieve element, the insect inoculates a small quantity of watery saliva during a brief period and then latter ingests phloem sap for longer periods (Prado and Tjallingii, 1994). This process is directly linked to the inoculation and acquisition of ‘*Candidatus Liberibacter solanacearum*’ (Mustafa et al., 2015; Sandanayaka et al., 2014). This process

cannot be seen directly but can be monitored very closely by using the Electrical Penetration Graph (EPG) technique (McLean and Kinsey, 1964; Tjallingii, 1978). This is an electrical system that records voltage fluctuations produced by different stylet penetration activities of the insect in different plant tissues. These voltage fluctuations are called “waveforms” and can be correlated with the position of the stylets in different tissues. Furthermore, EPG allows discriminating between intracellular and extracellular environments. Thus it is possible to identify when the insect stylets puncture plant cell membranes (Tjallingii 1985). Because of this, EPG have been used to correlate insect-feeding activities with the transmission of intracellular plant pathogens such as Lso, viruses or phytoplasmas (Sandanayaka et al., 2014;; Mustafa et al., 2015; Munyaneza et al., 2016).

The following EPG waveform patterns for economically important psyllids such as the Asian citrus psyllid *Diaphorina citri* Kuwayama, the pear psyllid *Cacopsylla piri* L. and the potato psyllid *B. cockerelli* have been described:

Waveform A: Irregular and short duration waveform associated with the contact of the stylet with the plant epidermis.

Waveform C: This waveform is characterized by an extracellular voltage and high frequency. It is believed that it is associated with multiple activities performed during the stylet pathway phase.

Waveform G: High amplitude extracellular waveform associated to xylem sap ingestion.

Waveform D: Short waveform composed of two separate phases. So far it has been described exclusively in psyllids and Bonani et al (2010) associated it with phloem tissues. However its biological role is still uncertain.

Waveform E: This waveform family is characterized by its intracellular potential and is divided in two types of waveforms:

Waveform **E1:** this waveform is associated with the inoculation of watery saliva in the phloem sieve elements. Recent studies suggest a correlation of this waveform with the inoculation of different species of *Candidatus Liberibacter* (Bonani et al., 2010; Sandanayaka et al., 2014; Mustafa et al., 2015).

Waveform **E2:** is associated with phloem ingestion and has been correlated with the acquisition of different species of *Candidatus Liberibacter* (Bonani et al., 2010; Sandanayaka et al., 2014; Mustafa et al., 2015).

Currently the biological significance of different EPG waveforms as well as their role in transmission is not clearly understood. To understand the transmission process of Lso, the feeding behaviour of current and potential vectors of was studied in detail in this Thesis.

CHAPTER II. Objectives

The main objective of this Thesis is the study of Vector-mediated transmission of ‘*Candidatus Liberibacter solanacearum*’ and the epidemiological consequences.

Specific objectives are:

1. To characterize the feeding behaviour of the psyllid *B. trigonica* by the EPG in order to correlate each EPG waveform with different feeding activities associated to the transmission of Lso.
2. To compare the feeding behaviour of males and females of the psyllid *Bactericera trigonica* and to determinate if such differences could affect the transmission, titre and symptoms caused by Lso in carrot plants.
3. To evaluate the feeding preferences of *B. trigonica* and *B. tremblayi* in different host plants to assess the risk of transmission of Lso from Apiaceae to other economically important crops.
4. To monitor the seasonal abundance of psyllid species in carrot and potato in commercial fields in Spain.

CHAPTER III. Characterization of the electrical penetration graphs of the psyllid *Bactericera trigonica* on carrots¹

Introduction

The psyllid *Bactericera trigonica* Hodkinson (Hemiptera: Triozidae) is a pest of carrot and celery. It is currently found in various countries across southern and central Europe, northern Africa, and Asia. In 2012, it was identified as a vector of the phloem-restricted pathogenic bacterium ‘*Candidatus Liberibacter solanacearum*’ (Lso) on carrots (*Daucus carota* L.) (Alfaro-Fernández et al., 2012b) and later identified as an Lso-vector on celery (*Apium graveolens* L.) (Teresani et al., 2014). Lso is an emerging vector-borne pathogen first described in America as causing disease in Solanaceae transmitted by *Bactericera cockerelli* Sulc (Secor et al., 2009) but later associated to Apiaceae in Europe and Africa (Munyanze et al., 2010a; Alfaro-Fernández et al., 2012a; Loiseau et al., 2014; 2015; Tahzima et al., 2014; Teresani et al., 2014). In northern Europe, Lso is transmitted by the psyllid *Trioza apicalis* Foerster, whereas in the southern Mediterranean, Lso is transmitted by *B. trigonica*. Damages caused by these psyllid-Lso complexes have caused severe economic losses to the fresh carrot market in Norway, Finland, Germany, and Spain (Laska, 2011; Munyanze et al., 2010a; Bertolini et al., 2015; Teresani et al., 2015).

Developing management practices aimed at reducing losses caused by Lso require a deep understanding of Lso transmission mechanisms and the relationships between the pathogen

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and its vectors. Lso transmission mediated by vectors is divided into three main steps. First, a psyllid must acquire the bacterium while ingesting from phloem sieve elements of an infected plant (Sandanayaka et al., 2014). Second, the bacteria must replicate and circulate in the vector for a latency period. Finally, Lso must be inoculated during salivation before sap ingestion in the sieve elements of a non-infected plant (Cooper et al., 2014; Sengoda et al., 2014; Mustafa et al., 2015;). Therefore, detailed knowledge of vector stylet penetration activities is important for developing control strategies to prevent Lso transmission. Nevertheless, despite the importance of Lso, the plant penetration activities of its vector species currently distributed in Europe are poorly documented.

The most powerful technique to study the plant penetration activities of sap-sucking insects is the electrical recording system developed by Mclean & Kinsey (1964), later modified and called electrical penetration graph technique (EPG) (Tjallingii, 1978, 1985). EPG is an electrical system in which an insect with piercing mouthparts and a plant are made part of an electrical circuit as soon as the insect inserts its mouthparts (stylets) into the plant (Tjallingii, 1978). As a result, voltage fluctuations are produced and can be recorded as waveforms, thus monitoring the insect's activities and the stylet tip positions in the plant. These voltage fluctuations are due to resistance (R) or electromotive force (emf) changes occurring during stylet penetration. EPG recording has allowed the study of the stylet penetration activities of insect vectors in real time and facilitated correlation of the insect's probing activities with inoculation or acquisition of various plant pathogens (Prado and Tjallingii, 1994; Bonani et al., 2010; Sandanayaka et al., 2014) . Such correlations can only be achieved based on a range of experiments that satisfy a set of conditions termed 'the triangle of correlations' (Walker, 2000). To meet these conditions, the EPG waveforms obtained must first be identified and described in terms of electrical origin, frequency, relative amplitude, and

appearance. Then, the stylet position on different plant tissues during each waveform must be determined by thin sectioning and light or electron microscopy. Finally, experimental evidence on the occurrence of probing activities such as salivation or ingestion should be demonstrated by honeydew excretion, membrane feeding, or pathogen transmission studies (Walker, 2000). Only after all these correlations are performed can the biological meaning of each waveform be defined and the data interpretation be deemed reliable (Pearson et al., 2014).

Due to the losses to the carrot industry caused by *B. trigonica*, a characterization of its plant penetration activities is needed. Previous characterizations of stylet penetration activities of other psyllid species have reported the following EPG waveform patterns: waveforms A, B, and C for stylet pathway activities, G for putative xylem ingestion, and three waveforms for phloem related activities: D for initial phloem contact, E1 for putative salivation into sieve elements, and E2 for phloem ingestion from sieve elements (Bonani et al., 2010; Civolani et al., 2011; Pearson et al., 2014; Sandanayaka et al., 2014). However, a direct correlation between E1 and Lso inoculation has not been established for psyllids so far. The close association of waveform D with waveform E1 has impeded the analysis of both waveforms separately, thus leaving a possible (but unlikely) role of waveform D in Lso inoculation unknown.

Our main goal was to characterize the EPG waveforms produced by *B. trigonica* when probing on carrots, and to correlate the waveforms with salivary sheath termini in plant tissues and with Lso inoculation. Based on the evidence obtained, we intended to infer the biological meaning of different waveforms and describe the probing behaviour of *B. trigonica*. In addition, comparing Lso-free and Lso-infected psyllids, we aimed to identify

whether Lso infected *B. trigonica* directly influences the stylet penetration activities and its possible impact on Lso epidemiology.

Materials and methods

Plant materials

Carrot (*D. carota* cv. Bangor) seeds (Bejo Zaden, Warmerhuizen, The Netherlands) were germinated in Petri dishes under dark conditions at 24 °C for 4 days. Carrot seedlings were then individually grown in 8-cm-diameter pots using a mixture of equal parts of vermiculite (no. 3; Asfaltex, Barcelona, Spain) and soil substrate (Kekilla Iberica, Almería, Spain) until the plants reached the three-true-leaf stage used for the experiments. The batch of seeds and seedlings used for experiments tested negative for Lso as revealed by real-time PCR according to Bertolini et al. (2015). Plants were maintained in a growth chamber under L16(24 °C):D8(20 °C) photo-thermoperiod. Plants were watered 3× a week, and a nutritional complex 20-20-20 (N:P:K) of Nutrichem fertilizer (Miller Chemical & Fertilizer, Hanover, PA, USA) was added once a week to the irrigation water (0.25 g l⁻¹).

Generation of Lso-infected and Lso-free *Bactericera trigonica* colonies

Lso-infected colony. A *B. trigonica*-Lso colony was established by collecting insects from a population in a carrot field that was exhibiting moderate leaf yellowing in Gomezserracín, Segovia, Spain, in 2014. A sample of 30 psyllids was taken from those collected in the field to determine the percentage of Lso-positive psyllids by real-time PCR. Fourteen psyllids were infected (42%). The remaining insects from the field population were reared on Lso-free carrot plants in 47.5 × 47.5 × 47.5 cm (nylon mesh 150 µm) insect cages under greenhouse conditions L14 (26°C): D10 (18°C) photo-thermoperiod]. Psyllids were

maintained under greenhouse conditions for several generations and the colony was periodically tested to confirm the presence of Lso-infected individuals. After five generations, 97 % of the population was Lso-positive.

Lso-free colony. The Lso-free colony was established by taking a group of 40 females from the original population collected in field. Females were then separated in Eppendorf tubes (one per tube) and released onto healthy young carrot plants (one female per plant). Each plant and psyllid combination was enclosed in a plastic cylinder. Females were allowed to oviposit for a period of 5 days and then set apart to be tested by real-time PCR. Combinations of plant/female where oviposition did not take place or where the female was positive for Lso were discarded. Plants with eggs originating from Lso-free females were monitored until egg eclosion and throughout the nymphal and adult stages (F1). A sample of 10 new adults from each of the plastic cylinders was analysed to confirm the absence of Lso by real-time PCR. Insects from the Lso-free cylinders were then transferred to insect cages (similar to the cages described above for Lso-infected psyllids) with Lso-free carrot plants. The Lso-free colony was then maintained in a separate greenhouse with conditions similar to the ones described for the infected colony. Psyllids were maintained for several generations and were periodically tested by PCR to confirm the absence of Lso.

Molecular detection of Lso

Molecular detection of Lso was performed on leaves and individual psyllids. For detection of Lso on plants DNA was extracted from 0.5 g of carrot leaves following the cetyltrimethyl ammonium bromide (CTAB) protocol (Murray and Thompson, 1980). DNA from insects was obtained following the print-capture protocol described by Olmos et al. (1996). Lso was detected by real-time PCR using primers, probe, and conditions previously described by Bertolini et al. (2015). The PCR reaction was performed on a 7300 Applied Biosystems

Foster city CA, USA thermal cycler.

Electrical penetration graph recordings

EPG recordings were obtained using an eight-channel DC EPG system (type Giga-8; EPG Systems, Wageningen, The Netherlands). The signal was digitized (100 Hz) using a DI-710 board (Dataq Instruments, Akron, OH, USA), stored onto a PC hard disk, and analysed with Stylet+ software (EPG Systems). Psyllids were anesthetized with CO₂ for 4 s and immobilized using a vacuum-operated plate (Eyela Aspirator A3S; Rikakikai, Tokyo, Japan) under a binocular microscope. Then, a gold wire (18.5 µm diameter) was attached to the psyllid pronotum using a small drop of water-based silver glue (EPG Systems). The wired insect was placed on the abaxial surface of a fully expanded carrot leaf. After the recording was started the output voltage was optimised during the first probes for each channel by adjusting the plant voltage (Giga manual; EPG Systems).

For waveform identification and characterization, 20 EPG recordings of Lso-infected females and 20 of Lso-free females were obtained and analysed. The following characteristics were used to describe the waveforms: (1) relative amplitude, (2) frequency or repetition rate (Hz), (3) voltage level, extra- or intracellular, and (4) main electrical origin, resistance (R) or electromotive force (emf). The relative amplitude was calculated based on a percentage of the voltage range of each waveform as compared to range of the waveform with the largest range (= 100%). The voltage range is the voltage difference between baseline (0 V) and the waveform's maximum voltage during a positive output voltage adjustment (Civolani et al., 2011; Pearson et al., 2014; Walker, 2000). To assess the electrical origin of each waveform, recordings with a positive, a negative, or a zero output voltage level were performed during recording of each waveform (Tjallingii, 1985, 2000; Civolani et al., 2011). Resistance originated waveforms or waveform aspects will be inverted when changing from a positive to

a negative output voltage and diminish at 0 V output voltage. In contrast, the shape and amplitude of emf originated waveforms or waveform aspects will not be affected by plant (output) voltage adjustments (Tjallingii, 1985).

In addition to the characterization described above, a series of additional recordings were obtained to compare the stylet penetration activities between Lso-infected and Lso-free individuals. A number of non-sequential and sequential EPG variables were processed using the MS Excel Workbook for automatic EPG data compilation developed by Sarriá et al. (2009). Next, the proportion of individuals who produced a specific waveform type (PPW), the number of waveform events per insect, the waveform duration per insect (WDI), and the total waveform duration per event were calculated as described by Backus et al. (2007). Descriptive statistics for each of the EPG variables were calculated using the statistical package SPSS v.21 (IBM-SPSS, Armonk, NY, USA, 2012). Direct comparisons between treatments (Lso-infected vs. Lso-free psyllid populations) were made by a nonparametric Mann-Whitney U test for variables that did not follow a Gaussian distribution and by Student's t-test for variables following a normal distribution before or after transformation by $\ln(x+1)$. Statistical tests were conducted using SPSS v.21. We also performed an analysis of the sequence of events during stylet penetration for Lso-free and Lso-infected psyllids by calculating the probability of each waveform pattern being followed by another (Wayadande and Nault, 1996).

Plant tissue histology

A plant histological thin-section analysis was conducted to correlate the positions of salivary sheath termini and the occurrence of the various types of waveforms observed during the EPG recordings. Additional adult females of *B. trigonica* were monitored on young carrot

leaves under conditions similar to those used for the first set of EPG data acquisition. Insect probing was artificially interrupted when each of the distinct EPG waveforms of interest was observed. An area of ca. 0.25 cm² of leaf tissue around the insect-probing site was removed from each plant using a scalpel. Then, the tissue was immersed in acid fuchsin (1%) for 3 min to stain the salivary flange produced by the insect during the probe and, thus, determine the stylet insertion point (Miranda et al., 2009). Afterwards, the leaf tissue was washed in distilled water and dried on filter paper. Tissues were then fixed in Karnovsky solution (Karnovsky, 1965), dehydrated in an increasing ethanol series (10-100%), and embedded in plastic resin following the manufacturer's instructions (Leica Histoiresin, Heidelberg, Germany). Serial transverse sections (9 µm thick) were cut on a Leica RM 2045 rotary microtome and stained with toluidine blue O (Sakai, 1973). Permanent slides were mounted in the synthetic resin Entelan® (Merck, Darmstadt, Germany). For image analysis, images were digitally captured with a Leica DMLB microscope using a Leica DFC 310FX video camera coupled to a computer running Las v.4.0 software (Leica, Wetzlar, Germany).

Correlations between EPG waveforms and Lso inoculation

This additional experiment was intended to identify which EPG waveforms were related to Lso inoculation. Females from the Lso-infected colony were wired and attached to the EPG amplifier as described earlier. Then, insects were placed on young leaves of healthy carrot plants. We monitored insect stylet penetration activities in real time, interrupting the registers after specific waveform patterns were recorded. The waveform patterns recorded were (1) waveform C for 15 min; (2) waveforms C + D (the probe was interrupted 30 s after the beginning of the second phase of waveform D, see the waveform description in the Results section); and (3) waveforms C + D + 30 s of sustained E1. After the interruption of EPG collection, the wired insect was collected to confirm the presence of Lso by real-time PCR.

Plants exposed to uninfected psyllids were discarded from the analysis. Test plants were sprayed with Confidor (Bayer Crop Science, Monheim am Rhein, Germany) (1 g l^{-1}) at days 0 and 10 after interruption of the recordings and were maintained in insect-proof cages in greenhouse conditions. Five weeks later, completely developed apical leaves from the test plants were collected for testing by real-time PCR.

Results

Waveform characterization of the EPG recordings of *Bactericera trigonica*

Young females of *B. trigonica* produced eight types of EPG waveforms labelled as A, C1, C2, pseudo-pd, G, D, E1, and E2 (Figures 1 and 2), according to the labels used for other psyllids (Bonani et al., 2010; Pearson et al., 2014). Psyllids from Lso-free and Lso-infected colonies produced similar waveforms, thus these waveform descriptions apply to both colonies tested. All characteristics and their correlations with feeding activities are summarized in Table 1. The waveform descriptions are as follows.

Waveform A. After a period of baseline non-probing (np), the first stylet contact with the plant epidermis generates a short high amplitude (100%) waveform (Figure 1). Due to its short duration, this waveform was not further characterized as such but was considered as part of the waveform C pathway activities in EPG analyses.

Waveform C (C1 and C2). Shortly after waveform A, the next waveform observed was C1 (Figure 1). This waveform is complex and mainly represented by a pattern of high-frequency fluctuations (12–16 Hz) (Figure 1, detail; Table 1). The main electrical origin of this waveform is emf because it remains unaltered by changes in output voltage (Figure 3A). This

waveform could be followed by waveforms G, D, or pseudo-pds (see below) but most commonly alternates with C2. All waveforms observed in our recordings could be followed by C1 (Figure 4).

Pseudo potential drop (pseudo-pd). We introduce the term ‘pseudo-potential drop’ according to Cid & Fereres (2010) to describe short lowered voltage periods that were occasionally observed during waveform C1 (Figures 2A,B). These pseudo-pds are characterized by a sharp drop of the voltage level, reaching values close to zero after which a low-amplitude saw tooth pattern is maintained (7-16 Hz) for 2-11 s, followed by a return to the level and waveform of C1 (Figure 2B). Interestingly, when changed to a negative output voltage, this waveform appears inverted and, therefore, an R originated waveform (Figure 3G); not due to stylet puncture and caused by an intracellular potential – emf-originated – as in the normal pd waveform occurring in other Sternorrhyncha.

Waveform C2. This waveform is not present in all recordings and mostly showed a shorter duration than C1. It is characterized by single downward spikes followed by irregular intervals of flat periods with low frequencies (0-2 Hz) (Table 1, Figure 3B). C2 does not precede any of the other waveforms observed and it is only found alternating with C1. Rarely, *B. trigonica* withdraw its stylets at the end of this waveform (Figure 4). Similar to C1, the main electrical origin of C2 is emf (Table 1; Figure 3B).

Waveform G. This waveform is characterized by a regular sinusoidal pattern with small positive spikes at regular intervals (Figure 3C). The main sine-wave aspect remains unaltered by output voltage changes and is emf-originated, but the small spikes diminish at 0 V and are inverted at negative output voltage. Therefore the sine waves are emf-originated and the

spikes have an R origin (Figure 3C). This waveform is always preceded by C1 and can return to C1 or terminate in np (Figure 4).

Waveform D. This waveform is composed of two phases: an initial undulating phase with square crested waves at 4-8 Hz and a second flat phase with positive spikes at regular intervals with repetition rate of about 2 s^{-1} and a somewhat decreasing voltage (Figure 2C). When the output voltage is changed from positive to negative, the undulations in the first phase, as well as the spikes in the second phase, seem inverted but at a 0-V adjustment they are still showing smaller spike-like remnants. The inverted parts represent the main R component, whereas the small volt remnants represent a certain emf origin as well. Based on the main R component, the amplitude range seems about 20-30% (Table 1, Figure 3D). Waveform D is always preceded by C1 and usually finishes in an abrupt potential drop that marks the start of E1 (Figure 2C). However, waveform D occasionally returns to C1 (Figure 4).

Waveforms E1 and E2. Waveform E1 is characterized by a lowered intracellular voltage level with a frequency of 4-5 Hz (Table 1). The potential drop observed at the end of D was the beginning of E1 (Figure 2C). This waveform is characterized by a regular pattern of low amplitude positive peaks (Figure 2E and 3E). When the output voltage is changed, E1 remains largely unaffected, indicating that the main electrical origin is emf (Figure 3E). E1 is always preceded by D and is usually followed by E2. However, a return to pathway activities (C1) or withdrawal of the stylet at the end of this waveform was also observed (Figure 4). Waveform E2 is always preceded by E1, and it presents characteristics similar to E1 in terms of the intracellular voltage, frequency, and amplitude, but resembles an inverted E1 with a characteristic downward spike pattern (Figure 3F, Table 1). It is common for E2 to appear

mixed with E1, creating a superimposed pattern with marked positive and negative peaks (Figure 2D). For this reason, we proposed to measure this mixed signal (E1-E2) as E2. Additionally, alternating periods of E1 and E2 frequently occur during the same phloem probe (Figure 2E). The waveform's appearance is not affected by voltage changes, which means that its electrical origin is mainly emf (Figure 3F).

Comparison between EPGs of Lso-free and Lso-infected psyllids

There were no significant differences in any of the number of waveform events per insect variables between the two *B. trigonica* colonies tested (Table 2). The analysis of waveform duration per insect showed that Lso-free psyllids spent less time in non-probing activities ($t = -2.13$, d.f. = 1, $P = 0.03$) and more time on C ($t = 2.84$, d.f. = 1, $P < 0.01$) compared to Lso-infected psyllids (Table 2). There were no significant differences in waveform duration per insect between the colonies for the remainder of the analysed waveforms. For the total waveform duration per event, Lso-free psyllids spend less time in np ($U = 54\ 140$, d.f. = 1, $P < 0.01$) and more time in C ($U = 72\ 657$, d.f. = 1, $P < 0.01$) and D ($t = 2.08$, d.f. = 1, $P = 0.04$) compared to Lso-infected psyllids (Table 2). Neither Lso-free nor Lso-infected psyllids showed any differences for any of the sequential parameters analysed (Table 2).

Stylet tip positions correlated with EPG waveforms

The salivary sheath terminus during the EPG waveforms C1, C2, G, D, E1, and E2 was determined after gently pulling out the insect during each waveform. Due to the low occurrence and the ephemeral duration of pseudo-pds their correlation with salivary sheath termini was not performed. Plant histological analyses showed that prior to start of the probe, the insect usually produces a small salivary flange on the leaf epidermal surface (Figure 5E). Next, the insect inserts its stylets perpendicularly to the leaf surface and the stylets cross the

mesophyll to the vascular bundle without causing LM-detectable damage to the cells. Salivary sheaths of waveforms C1 (n = 5) and C2 (n = 4) always ended in palisade, spongy, or vascular parenchyma (Figure 5A,B). Sections related to waveform G (n = 4) showed the salivary sheaths ending in the xylem tracheary elements: two of these were branched and two were not (Figure 5C). Waveform D (n = 2) salivary sheaths ended in the phloem (Figure 5D). For waveforms E1 (n = 3) and E2 (n = 3), all sections showed salivary sheath termini in phloem tissues (Figure 5E). One of the salivary sheaths during the E2 waveform was branched, revealing that psyllids may search for different sieve elements to feed on the phloem (Figure 5F).

Correlations between EPG waveforms and Lso transmission

Lso inoculation was achieved only when *B. trigonica* was able to salivate in phloem sieve elements. Seventeen plants out of 35 (48%) were infected with Lso when *B. trigonica* probing was artificially terminated after 30 s of sustained E1. In contrast, none of the plants was infected with Lso when probing was artificially terminated during the C (0/30) or the C + D waveforms (0/32).

Discussion

Several previous studies have defined and correlated EPG waveforms with stylet penetration activities of psyllids (Bonani et al., 2010; Civolani et al., 2011; Pearson et al., 2014; Sandanayaka et al., 2014). Nevertheless, not all biological backgrounds of the waveforms are completely understood. The present study provides new information and the first description of *B. trigonica* waveforms on carrot plants, including a new pseudo-pd waveform, not described earlier for a psyllid species.

In general terms, the waveforms produced by *B. trigonica* closely resemble the waveforms reported for other psyllids (Bonani et al., 2010; Civolani et al., 2011; Pearson et al., 2014). Our results demonstrated that waveforms C1 and C2 were histologically correlated with stylet tracks ending in the mesophyll, indicating that both waveforms represent stylet pathway activities. Histological preparations also showed that stylet tracks made during pathway activities did not cause cellular damage detectable by light microscopy, suggesting that most of the time stylet pathway is intercellular. Moreover, no potential drop waveforms – intracellular stylet punctures – as occurring in other hemipterans were observed; all the waveforms related to stylet pathway were maintained on an extracellular voltage level, which is consistent with the stylet penetration described in other psyllid species (Bonani et al., 2010; Civolani et al., 2011; Pearson et al., 2014). Nevertheless, it has been shown using light or electron microscopy that some psyllid species can occasionally present intracellular stylet tracks (Ullman and McLean, 1988a,b; Civolani et al., 2011). Our data showed that when EPG recordings were interrupted during stylet pathway activities (C waveforms), *B. trigonica* was unable to inoculate Lso. This is also consistent with transmission studies conducted with Lso and the American potato psyllid *B. cockerelli* (Sandanayaka et al., 2014).

The new pseudo-pd waveform has not been reported previously for psyllids, although these waveforms resemble the pseudo-pds described for the mealybug *Planococcus citri* (Risso) in grapevines (Cid and Fereres, 2010). Our work showed that the pseudo-pd waveforms are not related to the real potential drops waveforms described for aphids, whiteflies, or mealybugs (Tjallingii, 1985; Janssen et al., 1989; Johnson and Walker, 1999; Jiang et al., 2000). The real potential drops are caused by the transmembrane potential present when stylet punctured representing an emf origin (Tjallingii, 1985). Conversely, our study clearly showed that the

pseudo-pds by *B. trigonica* had an increased R origin because they appeared inverted when the output voltage was adjusted negatively. Whereas various studies have shown that potential drops represent intracellular punctures (Tjallingii, 1985; Powell, 1991), the biological meaning of the pseudo-pd remains unknown. What activity is causing this R increase remains uncertain. One putative cause may be a period of close contact of the stylet tips with a cell membrane, known as giga-seal in patch clamp studies (Priel et al., 2007), this may explain voltage changes similar to the pseudo-pds. However, more detailed studies are needed to correlate specific stylet activities with the pseudo-pds.

Waveform G was commonly detected after variable periods of stylet pathway. The histological evidence correlated this waveform with salivary sheath termini in the xylem. Our work did not provide any direct evidence of xylem sap ingestion; however, interpretation of the biological meaning of waveform G could be similar to that reported for its homologous waveform in aphids and sharpshooters. In such studies, waveform G was correlated with cibarial pump activity during xylem sap ingestion (Dugravot et al., 2008; Spiller et al., 1990). In our study, waveform G was observed in a high percentage of psyllids suggesting that *B. trigonica* commonly alternates phloem with xylem sap ingestion probably as a strategy to regulate osmotic potential, as has been proposed for other phloem sap feeders (Pompon et al., 2011).

Our histological data and EPG signals suggest that waveform D occurs when *B. trigonica* stylets reach the phloem but just before intracellular insertion into the sieve elements because EPG signals remain at the extracellular level. However, the biological meaning of waveform D is still unknown. It has been hypothesized that it could represent recognition of phloem tissues, just in the transition phase from the extracellular to the intracellular environment

(Civolani et al., 2011; Pearson et al., 2014). Also, Pearson et al (2014) suggested that waveform D could represent secretion of highly conductive sheath saliva during sieve element penetration. Our results of the transmission experiments support that waveform D represents extracellular stylet activities prior to E1, because no Lso transmission was observed when insects were removed from plants during the D waveform phase. Successful inoculation of Lso was achieved only when EPG recordings were interrupted during the E1 phase.

Our work shows that the saliva containing Lso secreted by *B. trigonica* reaches sieve element's cytosol just after the beginning of E1, when the potential drop indicative of transmembrane potential was observed. This result, combined with the additional evidence provided in our study such as the intracellular voltage observed during E1 as well as the correlation between E1 and salivary sheath termini on phloem tissues, strongly indicates that the biological meaning of waveform E1 for *B. trigonica* is salivation into the sieve element. Our results also showed that *B. trigonica* was able to efficiently inoculate Lso at high rates (48%) in a single period of E1 with a duration as short as 30 s. This high vector efficiency, accompanied by high vector abundance such as the ones reported by Teresani et al. (2015), could explain the high incidence of this pathogen reported in the field (Teresani et al., 2014).

Waveform E2 of *B. trigonica* was correlated with salivary sheaths in the phloem. Previous studies provided evidence of phloem ingestion during E2 by demonstrating the acquisition of *Candidatus Liberibacter asiaticus* (CLas) by *Diaphorina citri* Kuwayama (Bonani et al., 2010) and of Lso by *B. cockerelli* (Sandanayaka et al., 2014). Thus, based on this previous evidence, we hypothesize that phloem ingestion could also match as a main activity during the E2 waveform of *B. trigonica*.

Regarding the effects of Lso in vector behaviour it has been shown that this pathogen can affect its own transmission directly by inducing changes in vector behavioural traits or indirectly by altering the traits of its primary host (Mauck et al., 2012). Those changes could be advantageous, neutral, or disadvantageous for transmission. It is well known that changes in host plants mediated by Lso affect host preference by its vectors, which increases Lso transmission (Davis et al., 2012; Mas et al., 2014). Nevertheless, the direct effects of Lso on its vectors have received little attention, and this study proves that Lso may directly modify the stylet penetration activities of its vector, *B. trigonica*.

Our data revealed differences in stylet penetration activities before reaching phloem tissues between Lso-free and Lso-infected psyllids. However, none of these differences are related to Lso transmission and therefore, these are unlikely to affect it. However, Lso effects on other behavioural steps, previous to plant probing could be important too for Lso dispersion. For example, a recent study indicated that the closely related CLas directly affects the behaviour of its vector *D. citri*, causing females to move over longer distances and making them more attractive to males, thus increasing CLas dispersion (Martini et al., 2015). It is unknown whether Lso could affect its vectors in a similar way; nevertheless, this hypothesis seems interesting and should be tested in further investigations. This also holds for the impact of Lso infected plants on vector probing. We only evaluated the effects of Lso infection in the insect.

In summary, our study provides a solid framework for future work on vector-bacteria interactions and the transmission of Lso by psyllids. Our work also provides information on *B. trigonica* vector efficiency that could be incorporated into models for predicting the incidence of Lso in the field or used as a reference for future studies using EPGs as a tool to evaluate pesticide action against *B. trigonica*.

Figures

Figure 1. Overview of electrical penetration graphs produced by *Bactericera trigonica* on young carrot leaves. The inset box shows an expanded view of waveforms A and C1. np, non-probing baseline; C1, saliva secretion on parenchyma tissues during stylet pathway to conductive tissues; D, initial contact with phloem cells; E1, saliva secretion into the phloem; E2, ingestion from phloem; G, ingestion from xylem tissues. C2 and pseudo-pds are not shown due to their infrequent occurrence. Y-axis scale represents voltage; X-axis represents time in minutes (inset box; time in seconds)

Figure 2. Details of waveforms produced by *Bactericera trigonica* on carrot leaves. (A) General overview of waveform C1, showing two consecutive pseudo-potential drops (pseudo-pd) (arrows). (B) Detail of a pseudo-pd. (C) Detail of waveform pattern D – preceded by C1 and followed by E1 – divided into two phases. (D) Superimposed pattern E1–E2. (E) Alternating periods of E2 and E1. Y-axis scale represents voltage; X-axis represents time in seconds.

Figure 3. Details and changes in appearance of waveforms obtained by changing the applied plant voltage. Unaltered appearance at different applied voltages reveals strong electrical origin caused by electromotive force (emf). Conversely, variable appearance reveals an resistance force (R) origin. The Y-axis represents plant voltage at zero (0), positive (+), or negative (-) values. (A) Waveform C1; (B) waveform C2; (C) waveform G; (D) waveform D; (E) waveform E1; (F) waveform E2; (G) pseudo-potential drop. X-axis represents time in seconds.

Figure 4. Flow diagram showing (sequences of) behavioural events of (A) *Candidatus Liberibacter solanacearum* (Lso)-free and (B) Lso-infected *Bactericera trigonica* in carrot leaves during the 8-h recording period. Values near the arrowheads correspond to the likelihood (%) that a certain waveform will be followed by any other. See Results for a description of the various encircled waveforms.

Figure 5. Cross-sections of salivary sheaths of *Bactericera trigonica* in carrot leaves when probing was interrupted. (A,B) Salivary sheath ending on parenchyma tissues during waveform (A) C1 and (B) C2. (C) Branched salivary sheath with a branch ending in xylem tracheary element during waveform G. (D) Salivary sheath in phloem tissues area during waveform D. (E) Salivary sheath starting from a salivary flange on the leaf surface (red arrow) and ending in phloem tissues during E1. (F) Branched salivary sheath ending in phloem during E2, red arrow shows a detail of a sieve element. Ep, Lower epidermis; Pa, parenchyma; Ph, phloem; Xy, xylem; SC, secretory canal. A line was added surrounding the salivary sheath to indicate its limits.

1 **Tables**

2 **Table 1.** Summary of main characteristics and correlations of *Bactericera trigonica* electrical penetration graph (EPG) waveforms on carrot
3 leaves

Waveform	Frequency (Hz)	Relative amplitude ¹ (%)	Voltage level	Electrical origin ²	Salivary sheath position	Activity
C1	12-16	100	Extracellular	emf	Parenchyma	Pathway activities
Pseudo-pd	7-16	-	Extracellular	R	Parenchyma	Unknown
C2	0-2	-	Extracellular	emf	Parenchyma	Pathway activities
G spikes	6-9	20	Extracellular	R-emf	Xylem	Xylem ingestion
D	4-8	20-30	Extracellular	R-emf	Phloem	Phloem contact
E1	4-5	-	Intracellular	emf	Phloem	Phloem salivation
E2	4-6	-	Intracellular	emf	Phloem	Phloem ingestion

4 ¹C1 includes waveform A = 100% amplitude. This scale is applicable only for waveforms with a main R origin. (-) not determined.

5 ²emf, electromotive forces; R, resistance

Table 2. Sequential and non-sequential electrical penetration graph (EPG) variables (mean $\pm$ SE) describing the probing and feeding behaviour of *Candidatus Liberibacter solanacearum* (Lso)-free and Lso-infected *Bactericera trigonica* on carrot plants on 8 h of recordings. Value ranges are given in parentheses. PPW, proportion of individuals that produced the waveform.

EPG Parameter	Psyllid colony	PPW	No. waveform events per insect	P	Waveform duration per insect (min)	P	Waveform duration per event (min)	P
Np	Lso-free	20/20	18.65 $\pm$ 2.52 (4-42)	0.98 ¹	91.14 $\pm$ 11.50 (33.21-191.57)	0.03 ²	4.87 $\pm$ 0.58 (0.08-111.79)	<0.01 ³
	Lso-infected	20/20	18.60 $\pm$ 2.18 (3-39)		138.18 $\pm$ 17.87 (37.78-333.17)		7.48 $\pm$ 0.80 (0.08-152.99)	
C (C1+C2)	Lso-free	20/20	19.05 $\pm$ 2.62 (3-42)	0.97 ¹	284.51 $\pm$ 20.60 (52.40-438.68)	<0.01 ¹	14.26 $\pm$ 1.36 (0.06-261.18)	<0.01 ³
	Lso-infected	20/20	19.15 $\pm$ 2.25 (3-40)		202.49 $\pm$ 21.13 (39.84-378.37)		9.97 $\pm$ 0.84 (0.02-148.53)	
D	Lso-free	14/20	1.3 $\pm$ 0.32 (0-5)	0.58 ³	1.71 $\pm$ 0.39 (0-5.71)	0.44 ³	1.31 $\pm$ 0.18 (0.33-3.76)	0.04 ²
	Lso-infected	14/20	1.4 $\pm$ 0.26 (0-3)		1.16 $\pm$ 0.26 (0-3.98)		0.83 $\pm$ 0.07 (0.41-1.96)	
E1	Lso-free	11/20	2.0 $\pm$ 0.82 (0-16)	0.50 ³	8.22 $\pm$ 4.17 (0-64.51)	0.36 ³	4.11 $\pm$ 1.66 (0.02-48.49)	0.64 ³
	Lso-infected	13/20	1.80 $\pm$ 0.40 (0-6)		6.01 $\pm$ 2.21 (0-35.06)		3.34 $\pm$ 0.81 (0.01-17.46)	
E2	Lso-free	11/20	1.85 $\pm$ 0.76 (0-15)	0.58 ³	52.76 $\pm$ 18.90 (0-239.98)	0.41 ³	29.52 $\pm$ 10.12 (0.76-239.98)	0.10 ³
	Lso-infected	13/20	1.55 $\pm$ 0.36 (0-6)		76.21 $\pm$ 18.97 (0-276.88)		49.16 $\pm$ 11.93 (0.52-240.47)	
G	Lso-free	14/20	1.05 $\pm$ 0.18 (0-2)	0.81 ³	39.03 $\pm$ 8.33 (0-145.27)	0.38 ³	38.21 $\pm$ 6.38 (0.93-127.38)	0.15 ³
	Lso-infected	19/20	1.55 $\pm$ 0.16 (0-3)		50.98 $\pm$ 10.19 (0-190.40)		32.89 $\pm$ 5.67 (9.12-163.49)	
Time elapsed to:								
First E1 from start of EPG	Lso-free						367.90 $\pm$ 30.28 (74.47-479.99)	0.23 ³
	Lso-infected						321.05 $\pm$ 32.44 (53.28-479.99)	
First E1 from start of first probe	Lso-free						350.95 $\pm$ 31.03 (72.35-479.70)	0.38 ²
	Lso-infected						307.56 $\pm$ 32.24 (50.17-477.77)	
First E1 from start of that probe	Lso-free						31.08 $\pm$ 10.89 (5.83-100.63)	0.96 ²
	Lso-infected						30.42 $\pm$ 7.00 (4.95-148.53)	

P-values are based on ¹Student's t-test for Gaussian distribution variables, ²Student's t-test for transformed variables, or ³Mann-Whitney U-test

CHAPTER IV. Sex-specific probing behaviour of the carrot psyllid *Bactericera trigonica* and its implication in the transmission of ‘*Candidatus Liberibacter solanacearum*’²

Introduction

Candidatus Liberibacter solanacearum (Lso) is a phloem-limited plant pathogenic bacterium transmitted by psyllids (Hemiptera: Psylloidea). On potatoes, Lso has been associated with a vegetative disorder called zebra chip. This disorder causes millions of dollars in losses to the potato industry in North and Central America as well as in New Zealand (Liefting et al., 2008; Munyaneza, 2012, 2015). In addition to potatoes, Lso also infects tomatoes, peppers, tamarillos and other economically important solanaceous plants (Liefting et al. 2008b; 2009). The American potato psyllid, *Bactericera cockerelli*, is the known vector of Lso in solanaceous plants (Munyaneza et al., 2007; Secor et al., 2009).

In Europe, Lso was first described in 2010, causing vegetative disorders in carrots in Finland and other Scandinavian countries (Munyaneza et al., 2010a; Nissinen et al., 2014). Recently, Lso has also caused severe carrot crop losses in several locations in the Mediterranean as well as in Germany (Alfaro-Fernández et al., 2012a; Loiseau et al., 2014; Tahzima et al., 2014; Teresani et al., 2014; Munyaneza et al., 2015). Two different psyllid species have been reported so far as vectors of Lso in carrots: *Trioza apicalis* Foerster in

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northern Europe (Munyaneza et al., 2010b; Nissinen et al., 2014; Munyaneza et al., 2015b) and *Bactericera trigonica* Hodkinson in the Mediterranean region (Alfaro-Fernández et al., 2012b). Due to the recent emergence of the bacterium, the mechanism by which carrot psyllids transmit Lso is not completely understood.

Lso is transmitted in a propagative-circulative manner. It is acquired by its vector while ingesting from phloem sieve elements of an infected plant and needs to replicate in the vector body. Once Lso reaches the vector's salivary glands it can be inoculated into the phloem sieve tubes of a healthy plant during short periods of salivation (Sandanayaka et al. 2014; Mustafa et al. 2015). Under this scenario, vector salivation and sap ingestion into the phloem sieve tubes are an absolute prerequisite for pathogen transmission.

Vector sex has been reported to influence the probing behaviour of the Asian citrus psyllid *Diaphorina citri* Kuwayama (Serikawa, 2011). It is not known if such sex based behavioural differences are present in all psyllid species, nor is it clear how this could impact pathogen transmission. Interestingly, it has been reported that Lso infections in carrots, when mediated by females of the psyllid *T. apicalis*, produced more severe root damage than infections mediated by males (Nissinen et al. 2014). The reasons for such differences remain unexplained. However, it has been hypothesized that differences in the observed symptom severity could be the result of higher Lso titre inoculated due to an increased salivation time by females (Nissinen et al. 2014). Despite its importance, the connection between variation in the probing behaviour of carrot psyllids and Lso delivery into the plant remains unclear. More specifically, whether there is a sex difference in probing behaviour of the Mediterranean carrot psyllid (*B. trigonica*), similar to that reported for *T. apicalis* in Northern Europe, is also unknown. This study aimed to help fill

this knowledge gap by investigating the probing behaviour of both female and male *B. trigonica* when transmitting Lso in carrots. Using the DC Electrical Penetration Graph (EPG) technique (Tjallingii 1985), we monitored stylet penetration activities of *B. trigonica* and quantified Lso titre inoculated by each sex in carrots using real-time PCR. Finally, symptoms shown by carrot plants when males and females transmitted Lso were compared.

Materials and methods

Plant and Insect Material

Seeds of carrot (*Daucus carota* cv Bangor) (Bejo Zanden b. v. The Netherlands) were germinated in petri dishes in the dark at 24° C for four days. Carrot seedlings were then individually grown in 8-cm diameter and 18-cm height plastic pots using a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil substrate (Kekilla Iberica, Almería, Spain). Batches of seeds and seedlings used for experiments tested negative for *Candidatus Liberibacter solanacearum* (Lso) as determined by real-time PCR according to Bertolini et al. (2015). Plants were maintained in a growth chamber with the following conditions: temperature 24:20° C (L:D), photoperiod of 16:8 h (L:D). Plants were watered three times a week and a nutritional complex 20-20-20 (N:P:K) of Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., PA, USA) was added once a week to irrigation water at 0.25 g/l. For transmission experiments, 1-leaf stage carrot plants were transferred to greenhouse conditions inside insect proof cages.

A *Bactericera trigonica* Lso-infected colony was established from samples kindly supplied by Estrella Hernandez (Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife,

Spain), obtained from a population collected from commercial carrot fields in Tenerife in 2012. Psyllids were maintained on carrot plants in 47.5 x 47.5 x 47.5 cm (length x width x height) (nylon mesh 150 µm) insect cages at greenhouse conditions. Greenhouse conditions were 28:18° C temperature (L:D) and a photoperiod of 10:14 h (L:D). The psyllid colony was periodically tested using real-time PCR, and the percentage of Lso-infected psyllids was always above 98 %.

Transmission of Lso by males and females.

The Lso percentage of transmission by males and females was compared using two different inoculation access periods (IAP) and psyllid densities. Further, the symptoms caused by Lso to carrot plants when transmitted by both males and females were also compared. In the first set of transmission experiments, an IAP of six hours and eight psyllids per test plant were used. Groups of psyllids were collected from the Lso-infected colony cages with a handmade vacuum aspirator 24 h before conducting transmission assays. Individuals were anesthetized with CO₂ for 4 s in order to be able to separate males and females. All males and females were separated and transferred to different cages containing two carrot plants each and kept under greenhouse conditions. Groups of eight individuals from each sex were collected and placed in Eppendorf tubes just before the transmission test was conducted. Each group of 8 psyllids was released onto a 1-leaf stage carrot receptor seedling by opening and inserting the Eppendorf tube into the soil next to the carrot plant, which was then enclosed in a transparent plastic cylindrical cage following a procedure similar to the one described by Nissinen et al. (2012). Psyllids started to leave

the Eppendorf almost immediately after opening the lid and were able to access and remain on the test plant for 6-h IAP. There were three different treatments: i) 20 carrot plants exposed to groups of eight males; ii) 20 carrot plants exposed to groups of eight females; and iii) 20 plants that served as negative controls (carrot plants that were never exposed to psyllid probing). This assay was repeated three times, and treatments were arranged in a completely randomized design.

For the second set of transmission experiments, the methodology was similar to that described above, but just one psyllid was released for an IAP of 8 h on the receptor plant. In this case, the number of replicates was also three, but 30 plants were used for each treatment. This assay was also repeated three times and plants were arranged in completely randomized design. In this assay, we shifted the IAP from six to eight h to increase the chances of Lso transmission, as the psyllid number was decreased from eight to one.

For both sets of experiments, psyllids were removed from the receptor plants at the end of the IAP and were tested to confirm the presence of Lso by real-time PCR (insects were individually tested in the one psyllid assay and pooled in groups of eight in the eight psyllid assay). Test plants were sprayed with 1 g l⁻¹ of Confidor ® (Bayer, Kansas City MO USA). A second insecticide spray was repeated 10 d later to ensure that possible nymphs were removed. Symptom development was monitored for 60 d post inoculation; then plants were collected and the number of yellow leaves and root weight were quantified. Leaf samples were also taken to perform real-time PCR for detection of Lso.

Lso detection by real-time PCR.

DNA was purified for each plant and insect used in the experiment. A crude plant extract was obtained by grinding 0.6 g of symptomatic plant material (leaves) in 4 ml of extraction buffer. Crude plant extract was stored at -20 °C until use. DNA purification from 200 ml of crude extract was performed following the CTAB (cetyltrimethyl ammonium bromide) protocol (Murray & Thomson 1980). For Lso detection on psyllids, individual insects were squashed onto Whatman 3MM paper using the round bottom of an Eppendorf tube (Bertolini et al., 2014; Olmos et al., 1996). Whatman paper containing the sample was placed inside an Eppendorf tube and 100 µl of triton 100X were added. Then, tubes were placed in boiling water at 100 °C for 10 min to allow the release of the DNA from the paper to the triton.

Lso was detected by real-time PCR according to Bertolini et al. (2015) for both plant and insect samples, using universal primers for Liberibacters, CaLsppF (5'-GCAGGCCTAACACATGCAAGT-3') and CaLsppR (5'-GCACACGTTTCCATGCGTTAT-3'), and a '*Ca. L. solanacearum*'-specific TaqMan probe (5' FAM-AGCGCTTATTTTAAATAGGAGCGGCAGACG-3' TAMRA). An Applied Biosystems 7300 thermal cycler was used; PCR conditions were as follows: an initial step of 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

Quantification of bacterial titre inoculated by males or females.

The bacterial titre inoculated by a single male or female in a receptor plant was quantified by quantitative real-time PCR in an independent assay. Single Lso-infected psyllids (male

or females) were placed on one leaflet of a one-leaf age Lso-free carrot plant and enclosed on a restricted area (0.5 cm diameter) by using a small clip cage. After an IAP of 8 h, psyllids were removed from the leaflet and processed for quantification of Lso by real-time PCR as described above. A total of 90 test plants (50 for males and 40 for females) were used in this experiment. The restricted area of the leaflet where the psyllid was probing was cut from the test plant with a lid of an Eppendorf tube after the psyllid was removed from the receptor plant. Leaf disks of each plant were sampled and processed for real-time PCR as described previously. Samples that were PCR-negative were discarded from the analysis. To establish the standard curve for real-time PCR, plasmid DNA was kindly provided by Dr Mariano Cambra from Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Avogadro's constant was used to estimate the number of plasmid DNA copies. Then, eight 10-fold plasmid dilution series were obtained that ranged from 1.3×10^8 to 1.3 ng/ml according to Bertolini et al. (2015).

Comparison of the probing behaviours of males and females.

The probing behaviour of Lso-infected *B. trigonica* males and females was compared by analysis of different EPG variables. Twenty EPG recordings were conducted on different carrot plants (one plant for each insect) and for each of the two treatments (either males or females). EPG recordings were obtained using a 4-channel DC EPG system (type Giga-4, Wageningen University, The Netherlands). Young adult Lso infected psyllids, 8 d of age, were collected from the insect rearing colonies and placed individually inside an Eppendorf tube. Psyllids were anesthetized with CO₂ for 4 s, divided by sex and then immobilized

using a vacuum-operated plate (Eyela Aspirator A3S, Tokyo Rikakikai 6 Co.LTD, Japón) under a dissecting microscope. A thin gold wire (diameter 18µm) (Coining ,Montvale, NY USA) was placed on the psyllid mesothorax and glued by a small drop of conductive water-based silver glue solution (EPG Systems, Wageningen. The Netherlands). The opposite end of the gold wire was attached to a copper electrode (input electrode). Then the attached insect was placed on the abaxial surface of a fully expanded carrot leaf. Another electrode (copper, 10 cm long, 2 mm wide) was inserted in the substrate of the pot containing the carrot plant to complete the electrical circuit. Prior to EPG all plants used tested negative for Lso by real-time PCR. The monitoring system (plant, insect and amplifier) was placed inside a Faraday cage to avoid external electrical noise. Each insect was monitored for 8 h under laboratory conditions (22-24°C) starting immediately after insects were placed on the leaf. Data were analysed using Stylet+ software for Windows (EPG Systems, Wageningen, The Netherlands). EPG waveforms previously described for psyllids (Bonani et al. 2010; Civolani et al. 2011; Pearson et al. 2014), were identified as follows: non-probing (np), intercellular stylet pathway (C), initial contact with phloem tissue (D), salivation into phloem sieve elements (E1), passive phloem sap uptake from the phloem sieve elements (E2) and active intake of xylem sap from xylem elements (G). The behavioural data was compiled using the MS Excel Workbook for automatic EPG data compilation developed by (Sarria et al., 2009). Selected EPG variables (mean $\pm$ SE) (listed in Supplementary material 1) were calculated using the SPSS statistical package. The following variables were calculated as described in Backus et al. (2007): PPW, proportion of individuals that produced a specific waveform type; NWEI, number of waveform events per insect, that is the sum of the number of events of a particular waveform divided by the total number of

insects under each treatment; NPI, number of probes per insect (the number of stylet insertions into the plant tissue regardless of the activity performed); WDI, total waveform duration (min) (TWD) per insect (that is the sum of durations of each event of a particular waveform) divided by the total number of insects under each treatment; and WDE, total waveform duration (min) per event (that is the sum of the duration of the events for a particular waveform) divided by the total number of events of that particular waveform under each treatment. We calculated also WDEI (mean waveform duration per event per insect) for the waveforms related to phloem activities (E1 and E2) because they are associated to the transmission of Lso.

Statistical analysis

Pairwise comparisons between the Lso percentage of transmission under the different treatments were analysed using a chi-square test and by Fisher's exact test when expected values were lower than 5 cases. Root weight and number of yellow leaves obtained from plants inoculated by either Lso-infected males or females were compared by means of a one-way ANOVA followed by pairwise comparisons for least significant differences (LSD). If data did not follow the ANOVA assumptions, a Kruskal-Wallis test was performed followed by a Mann-Whitney test on each pair of groups, adjusting *P* values with the Bonferroni method. An adjusted general linear model was carried out to compare the concentration of Lso inoculated in carrot leaves by males or females. Concentration of Lso in psyllid bodies was handled as a fixed factor and divided in tertiles ($n=11$) with three different levels (low, medium and high). Comparisons between probing behaviour of males

and females were made using a Student's t-test for Gaussian variables or a Mann-Whitney U test when normality was not achieved. Data were previously transformed with $\sqrt{x + 0.5}$ or $\ln(x + 1)$ if needed to reduce heteroscedasticity and achieve normality. All data were processed with the statistical software SPSS 21 (SPSS 2013).

Results

Transmission of Lso by males or females.

None of the uninfected control plants tested positive for Lso. Transmission experiments showed that there were no significant ($P > 0.05$) differences in the percentage of transmission of Lso between the two sexes of *B. trigonica* when testing different number of insects per test plant and different IAP (Fig. 1). When assays were performed using a psyllid density of eight individuals per plant and an IAP of 6 h, Lso percentages of transmission for both sexes were similar (81.66 ± 7.26 (49/60) for males and 80.00 ± 12.58 (48/60) for females) ($\chi^2 = 0.03$, $df = 1$, $P = 0.85$) (Fig. 1).

For the second transmission assay, a single psyllid per test plant and an IAP of 8 h were used. The results again demonstrated no differences in the percentage of transmission between sexes, although efficacy decreased as expected (23.33 ± 6.65 (21/90) for males and 20.00 ± 5.23 (18/90) for females) ($\chi^2 = 0.46$, $df = 1$, $P = 0.49$) (Fig. 1).

Symptoms caused by Lso when inoculated by males or females.

None of the control plants showed symptoms. However, all plants positive for Lso showed stunting of roots and shoots and leaf yellowing 60 d post inoculation. Root weight of the male- and female-inoculated carrot plants was reduced by almost half compared to the healthy control plant for one and eight psyllids (Table 1). However, there were no differences in root weight between male and female-inoculated carrots, and results were similar for the two psyllid densities used ($P>0.05$) (Table 1).

Interestingly, when the numbers of yellow leaves were compared, differences between all treatments were found when eight individuals per plant were used (Table 1). Female-inoculated carrots showed a higher number of yellow leaves when compared with male-inoculated carrots (Table 1). On the other hand, when a psyllid density of one was used, the number of yellow leaves was not significantly different between carrots inoculated by males or females (Table 1).

Bacterial titre inoculated by males and females.

Real-time PCR indicated that the Lso titre in psyllid body was higher for males than for females (number of Lso amplified targets per psyllid: $557,135.48 \pm 260,237$ in males and $155,882.48 \pm 62,172$ in females, $t=2.26$, $df=1$, $P=0.03$), however the overall Lso titre inoculated was similar between sexes ($F=5.35$, $df=2$, 31 , $P=0.10$) (Table 2) (Fig. 2). Interestingly, our data showed that Lso titre inoculated in carrot leaves was statistically affected by psyllid titre ($F=13.93$, $df=2$, 31 , $P < 0.0001$) and there was also a significant interaction between sex and psyllid titre ($F=5.35$, $df=2$, 31 , $P=0.01$) (Table 2) (Fig. 2).

Thus, females with high Lso titres inoculated significantly more bacteria compare to males in the same category (Table 2), (Fig. 2) (supplementary material 2), suggesting that females with high Lso titres were more efficient inoculators than males.

Probing behaviour of males and females.

Analysed sequential variables were not significantly different between males and females. The analysis of non-sequential EPG variables demonstrated that females (NPI: 16.85 ± 1.60) probed more frequently than males (NPI: 11.45 ± 1.71) ($t=0.74$, $df=1$, $P=0.04$). The duration of non-probing events was longer for males (WDE: 17.29 ± 2.37 minutes) than for females (WDE: 10.07 ± 1.44 minutes) ($U=33607.50$, $df=1$, $P=0.01$) (supplementary material 3). Males also showed a higher proportion of xylem activities than females. While waveform G (PPW) was present in all recordings for males, only 15/20 (75%) of females performed this waveform ($\chi^2=5.71$, $df=1$, $P=0.01$). NWEI mean values for G were also significantly higher for males (1.45 ± 0.13) compared to female values (1.00 ± 0.17) ($U=132.00$, $df=1$, $P=0.03$) (Supplementary material 3). In our EPG recordings, phloem salivation (E1) and phloem ingestion (E2) frequently alternate in the same probe. As a result, the mean number of E1 per insect (NWEI) could not be used to accurately describe frequency of phloem contacts within a single probe. Therefore, we used the number of E1 at the beginning of each phloem probe (Initial E1) to describe frequency of phloem contacts that could lead to inoculation of Lso. Data showed that initial E1 numbers were significantly higher for females than for males (NWEI: 0.50 ± 0.18 for males and 1.65 ± 0.39 for females) ($U=117.50$, $df=1$, $P=0.01$) (Fig. 3a). Also waveform D numbers were

higher for females than for males (NWEI: 1.70 ± 0.40 for females and 0.55 ± 0.19 for males) ($U=121.50$, $df=1$, $P= 0.02$) (Fig. 3b). However, the mean number (NWEI) and the mean duration per event (WDE) of E1 and E2 were not significantly different between sexes. Moreover, there was no difference for the duration per event per insect (WDEI) for E1 or E2 between males and females of *B. trigonica* (WDEI E1: 2.22 ± 0.90 for males and 3.23 ± 0.81 for females ($t=-0.78$, $df=1$, $P=0.41$) and WDEI E2: 25.23 ± 10.94 for males and 45.07 ± 11.41 for females ($t=-1.25$, $df=1$, $P=0.22$)). The proportion of individuals reaching phloem tissues at least once (PPW) was also not different between males and females (8/20 males; 13/20 females) ($\chi^2=2.50$, $df=1$, $P=0.13$) (Supplementary material 3). Further, other relevant phloem-related variables were significantly higher for females than males. For example, the duration per insect for waveform D (WDI: 0.59 ± 0.24 for males and 1.72 ± 0.41 for females) ($U=123.50$, $df=1$, $P= 0.02$) and E2 (WDI: 15.14 ± 7.16 for males and 55.08 ± 16.14 for females) ($U=122.50$, $df=1$, $P= 0.02$) were significantly higher for females than for males (Fig. 3c, e). Also, the number of single E1 (NWEI: 0 for males and 0.3 ± 0.14 for females) ($U=160.00$, $df=1$, $P=0.03$) and the number of sustained E2 (E2 events longer than 10 minutes) (NWEI: 0.25 ± 0.14 for males and 0.75 ± 0.19 for females) ($U=125.50$, $df=1$, $P= 0.01$) were higher for females than for males (Fig. 3d, f).

Discussion

Most sex-specific responses of psyllids reported so far refer to settling and host selection behaviour prior to probing and ingestion (Gharaei et al., 2014; Martini et al., 2015). Less

importance has been given to sex-specific probing behaviours associated with pathogen transmission. Our work demonstrates that probing behaviour of male and female *B. trigonica* differs in traits associated with inoculation of Lso. Although we found no differences in the percentage of transmission between sexes, different probing behaviours were correlated with differences in observed Lso transmission efficiency between males and females.

Current experimental evidence suggests that Lso is transmitted in a propagative-circulative manner, which means the bacteria replicate in their vectors and are inoculated in saliva secreted before phloem ingestion (Sandanayaka et al., 2014; Sengoda et al., 2014 Cooper et al., 2014; Cicero et al., 2016a; Mustafa et al., 2015). Previous reports have shown that Lso could be successfully inoculated in a single salivation event in a phloem sieve element by *B. cockerelli* (Mustafa et al. 2015); Thus, it is likely that only small titres of bacteria inoculated are needed to achieve infection. Therefore, similar proportions of males and females reaching phloem sieve elements would lead to similar inoculation rates between sexes as observed in our study. Our results are consistent with a previous study conducted with *Bemisia tabaci* biotype B transmitting *Tomato yellow leaf curl virus* (TYLCV), where similar percentage of males and females reached the phloem of tomato plants and caused similar percentages of transmission of TYLCV for both sexes (Ning et al. 2015). In addition, our data also agree with Nissinen et al. (2014), who reported similar Lso percentages of transmission for male and females of the carrot psyllid *T. apicalis*.

On the other hand, the Lso titre in a psyllid body was positively correlated with Lso titre inoculated into the plant. Despite the lack of differences in the mean values for Lso titre inoculated between sexes, there was a significant interaction between sex and psyllid titer.

Hence, females with higher Lso titres tend to inoculate more bacteria into the plants than males. This suggests that at high Lso titers, females might be more efficient at delivering Lso than males. However, this result should be analysed carefully considering that our sample size is small and larger data would be needed in order to give a stronger support to our hypothesis. Different factors can affect the efficiency of pathogen inoculation. For example, it is well known that the capacity to inoculate propagative-circulative pathogens increases with higher phloem contacts as well as with longer salivation events (Moreno-Delafuente et al. 2011; Jiang et al. 2000; Mustafa et al. 2015). In our study, duration of psyllid salivation in phloem sieve elements (E1) did not significantly differ between males and females, but numerically were two times greater for females than for males. Also, females showed a significantly higher frequency of phloem contacts, which is correlated with the higher Lso transmission efficiency showed by females. Thus, it is likely that an increased frequency of initial E1 as observed for females may play an important role explaining the higher Lso transmission efficiency observed by females.

In addition to an increased frequency of phloem contacts, a higher number of sustained phloem ingestion events as well as longer phloem ingestion events were observed for females, suggesting that they ingest phloem sap more actively than males. These results are consistent with related studies where increased phloem ingestion by females has been observed in other psyllid species as well as other hemipterans such as whiteflies, planthoppers, leafhoppers and thrips (Swenson, 1971; Narayana and Muniyappa, 1996; Serikawa 2011; van de Wetering et al., 1999; Ning et al., 2015). Increased phloem ingestion could be explained because females commonly require a high amount of nutrients to supply energetically costly processes such as egg production (Swenson, 1971; Nissinen

et al. 2014). As a result, this increased phloem ingestion would result in increased efficiency to acquire a phloem-restricted pathogen such as Lso.

Previous studies have correlated symptom severity with Lso titre in potato and carrots (Alvarado et al., 2012; Nissinen et al., 2014). In our study, symptom severity caused when males or females transmitted Lso was similar except for the number of yellow leaves at a psyllid density of eight. This lack of differences in symptoms using single psyllid densities is consistent with the similar Lso titres inoculated by both sexes. Our results contrast with Nissinen et al (2014), who reported more severe root damage when single females of *T. apicalis* transmitted Lso compared to single males. Factors that may account for divergent results between our study and Nissinen et al. (2014) may include differences in inoculation efficiency between vector species, different inoculation access periods as well as different plant varieties or genetic variants of the pathogen used.

On the other hand, our data revealed that Lso caused a greater number of yellow leaves when inoculated by groups of eight females. We hypothesize that groups of psyllids increase the Lso titre inoculated and could also synergize to magnify the differences observed in inoculation efficiency between males and females. Thus, if females are more efficient at inoculating Lso, it is expected that groups of females could inoculate higher titres than males, explaining the differences in symptoms obtained. Actually, this observation is consistent with previous studies that reported a positive correlation between the number of yellow leaves and Lso titre (Nissinen et al., 2014), as well as with reports of increased Lso titres inoculated by groups of four psyllids versus single ones (Rashed et al., 2012). Despite the supporting evidence, our hypothesis still requires further research to be confirmed.

Finally, it is important to highlight that direct damage from psyllid feeding could have contributed to the symptoms observed. However, because above-ground symptoms appeared on average 40 days after psyllids were removed, we believe that they were caused by Lso infection rather than by direct psyllid feeding as proposed by Nissinen et al. (2014). However, discriminating between the effects of Lso and direct psyllid feeding on below-ground symptoms is complicated because it has been reported that psyllid feeding could affect root development in carrots (Nissinen et al., 2007). Thus, the possibility that part of the root reduction observed could be due to direct psyllid feeding should not be completely disregarded.

In summary, we can conclude that differences in probing behaviours observed between males and females might impact the transmission of *Candidatus Liberibacter solanacearum*. Further research is still needed in order to establish a direct relationship between symptom severity and Lso titre in the plant. This study provides key information to understand the transmission process of Lso mediated by its vector *B. trigonica*. Our results also highlight the importance of taking psyllid sex into consideration for subsequent transmission or probing behaviour studies.

Figures.

Figure 1. Percentage of Lso infected plants when Lso is transmitted by *Bactericera trigonica* males or females at densities of one or eight psyllids per plant. Values represent the mean of three different assays. Error bars represent standard error.

Figure 2. Analysis of psyllid vs receptor plant Lso titres. Y-axis values represent the Ln of Lso-amplified targets per milligram of plant tissue and X-axis values represent three different categories of psyllid titre (Low, Medium and High). Range of values for the Ln of Lso amplified targets per psyllid in each category: Low=8.86 - 10.97, Medium =10.98 - 12.67, High=12.68 - 15.33, each category contains eleven individuals. Error bars represent standard error.

Figure 3. Variables describing probing behaviours (Mean $\pm$ SE) of males and females of *Bactericera trigonica* on carrot plants. (a) Number (NWEI) of initial E1 (First salivation period in a phloem probe); (b) Number (NWEI) of D (initial contact with phloem); (c) Duration (WDI) of D (initial contact with phloem); (d) Number (NWEI) of Single E1 (salivation not followed by phloem ingestion); (e) Duration (WDI) of E2 (phloem ingestion); (f) Number (NWEI) of Sustained E2 (phloem ingestion longer than 10 minutes). Only the EPG variables associated with phloem activities that showed significant differences are shown. Information on all calculated variables is provided in supplementary material 3. NWEI = Mean Number of Waveform Events per Insect. WDI = Mean Waveform Duration per Insect. WDE = Mean Waveform Duration per Event.

WDI and WDE are expressed in minutes.

*Significant differences according to Student's t-test (for Gaussian variables) or Mann-Whitney U test (for non-Gaussian variables) ($P < 0.05$).

1 **4. 6. Tables**

2 **Table 1.** Symptoms observed in carrot plants after the inoculation of Lso by single insects or groups of eight males or females of
3 *Bactericera trigonica*. Root weight is represented in grams (g).

	one psyllid								eight psyllids							
	Root weight ¹	Df	F	P*	Number of yellow leaves ²	df	H	P*	Root weight ²	df	H	P*	Number of yellow leaves ²	df	H	P*
Control	18.73 ± 1.34 a	2, 51	32.05	0.00	0.06 ± 0.06 a	2	12.49	0.00	17.16 ± 0.75 a	2	62.41	0.00	0.10 ± 0.45 a	2	78.97	0.00
Males	7.58 ± 1.04 b				0.85 ± 0.17b				9.39 ± 0.58 b				1.70 ± 0.19 b			
Females	6.06 ± 1.02 b				1.05 ± 0.26b				8.64 ± 0.66 b				2.80 ± 0.30 c			

4

5 Letters indicate statistically significant differences between groups in columns (P<0.05) according to (1) One way factor ANOVA or
6 (2) Kruskal-Wallis test.

7 *P < 0.0001

Table 2. Degrees of freedom (*df*) F-test (F) and significance (P) values for the comparison between the Lso titre inoculated by males or females of *B. trigonica* on carrots.

	<i>df</i>	F	Significance
Corrected model ⁺	5	6.45	0.01
Intercept	1	357.12	0.00*
Sex	1	2.78	0.10
Psyllid titre	2	13.93	0.00*
Sex*Psyllid titre	2	5.35	0.01
Error	26		
Total	32		

Dependent variable: Plant titre.

$R^2 = 0.55$ Adjusted R^2 0.468.

⁺This term represents the overall effect of all main effects and interactions included in the model.

* $P < 0.0001$

28 **Supplementary material**

29 **Supplementary material 1.** Sequential and non-sequential EPG variables analyzed to
30 compare the probing behaviours of males and females of *B. trigonica* (Lso +) on carrot
31 plants. Variables calculated represent the mean values for each cohort of insects used in the
32 study.

Non-sequential variables

Number of np

Number of Probes per Insect

Number of waveform events for C

Number of waveform events for E1

Number of waveform events for E2

Number of waveform events for G

Number of waveform events for D

Number of initial E1

Number of single E1

Number of single E2 > 10 min

Duration of np

Probing time

Waveform Duration per Insect for C

Waveform Duration per Insect for G

Waveform Duration per Insect for E1

Waveform Duration per Insect for E2

Waveform Duration per Insect for D

Waveform Duration per Event for C

Waveform Duration per Event for G

Waveform Duration per Event for E1

Waveform Duration per Event for E2

Waveform Duration per Event for D

Waveform Duration per Event per Insect for E1

Waveform Duration per Event per Insect for E2

Sequential variables

Total duration of E1 followed E2 sustained

Total duration of E1 followed E2

Time to 1st probe from start of EPG

Time from start of EPG to 1st E

Time from 1st probe to 1st E

Time from the beginning of that probe to 1st E

Time to from start of EPG 1st sustained E2 (10 minutes)

Time from 1st probe to 1st sustained E2 (10 minutes)

Time from the beginning of that probe to 1st sustained E2 (10 minutes)

Time from the beginning of that probe to 1st E2

Duration of np just after the probe of the first sustained E2

Number of probes after the 1st E1

Supplementary material 2. *t*-test comparison between the Lso titre inoculated in carrot plants by males and females with different quantities of Lso in insect body.

Psyllid titre	Levene´s test		t-test for equality of means				
	F	Sig	t	df	sig (2 tailed)	Mean difference	Standart error
Low	0.25	0.62	-1.14	9	0.28	-1.17	1.03
Medium	1.00	0.34	1.59	9	0.14	1.93	1.21
High	0.67	0.43	-4.14	8	0.00	-3.74	0.90

45 **Supplementary material 3.** Occurrence and duration (Mean $\pm$ SE) of non-sequential DC-Electrical Penetration Graph (EPG) waveforms of *B.*
46 *trigonica* (Lso +) carrot plants during an 8-h recording period. PPW, proportion of individuals that produced the waveform type; NWEI, number
47 of waveform events per insect. WDI, waveform duration per insect (minutes). WDE, waveform duration per event. (minutes)

Non sequential variables	Psyllid gender	PPW	NWEI	P	WDI	P	WDE	P
Non probe	Male	20/20	11.90 $\pm$ 1.72 (3-29)	<u>0.04</u> ¹	205.82 $\pm$ 22.71 (32.59-378.32)	0.31 ¹	17.29 $\pm$ 2.37 (0.18-282.26)	<u>0.01</u> ³
	Female	20/20	16.85 $\pm$ 1.60 (7-39)		169.75 $\pm$ 27.78 (33.97-431.43)		10.07 $\pm$ 1.44 (0.11-316.63)	
Probes*	Male	20/20	11.45 $\pm$ 1.71 (3-29)	<u>0.04</u> ¹	271.16 $\pm$ 22.49 (102.16 -447.40)	0.27 ¹	23.68 $\pm$ 2.54 (0.21-308.60)	0.14 ¹
	Female	20/20	16.85 $\pm$ 1.60 (7-39)		310.24 $\pm$ 26.78 (48.55-446.01)		18.85 $\pm$ 2.12 (0.15-292.01)	
C	Male	20/20	12.55 $\pm$ 1.81 (3-30)	0.09 ¹	224.86 $\pm$ 23.45 (55.93-400.86)	0.97 ¹	17.23 $\pm$ 1.94 (0.7-308.60)	0.05 ²
	Female	20/20	16.85 $\pm$ 1.68 (7-40)		226.08 $\pm$ 23.65(55.93-410.01)		12.25 $\pm$ 1.17 (0.3-214.46)	
D	Male	8/20	0.55 $\pm$ 0.19 (0-3)	<u>0.02</u> ³	0.59 $\pm$ 0.24 (0-2.17)	<u>0.02</u> ³	1.07 $\pm$ 0.21 (0.37-2.64)	0.65 ¹
	Female	13/20	1.70 $\pm$ 0.40 (0-6)		1.72 $\pm$ 0.41 (0-3.11)		1.01 $\pm$ 0.14 (0.31-4.92)	
E1	Male	7/20	1.35 $\pm$ 0.47 (0-7)	0.10 ²	3.97 $\pm$ 2.59 (0-50.73)	0.80 ³	3.45 $\pm$ 0.99 (0.3-17.56)	0.89 ²
	Female	13/20	2.1 $\pm$ 0.59 (0-10)		7.72 $\pm$ 3.13 (0-57.45)		3.68 $\pm$ 0.66 (0.1-18.77)	
E2	Male	7/20	1.15 $\pm$ 0.39 (0-6)	0.10 ³	15.14 $\pm$ 7.16 (0-98.78)	<u>0.02</u> ³	15.14 $\pm$ 5.86 (0.31-88.16)	0.22 ¹
	Female	13/20	1.75 $\pm$ 0.46 (0-8)		55.08 $\pm$ 16.14 (0-250.98)		31.47 $\pm$ 9.50 (0.35-234.9)	
G	Male	20/20	1.45 $\pm$ 0.13 (1-3)	<u>0.03</u> ³	27,18 $\pm$ 3.63 (5.71-87.48)	0.25 ²	18.74 $\pm$ 3.02 (0.39-87.48)	0.16 ²
	Female	15/20	1 $\pm$ 0.17 (0-3)		21.43 $\pm$ 3.55 (0-48.82)		21.32 $\pm$ 2.42 (1.14-48.82)	
Initial E1	Male	8/20	0.50 $\pm$ 0.18 (0-3)	<u>0.01</u> ³				
	Female	13/20	1.65 $\pm$ 0.39 (0-6)					
Single E1	Male	0/20	0	<u>0.03</u> ³				
	Female	4/20	0.3 $\pm$ 0.14 (0-2)					
Sustained E2	Male	3/20	0.25 $\pm$ 0.14 (0-2)	<u>0.01</u> ³			52.60 $\pm$ 13.49 (10.61-88.16)	0.57 ¹
	Female	11/20	0.75 $\pm$ 0.19 (0-3)				71.09 $\pm$ 17.77 (10.73-234.90)	

48 Waveforms: **C**, intercellular stylet pathway; **G**, xylem ingestion; **E** shows phloem-related activities: **E1**, correlates with salivation into phloem sieve elements; E2, ingestion
49 from phloem (Prado and Tjallingii, 1994); **sE2**: sustained E2 (longer than 10 minutes) Single E1 refers to E1 not followed by E2. Initial E1 refers to E1 preceded by D
50 waveform. Statistical comparisons for each variable were made by:

51 ¹ t-Student for Gaussian distribution variables.

52 ² t-Student for transformed variables.

53 ³ Non-parametric U-Mann Whitney test for non-Gaussian distribution variables.

54 * Number of probes per insect (NPI)

CHAPTER V. Risk assessment of ‘*Candidatus Liberibacter solanacearum*’ transmission by the psyllids *Bactericera trigonica* and *B. tremblayi* from Apiaceae crops to potato.³

Introduction.

Most plant pathogenic viruses and bacteria depend on insect vectors to spread to new areas or new hosts (Perilla-Henao and Casteel, 2016). Consequently, vector feeding preferences are strongly correlated with the epidemiology of vector-transmitted plant pathogens. The psyllids (Hemiptera, Psylloidea) are insect pests that ingest sap from the xylem and phloem tissues (Bonani et al., 2010; Pearson et al., 2014; Mustafa et al., 2015). Nevertheless, they are commonly known as phloem feeders because nutrients obtained from this tissue are mandatory in order to complete their life cycle on certain host plants (Hodkinson, 1974). Actually, psyllid host range is restricted to only a few species or genera of plants (Hodkinson, 2009; Burckhardt et al., 2014). Because of their feeding habits, psyllids could serve as vectors of phloem-restricted pathogens that cause economic losses in agriculture (Burckhardt et al., 2014).

In recent years, psyllids have been described transmitting the phloem-restricted bacterium *Candidatus Liberibacter solanacearum* (Lso), which is an emerging pathogen that causes severe losses primarily in potato and carrot (Alfaro-Fernández et al., 2012a; Munyaneza, 2012). Notably, different psyllid species transmit different haplotypes of Lso in different geographical areas and to different host plants (Nelson et al., 2011). In the Americas and New Zealand, Lso haplotypes A and B are transmitted to plants in Solanaceae by the potato psyllid *Bactericera cockerelli* Šulc (Nelson et al., 2011). In Europe and North Africa, Lso haplotype C is transmitted by *Trioza apicalis* Foerster, 1848 (Nissinen et al., 2014). Haplotypes D and E

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affects carrot and celery and have been associated to *Bactericera trigonica* Hodkinson, 1981 (Alfaro-Fernández et al., 2012b; Teresani et al., 2014). *Trioza apicalis* is found in northern and central European countries associated with Lso haplotype C, whereas *B. trigonica* is found in the Mediterranean region associated with Lso haplotypes D and E (Munyanzeza et al., 2010b; 2015). Despite the fact that it has been demonstrated that Lso could be transmitted through seeds, the main spread of this bacterium in the field is guided by psyllids that transmit the disease in a persistent-propagative manner (Munyanzeza et al., 2007; Bertolini et al., 2015; Cicero et al., 2016b). Accordingly, Lso needs to replicate in the psyllid body and then inoculated into the plant with the saliva, when the psyllid feeds from the phloem sieve elements of a susceptible plant (Cicero et al., 2016ab; Mustafa et al., 2015). Therefore, success and efficiency of Lso transmission by psyllids heavily depends on vector feeding behaviour and vector host range.

The most powerful tool to study feeding behaviour of hemipterans is the electrical penetration graph (EPG) technique (McClean and Kinsey, 1964; Tjallingii, 1978, 1985). This technique has been used to correlate psyllid-feeding activities with the transmission of different species of *Candidatus Liberibacter* (Bonani et al., 2010;; Sandanayaka et al., 2014; Mustafa et al., 2015; Antolínez et al., 2016; Munyanzeza et al., 2016). Recently, Munyanzeza et al (2016) used EPG to assess the risk of cross transmission of Lso from potato to carrot by the potato psyllid *B. cockerelli* (Munyanzeza et al., 2016). In that study, *B. cockerelli* had difficulty reaching the phloem tissues of carrot plants, which was correlated with very low percentages of Lso transmission in carrots. Additionally, Munyanzeza et al. (2016) also showed that Lso haplotype B from potatoes produces symptoms in carrots. Therefore, despite the fact that Lso haplotypes associated with potato can infect carrots, the movement of Lso from potato to carrot plants mediated by *B. cockerelli* is unlikely because carrot is not a host of this psyllid species.

While the risk of transmission from potato to carrot mediated by *B. cockerelli* has been assessed, the risk of Lso transmission from carrot to potato or to other crops by carrot psyllids in Europe is unknown. In the Mediterranean region for example, the carrot psyllid *B. trigonica* is

the only known vector of Lso (Teresani et al., 2014, 2015). Nevertheless, it is unknown if *B. trigonica* is able to feed on potatoes and subsequently can transmit Lso to this crop. In addition to *B. trigonica*, other psyllid species (*Bactericera tremblayi* and *Bactericera nigricornis*) are common in the Mediterranean region and have been suggested as potential vectors of Lso in Spain (Teresani et al., 2015). So far, *B. nigricornis* and *B. tremblayi* have been found in very low populations in carrot and potato fields (Teresani et al., 2015). However *B. tremblayi* has been reported as a serious pest in onion and leek crops (Ouvrard and Burckhardt, 2012), suggesting this psyllid may constitute a potential treat to carrot or potato because leek and onion fields are commonly grown in the vicinity of carrot and potato crops.

Despite of its economic importance, the potential risk of cross transmission of Lso from Apiaceae to potato by *B. trigonica* has not been evaluated. Moreover, the role of *B. tremblayi* as an Lso vector remains uncertain and must be determined. Thus, the main objective of this study was to evaluate the risk of Lso cross transmission from Apiaceae to potato and other crops mediated by *B. trigonica* and *B. tremblayi* two of the most abundant psyllid species in Spain. Our specific objectives were: i) to evaluate the potential of Lso transmission to potato, carrot, celery and leek by assessing the probing behaviour of *B. trigonica* and *B. tremblayi* using the EPG technique, ii) to assess whether *B. tremblayi* is a vector of Lso, and iii) to assess the settling preference and Lso transmission of *B. trigonica* (in carrot celery and potato) and *B. tremblayi* (in potato, carrot and leek) by performing non-choice and dual-choice assays.

Materials and methods

Plants and insects.

The following plant species were used in the assays: carrot (*Daucus carota*, variety ‘Bangor’), celery (*Apium graveolens*, cv ‘Pascal’), potato (*Solanum tuberosum*, cv ‘Monalisa’) and leek (*Allium porrum*, cv ‘Costeau’). Seeds of carrot (Bejo Zanden b. v. The Netherlands), leek (Royal Sluis SVS, Holland B. V), and celery (Battle S.A, Barcelona, Spain) and potato tubers

were germinated individually in 8 cm diameter pots with soil substrate (Kekkilä Iberica, Almería, Spain). Plants were grown in an insect-proof chamber at 24:18°C (L:D), 60-80% relative humidity (RH) and with a 16:8 h (L:D) photoperiod. Plants were watered three times a week with 20–20–20 (N:P:K) Nutrichem fertilizer (Miller Chemical & Fertilizer Corp., PA, USA) at a dose of 0.25 g l⁻¹. Plants were maintained in the growth chamber until reaching the developmental stage of three true leaves for carrot, celery and leek and four leaves for potatoes.

Colonies of *B. trigonica* and *B. tremblayi* were maintained in the greenhouse facilities at ICA-CSIC, Madrid, Spain. The *B. trigonica* colony was collected from carrot fields in Gomezserracín, Segovia, Spain, in 2014. Individuals for the *B. tremblayi* colonies were also collected in Gomezserracín from commercial leek fields in 2014. Colonies of *B. trigonica* were reared on carrot plants in 47.5 x 47.5 x 47.5 cm cages (length x width x height; nylon mesh, 150 µm), and colonies of *B. tremblayi* were reared on leek plants in similar insect cages. Attempts to rear *B. trigonica* on plants other than *Apiaceae* or *B. tremblayi* on plants other than *Allium* were unsuccessful. Colonies of *B. trigonica* and *B. tremblayi* were housed in different glasshouses under similar greenhouse conditions, i.e., 26:18°C (L:D) and a photoperiod of 16:8 h (L:D). Colonies were tested for Lso by real-time PCR; the percentage of infection of the *B. trigonica* colony was 97%, whereas *B. tremblayi* was Lso-free. Dr Jaime Cubero from the National Institute for Agronomic Research (INIA), Madrid, Spain, kindly identified the Lso haplotype in our *B. trigonica* colonies as haplotype E, according to the procedure described by Nelson et al. (2011). For the assays, 5-7-d-old, adult psyllids were collected from the psyllid colonies with a handmade vacuum aspirator one hour after the beginning of the experiments. Groups of insects used in the preference and transmission assays contained similar proportions of males and females.

EPG analysis of psyllid probing behaviour

The probing behaviour of *B. trionica* and *B. tremblayi* was monitored on different plants (carrot, celery and potato for *B. trionica* and carrot, leek and potato for *B. tremblayi*) using the electrical penetration graph (EPG) technique. A gold wire electrode (2 cm length, 20 μ m diameter) was attached to the insect pronotum following the procedure described by Antolínez et al. (2016). A second electrode (copper, 10 cm length, 2 mm diameter) was inserted into the soil of the plant container. The psyllids were starved for 1 h during acclimatization between the time of wiring and the beginning of EPG recording. Then, the psyllids were placed on the abaxial surface of a fully expanded leaf and were allowed to probe and feed on the test plant for 8 h. The EPG recordings were obtained using an eight channel DC EPG system (type Giga-8; Wageningen University, The Netherlands). The signal was digitized using a DI-710 board (Dataq[®] Instruments, Akron, OH, USA), and the digitized data were loaded onto a PC and analysed with Stylet+ software (EPG Systems, Wageningen, The Netherlands). A minimum number of 15 replicates were obtained for each plant and psyllid combination. EPG waveforms previously described for psyllids were identified according to Pearson et al. (2014)(Pearson et al., 2014) as follow: non-probing (NP), intercellular stylet pathway (C), salivation into phloem sieve elements (E1), phloem sap uptake from the sieve elements (E2) and active intake of xylem sap from xylem vessels (G). The following EPG parameters were calculated to describe pathway activity and phloem or xylem activity according to (Backus et al., 2007): probing duration per insect, PDI, is the amount of time an average insect has the stylet inserted; number of waveform events per insect, NWEI, is the sum of the number of events of a particular waveform divided by the total number of insects in each treatment; total waveform duration (min) per insect, WDI, is the sum of durations of each event of a particular waveform divided by the total number of insects in each treatment; and waveform duration per event by insect, WDEi, is the average duration of events of a given waveform by an insect in a cohort. The variable time from the first probe to the first E1 was calculated according to (Sarria et al., 2009).

Lso detection by real-time PCR

To detect Lso in plants, plant DNA was purified following the CTAB (cetyltrimethyl ammonium bromide) protocol (Murray and Thompson, 1980). To detect Lso in psyllids, psyllid DNA was obtained following the squash protocol (Bertolini et al., 2014). Then, Lso was detected by real-time PCR in plant and psyllids using the primers, TaqMan probe and procedure described by Bertolini et al. (2015).

Transmission of Lso by *Bactericera tremblayi*.

Adult psyllids of *B. tremblayi* were tested for both the acquisition of Lso from carrots and the inoculation of carrots with Lso. Groups of psyllids were exposed to Lso-infected carrot plants for an acquisition access period (AAP) of 72 h. Then, the psyllids were removed from carrots and transferred to leek plants for 15 d (latency period of Lso). After the 15 d, 400 psyllids were collected from the leek plants and transferred to 100 healthy carrot plants, each contained in a transparent, plastic cylindrical cage (4 psyllids/plant). The psyllids had access to the entire plant for an inoculation access period (IAP) of 24 h. Later, the psyllids were removed and tested individually using real-time PCR. Plants exposed to groups of insects that tested negative for Lso by real-time PCR (70 plants) were discarded from the analysis. Plants exposed to groups of insects that tested positive for Lso (30 plants) were sprayed with 1 g l⁻¹ of Confidor ® (Bayer, Kansas City, MO, USA) on days zero and 10 and were maintained under greenhouse conditions for eight weeks to test for Lso by visual inspection of symptoms and by real-time PCR.

Settling preference, oviposition and Lso transmission on different hosts.

Non-choice assays.

The assays used a set of three cages with each cage containing one treatment. The following three treatments were evaluated: (T1) 36 carrot plants, (T2) 36 celery plants and (T3) 36 potato

plants. Each treatment was replicated three times, and the replicates were rearranged to minimize location effects. Each cage contained potted plants arranged in a square (six rows and six columns, with 12.5 cm between plants). The cages were $1 \times 1 \times 1$ m and covered with an aphid-proof mesh net. On a flight platform similar to that described by (Feres et al., 1999), two hundred Lso-infected individuals of *B. trigonica* were released. The flight platform was placed 0.5 m above the test plants inside each cage. Insects were released at solar noon in greenhouse conditions similar to those described for insect rearing. All cages were rotated 180° daily to avoid orientation bias. The percentage of psyllids settled per test plant was determined, and the eggs per plant were counted 72 h after psyllid release. Settling preference was calculated as the percentage of insects settled on plants per cage. The percentage of plants infested by at least one insect was also calculated. Then, test plants were sprayed with 1 g L^{-1} of Confidor® (Bayer, Kansas City, MO, USA) and re-sprayed 10 d later to avoid further Lso transmission. Plants were maintained in a separate glasshouse under greenhouse conditions. Eight weeks after the experiment was completed, the percentage of plants infected with Lso was evaluated by visual inspection of symptoms and by real-time PCR as described above.

In a separate assay, the percentage of infested plants, settling and oviposition of *B. tremblayi* were also evaluated using a similar procedure to that described for *B. trigonica*. In these assays, the following treatments were evaluated: (T1) 36 leek plants, (T2) 36 carrot plants, and (T3) 36 celery plants. Because of the lack of transmission shown by *B. tremblayi* in the previous transmission experiments (see results section), the individuals of *B. tremblayi* used in this assay and in the dual-choice assay were Lso-free. Thus, Lso transmission was not evaluated for *B. tremblayi*. This assay was also repeated three times, and the parameters evaluated were identical to those described for *B. trigonica*.

Dual-choice assays.

For these assays, we used a similar procedure to that described for the non-choice assays. However, each experimental arena contained a combination of two different plant species (18

plants of each species were alternately arranged in a square 6 x 6 layout, with 12.5 cm between plants) (Supplementary material Fig. S1). To assess the preference of *B. trigonica*, the assay included three treatments of the following plant combinations, with each in a separate cage: (T1) carrot-celery, (T2) celery-potato and (T3) potato-carrot. Additionally, a separate assay was performed to assess the host plant preference of *B. tremblayi*. This assay included the following treatments: (T1) carrot-leek, (T2) carrot-potato, and (T3) leek-potato. The parameters that were evaluated and the methodology used in this assay were identical to those described for the non-choice assays.

Statistical analyses.

All behavioural variables were tested for normality using the Shapiro-Wilk W-test and were transformed when required by either $\sqrt{x+1}$ or $\ln(x+1)$. Comparisons among EPG treatments were performed using one-way ANOVA for Gaussian variables or with Kruskal-Wallis tests when normality was not achieved. For the preference assays, the percentage of insects settled per cage, the percentage of plants infected with Lso and the percentage of infested plants were transformed by $\arcsin\sqrt{x}$ when required to reduce heteroscedasticity and achieve normality. Following transformation, values were rechecked for normality using Shapiro-Wilk W-tests, and then the means for each treatment were compared using one-way ANOVA for the non-choice tests. For the dual-choice tests, pairwise comparisons between combinations of plants in each treatment were performed with a Student's t-test or with a Mann-Whitney U-test when normality was not achieved. Data were analysed using the SPSS 21 statistical software package (IBM Corp).

Results

Probing behaviour of *Bactericera trigonica* and *Bactericera tremblayi* on different hosts.

Stylet penetration process of *B. trigonica* and *B. tremblayi* in different host plants was monitored using the electrical penetration graph technique (EPG). The probing duration per insect (PDI) showed no differences between carrot and celery but was shorter when *B. trigonica* probed on potato than on carrot or celery, ($F=7.17$, $df=2$, 47 , $P=0.002$) (Table 1). In contrast to the PDI, the time from the first probe to the first phloem salivation (E1) was longer for insects probing on potato than for those on carrot or celery ($F=8.10$, $df=2$, 47 , $P=0.001$) (Table 1). *Bactericera trigonica* spent more time in non-probing activities (NP) on potato compared with celery or carrot ($F=5.71$, $df=2$, 47 , $P=0.006$). The duration of the stylet pathway (C) and the time to the first probe from the start of the EPG were not significantly different when *B. trigonica* probed on carrot, potato or celery (Duration of C: $F=0.97$, $df=2$, 47 , $P=0.385$; Time to the first probe from the start of EPG: $H=2.88$, $df=2$, $P=0.236$) (Table 1). On the other hand, *B. trigonica* probed in similar proportions from the xylem tissues (G waveform) of celery and potato but probed more from xylem of carrot compared to potato. (Number of G: $H=12.09$, $df=2$, $P=0.002$). Additionally, *B. trigonica* failed to reach the phloem tissues of potato plants because no phloem salivation (E1) or ingestion (E2) waveforms were detected. By contrast, E1 and E2 waveforms were commonly detected when *B. trigonica* probed carrot or celery (Table 1).

For *B. tremblayi*, the time to the first probe from the start of the EPG was different between leek and potato but not between leek and carrot or when comparing carrot and potato ($H=10.38$, $df=2$, $P=0.006$) (Table 1). The duration of stylet pathway (C) was similar in all plants tested ($F=2.00$, $df=2$, 47 , $P=0.140$). The duration of non-probing (NP) and the time from the first probe to the first phloem salivation (E1) were significantly shorter in leek than those in carrot or potato and no differences were observed between carrot and potato (Duration of NP: $H=23.85$, $df=2$, $P=0.000$; Time from first probe to first E1: $H=17.87$, $df=2$, $P=0.000$) (Table 1). Probing

duration per insect (PDI) was higher in leek than that in carrot or potato and no differences were detected between carrot and potato ($F=9.71$, $df=2$, 47 , $P=0.000$) (Table 1). Data showed that EPG variables related to xylem activities were similar among all species of plants tested (Number of G: $H=1.81$, $df=2$, $P=0.403$; Duration of G: $F=0.30$, $df=2$, 47 , $P=0.739$) (Table 1). Moreover, *B. tremblayi* reached the phloem tissues of all plants tested but showed a clear preference to probe longer and to spend more time in phloem-associated waveforms on leek than on carrot or potato (Number of E1: $H=21.27$, $df=2$, $P=0.000$; Duration of E1: $H=28.94$, $df=2$, $P=0.000$; Duration of E2: $H=30.50$, $df=2$, $P=0.000$) (Table 1). Additionally, although not statistically significant, *B. tremblayi* reached the phloem salivation phase (E1) more frequently on carrot than on potato (Table 1). Furthermore, *B. tremblayi* ingested phloem sap (E2) from carrots but failed to ingest phloem sap from potatoes (Table 1).

Transmission of Lso by *Bactericera tremblayi* from carrot to carrot.

Lso transmission efficacy by *B. tremblayi* from carrot to carrot was assessed in greenhouse tests. Only 11% of the *B. tremblayi* individuals acquired Lso (44 Lso positive insects/400 insects analysed) after 72 h exposure to Lso-infected carrots. Notably, after a 15 d post-acquisition period, none of the psyllids that acquired Lso inoculated healthy carrot plants with the bacteria (0 infected plants/30 receptor plants with at least one infected psyllid per plant). Therefore, although the results showed clearly that *B. tremblayi* acquired the bacterium, the pathogen was not transmitted to carrots by this species of psyllid.

Settling preference, oviposition and Lso transmission on different hosts.

***Bactericera trigonica*.**

Settling preferences and oviposition on carrot, celery and potato as well as Lso transmission by *B. trigonica* were evaluated in non-choice and dual-choice assays. Adult *B. trigonica* did not

show a preference between the Apiaceae plants tested in the non-choice and dual-choice assays. In both assays, the settling and oviposition preference of *B. trigonica* was similar for carrot and celery (Fig. 1a,b,e,f). However, settling and oviposition were significantly reduced on potato compared with carrot or celery in both the non-choice and dual-choice assays (Fig. 1a,b,c,d,g,h). The percentage of infested plants was also higher for carrot or celery than that for potato, but when comparing carrot and celery in both non-choice and dual-choice assays, no differences in the percentage of infested plants were observed (Supplementary material Table S1).

The transmission of Lso to the different plants tested in both non-choice and dual-choice assays was different for *B. trigonica* (Fig. 2). In non-choice tests, *B. trigonica* did not transmit Lso to potatoes (Fig. 2a). By contrast, *B. trigonica* efficiently transmitted Lso to carrot and celery, which showed similar percentages of transmission (Fig. 2a). In potato-carrot and potato-celery treatments in the dual-choice assay, Lso transmission was always significantly higher to carrot or celery than to potato (Fig. 2c,d). However, in the carrot-celery treatment, Lso transmission was not significantly different between the two plant hosts (Fig. 2b).

***Bactericera tremblayi*.**

Settling and oviposition of *B. tremblayi* on leek, carrot and potato were also evaluated in non-choice and dual-choice assays. Settling preference of *B. tremblayi* was significantly different among all host plants tested in the non-choice and dual-choice assays (Fig. 3). Our data indicated that *B. tremblayi* preferred to settle and oviposit on leek compared with carrot or potato in both types of assay (Fig. 3a,b,c,d,g,h). Additionally, *B. tremblayi* preferred to settle and oviposit on carrot compared with potato (Fig. 3a,b,e,f). Compared with potato, a higher percentage of leek or carrot were infested, although no differences were detected between carrot and leek (Supplementary material Table S2).

Discussion.

Lso is a persistently transmitted pathogen that has a close relationship with the psyllid vectors (Cicero et al., 2016a,b). For successful transmission, the stylet of Lso infected psyllids must reach and inoculate infected saliva into the phloem tissues of a susceptible host plant (Sandanayaka et al., 2014; Mustafa et al., 2015; Munyaneza et al., 2016). Because of this, the study of psyllid host preferences and stylet penetration process is fundamental for a complete understanding of the epidemiology of Lso in different crops. In this work, we showed that settling, oviposition and feeding behaviour patterns were related to the propensity of the vectors *B. trigonica* and *B. tremblayi* to transmit Lso to Apiaceae or potato plants, respectively.

In this study, *B. trigonica* clearly preferred to settle and oviposit on Apiaceae compared with potato plants. Settling and oviposition of *B. trigonica* on potato was very low, even when other plant species were not available (non-choice assay). The EPG data were consistent with this marked preference pattern for Apiaceae by *B. trigonica* and demonstrated that *B. trigonica* engaged in sustained feeding from the phloem of carrot and celery but failed to reach and feed from the phloem of potato plants. Since psyllids use the phloem sap as the primary source of sugars and amino-acids (Hodkinson, 1974), feeding from phloem tissues is fundamental for the reproduction and the ability of a psyllid species in order to colonize a given host. Therefore, this information suggests that *B. trigonica* cannot colonize potato because it could not continuously feed from the phloem tissues of potato plants.

On the other hand, phloem feeding is an absolute prerequisite for transmission, thus only colonizing vectors capable of sustained phloem feeding can efficiently spread phloem-restricted pathogens (Irwin et al., 2007; Moreno-Delafuente et al., 2013). Actually, inoculation success is directly correlated with the duration of the salivation phase performed by the vector just before phloem ingestion (Jiang et al., 2000; Mustafa et al., 2015). Our results support these

assumptions because high Lso transmission rates were detected in plants on which *B. trigonica* settled for long periods and salivated and ingested from the phloem. However, null or very low Lso transmission was obtained for potato on which *B. trigonica* showed poor settling, low oviposition and neither salivation nor phloem ingestion. Interestingly, despite no phloem related activities were observed by EPGs in an 8 h period, a very low percentage of potato plants tested positive for Lso in the dual-choice assays. This result suggest that eventually a few individuals of *B. trigonica* may have reached the phloem of potato in the extended 72 h period used in our dual-choice assays.

According to the results of this study, the risk of Lso transmission mediated by *B. trigonica* from Apiaceae to potato would be very low. Although highly infected carrot and celery crops commonly overlap in growing zones with potato, our data suggest that *B. trigonica* may land on but would not settle or feed from the phloem of potato. This result is consistent with few *B. trigonica* captured in potato crops growing near to carrot crops (Teresani et al., 2015). With these conditions, the primary transmission of Lso mediated by *B. trigonica* would be very unlikely. Moreover, if primary transmission does occur, secondary dispersion from the infected potato to other potatoes is likely be very low because *B. trigonica* cannot feed in a sustained way from the phloem tissues and therefore cannot colonize potato.

Generally, the results of this work were consistent with and complements well the work performed by Munyaneza et al. (2016). They showed that the risk of Lso transmission from potato to carrot is negligible because the potato psyllid *B. cockerelli* is not able to efficiently localize and feed from the phloem tissues of carrot plants. In our study, the risk of movement of Lso mediated by *B. trigonica* from carrots to potato was very low for a similar reason. However, assessing the risk of cross transmission from carrot to potato in Europe is complex because more than one vector could be involved in the transmission of this pathogen. For

example, the role of *T. apicalis* in cross transmission from carrot to other economically important crops in northern Europe remains unknown and requires further investigation.

Additionally, other psyllid species catalogued as potential vectors of Lso should receive special attention, e.g., *B. tremblayi* or *B. nigricornis* (Teresani et al., 2015). Our data showed that *B. tremblayi*, preferred to settle, oviposit and feed on leek but also settled and fed from phloem on carrots. This result was unexpected because the attempts to rear *B. tremblayi* on carrots were unsuccessful, and to our knowledge, the life cycle of this psyllid cannot be completed on carrot (Hodkinson, 2009). However, for psyllids, adults and nymphs may display different host plant specificity, and adults of some psyllid species may feed temporarily from plant species unsuitable for nymphal development (Burckhardt et al., 2014). Based on this scenario, our results suggest that in the absence of a plant suitable for reproduction, adults of *B. tremblayi* might use carrot as a temporary food plant. The EPG results support such use because *B. tremblayi* also ingested phloem sap from carrots. Although feeding from phloem was correlated with the capacity of this species to acquire Lso, we also showed that this psyllid species did not transmit the bacterium to carrot. Lack of transmission could be explained by different factors. For example, it has been reported that when Lso is acquired, the bacteria must pass through the insect midgut epithelium to infect the haemolymph and then enter the salivary glands (Cicero et al., 2016ab; Sengoda et al., 2014). It is uncertain if Lso is able to complete circulation in the body of *B. tremblayi* since this process has not been studied in this psyllid species. Finally, in this Lso-psyllid interaction, it is possible that longer latency periods than normal are required for the replication of Lso in the psyllid body, which provides another explanation for the absence of transmission. However, we did not test for latency periods in *B. tremblayi*, and the only reported latency period for Lso is in *B. cockerelli* (Sengoda et al., 2014); therefore, additional research is recommended to fully understand the Lso infection process in this psyllid species.

In conclusion, our results suggest that the host plant preferences of *B. trigonica* strongly influences the host range of Lso in the Mediterranean region. As a consequence, the risk of Lso transmission from carrot to potato mediated by *B. trigonica* is negligible, but tests of the transmission ability of other psyllid species that feed on potato might identify other risks. Additionally, we concluded that the hypothesis was not supported that *B. tremblayi* is a competent vector of Lso.

Figures

Figure 1. Settling and oviposition preferences of *B. trigonica* on potato, carrot and celery in non-choice assays: (a) Percentage of insects settled and (b) Number of eggs per plant. Different letters represent significant differences among treatments according to one-way ANOVA. Settling and oviposition preferences of *B. trigonica* in dual-choice assays: (c) Percentage of insects settled on celery and carrot; (d) Number of eggs per plant on celery and carrot; (e) Percentage of insects settled on celery and potato; and (f) Number of eggs per plant on celery and potato. Different letters represent significant differences according to Student's *t*-tests or Mann-Whitney U-tests. Error bars represent standard error.

Figure 2. (a) Percentage of infected plants when *B. trigonica* transmitted Lso in no-choice assays. Different letters represent significant differences according to one-way ANOVA. Percentage of infected plants when *B. trigonica* transmitted Lso in dual-choice assays: (b) Carrot-celery, (c) Potato-carrot, and (d) Potato-celery. Different letters represent significant differences according to Student's *t*-tests.

Figure 3. Settling and oviposition preferences of *B. tremblayi* on leek, carrot or potato in non-choice assays. (a) Percentage of insects settled and (b) Number of eggs per plant. Different letters represent significant differences among treatments according to one-way ANOVA. Settling and oviposition preferences of *B. trigonica* in dual-choice assays: (c) Percentage of insects settled on potato and leek; (d) Number of eggs per plant on potato and leek; (e) Percentage of insects settled on potato and carrot; (f) Number of eggs per plant on potato and carrot; (g) Percentage of insects settled on leek and carrot; and (h) Number of eggs per plant on leek and carrot. Different letters represent significant differences according to Student's *t*-tests. Error bars represent standard error.

Tables

Table 1. Means and SEs of probing behaviour variables for *Bactericera trigonica* on carrot, celery and potato and for *Bactericera tremblayi* on leek, carrot and potato. Different letters indicate significant differences among columns according to ANOVA or Kruskal-Wallis tests. Waveform definitions: **C**, intercellular stylet pathway; **G**, xylem ingestion; **E1**, salivation into phloem sieve elements; **E2** ingestion from sieve elements; and **NP**, non-probing. Number of waveforms performed by insect (NWEI); Waveform duration performed by insect (WDI); Probing duration per insect (PDI); Waveform duration per event by insect (WDEi).

	<i>B. trigonica</i>					<i>B. tremblayi</i>			
	EPG Parameter	Celery (n=17)	Carrot (n=18)	Potato (n=15)	P value	Leek (n=18)	Carrot (n=17)	Potato (n=15)	P value
Activities prior to vascular tissues	Time to 1 st probe from start of EPG	16.42 ± 6.74 A	12.30 ± 3.70 A	26.72 ± 5.99 a	0.236	5.85 ± 1.26 A	21.63 ± 9.84 ab	42.02 ± 9.26 b	0.006
	Duration of C (WDI)	163.02 ± 19.25 a	201.05 ± 21.73 a	202.25 ± 27.42 a	0.385	163.10 ± 27.14 a	238.44 ± 34.11 a	226.83 ± 24.74 a	0.140
	Duration of NP (WDI)	180.75 ± 20.25 a	140.57 ± 16.07 A	248.58 ± 27.42 b	0.006	54.60 ± 20.12 A	214.60 ± 25.28 b	226.07 ± 24.71 b	0.000
	Probing duration per insect (PDI)	299.21 ± 20.25 a	335.97 ± 18.89 a	219.44 ± 25.87 b	0.002	425.39 ± 20.12 a	293.62 ± 38.73 b	253.91 ± 24.71 b	0.000
	Time from 1 st probe to 1 st E1	249.41 ± 44.61 a	300.86 ± 35.39 a	445.23 ± 8.47 b	0.001	177.26 ± 35.09 a	397.97 ± 37.11 b	435.62 ± 8.82 b	0.000
Xylem activity	Number of G (NWEI)	1.17 ± 0.28 ab	1.50 ± 0.13 b	0.53 ± 0.16 a	0.002	1.00 ± 0.05 a	1.23 ± 0.16 a	1.00 ± 0.16 a	0.403
	Duration of G (WDI)	44.19 ± 12.45 Ab	50.00 ± 11.11 B	17.18 ± 5.14 c	0.040	26.69 ± 3.19 A	34.99 ± 4.59 a	33.85 ± 4.93 a	0.739
Phloem Activity	Number of E1 (NWEI)	2.47 ± 0.76 a	1.88 ± 0.44 a	0 c	0.000	2.55 ± 0.50 a	1.35 ± 0.68 b	0.06 ± 0.06 b	0.000
	Duration of E1 (WDI)	25.36 ± 9.60 a	6.11 ± 2.43 a	0 c	0.000	15.52 ± 3.56 a	0.66 ± 0.38 b	0.07 ± 0.01 b	0.000
	Number of E2 (NWEI)	2.05 ± 0.66 a	1.61 ± 0.39 a	0 c	0.000	2.1 ± 0.36 a	1.35 ± 0.68 b	0 c	0.000
	Duration of E2 (WDI)	66.62 ± 21.15 A	78.80 ± 20.61 A	0 c	0.008	220.06 ± 18.33 a	25.59 ± 34.58 b	0 c	0.000

Supplementary material

Supplementary figures.

Figure S1. Scheme of the experimental design used in the two-choice assays for (A) *B. trigonica* and (B) *B. tremblayi*.

A.

B.

Supplementary tables.

Table S1. Percentage of host plants infested by at least one psyllid of *B. trigonica* or *B. tremblayi* in non-choice assays. Different letters represent significant differences among treatments according to one-way ANOVA.

Non-choice	Potato	Carrot	Celery	P value
<i>B. trigonica</i>	25.00 ± 8.48 a	65.74 ± 7.91 b	49.07 ± 3.33 b	0.017
	Potato	Carrot	Leek	
<i>B. tremblayi</i>	21.29 ± 7.57 a	51.85 ± 3.33 b	57.40 ± 8.07 b	0.018

Table S2. Percentage of host plants infested by at least one psyllid of *B. trigonica* or *B. tremblayi* in dual-choice assays. Different letters represent significant differences among treatments according to Student's t-tests.

Dual-choice	T1	P value	T2	P value	T3	P value
<i>B. trigonica</i>	Carrot = 72.22 ± 1.85 a	0.613	Celery = 66.66 ± 8.48 a	0.051	Potato = 24.07 ± 16.45 a	0.007
	Celery = 66.66 ± 8.48 a		Potato = 18.51 ± 5.55 b		Carrot = 74.07 ± 11.26 b	
	T1	P value	T2	P value	T3	P value
<i>B. tremblayi</i>	Carrot = 37.03 ± 4.89 a	0.550	Potato = 18.51 ± 4.89 a	0.007	Leek = 66.66 ± 0.00 a	0.250
	Leek = 59.25 ± 6.67 a		Carrot = 55.55 ± 4.55 b		Potato = 27.77 ± 11.11 b	

CHAPTER VI. Seasonal abundance of psyllid species associated with carrot and potato fields in Spain.

Introduction

Vegetable crop production plays a major role in European Union agriculture, accounting for 13.6 % of its agricultural output. Carrots (*Daucus carota* L.) and potato (*Solanum tuberosum* L.) are among the most important vegetable products in the EU. The whole carrot production in the EU is estimated at 5.1 million tonnes per year and potato production is around 59 million tonnes per year. In recent years, psyllids (Hemiptera: Psylloidea) have emerged as important pests that threaten carrot and potato production in different regions of the world.

In Europe two different psyllid species affect carrot production. *Trioza apicalis* Foester 1848, in northern Europe and *Bactericera trigonica* Hodkinson 1941, in the Mediterranean region where it also affects celery. Both psyllid species cause direct damage to carrot and are also vectors of the phloem restricted bacterium ‘*Candidatus Liberibacter solanacearum*’ (Lso) (Alfaro-Fernández et al., 2012a,b; Munyaneza et al., 2010a). In carrots, the main symptoms of Lso are purple or yellow leaf discoloration and root reduction (Munyaneza et al., 2010a). The incidence of Lso infected plants ranges from 50 to 90% in carrot fields in Spain (Alfaro-Fernández et al., 2012a). So far, Lso has been associated to Apiaceae crops in Europe; however it is well known that this bacterium affects Solanaceae plants in America and New Zealand (Liefting et al., 2009). In this zones Lso is specially important in

potato where causes a vegetative disorder called “zebra chip” (Munyanzeza et al., 2007; Munyanzeza, 2012). This disease is characterized by a stripped pattern of necrosis in tubers that become more evident when chips are fried; as a result potato chips processed from infected tubers are commercially unacceptable (Munyanzeza, 2012). Due to this, the disease has caused the abandonment of entire potato fields and millionaire losses to the potato industry (Munyanzeza et al., 2007, 2008; Munyanzeza, 2012).

In Solanaceae Lso is transmitted by the potato-tomato psyllid *Bactericera cockerelli* that is an endemic pest of the Americas and recently introduced in New Zealand (Hansen et al., 2008; Teulon et al., 2009; Munyanzeza, 2012). *Bactericera cockerelli* does not overlap geographical distribution with the carrot psyllids *B. trigonica* or *T. apicalis* currently found in Europe (Munyanzeza, 2012; Burckhardt and Ouvrard, 2014;). Accordingly, despite of carrot and potato crops are commonly grown close to each other, experimental evidence suggest that risk of transmission of Lso from carrot to potato mediated by *B. trigonica* is very low (Antolínez et al., 2017a). Nevertheless, cross transmission of Lso from carrot to potato is still a big concern for potato producers in Europe (Soliman et al., 2013)

On the other hand, despite the economical losses associated with carrot psyllids in Mediterranean countries, population dynamics of these insects are poorly documented. This information is essential to minimize Lso incidence in field, because Lso management is primary based on the control of vector populations (Goolsby et al., 2007; Gharalari et al., 2009; Munyanzeza, 2012). Moreover, the timing of insecticide application is determined based on information of psyllid arrival into a crop as well as psyllid population peak density (Tsai et al., 2002; Goolsby et al., 2007; Munyanzeza et al., 2009). To date, just one study has monitored psyllid populations in mainland Spain, reporting the population

dynamics of (*Bactericera trigonica*, *Bactericera tremblayi* and *Bactericera nigricornis*) in celery and population dynamics of *B. trigonica* in carrots in Tenerife (Teresani et al., 2015). That study also reported occasional surveys in potato and carrot where *B. trigonica* and *B. nigricornis* were found on both crops. Therefore, to date monitoring of psyllid populations during the complete carrot and potato-growing season has not been reported in mainland Spain. In order to fill this knowledge gap our study aimed to monitor the population dynamics of psyllids associated with carrots and potato crops in Spain using different sampling methods.

Materials and methods

Sampling locations.

Adult psyllids were collected from plots in carrot and potato commercial fields using a sweep net in one sampling in the summer of 2015 and by both both sweep net and green tile traps during the whole 2016 growing season. Carrot and potato plots sampled in 2015. Carrot fields used for psyllid sampling in 2016 were located in: Gomezserracín (Segovia, Spain), Íscar (Valladolid, Spain) and Villena (Alicante, Spain). Potato fields used for sampling in 2016 were located in Gomezserracín and Aldearrubia (Salamanca, Spain). Sampling was conducted every two weeks from emergence to harvest. Details of field locations plot location and varieties grown are shown in Table 1.

Sweep net sampling.

The carrot and potato canopy were sampled for adult psyllids using a telescopic folding sweep net. (NHBS Ltd., 1-6 the Stables, Fort Road Totnes, UK). Each sample consisted of ten consecutive sweeps along a surface of 2 m² in ten different points, to cover a total distance of 100 m, obtaining a total of ten samples per plot per sampling date. Quadrants where psyllids were sampled were not treated with insecticides. Sweep net samples were transferred to plastic ziplog® bags, then were brought to the laboratory of Insect Vectors of Plant Pathogens at ICA-CSIC in Madrid-Spain, and frozen at -20°C until taxonomic identification.

Horizontal green tile traps

Horizontal green water tile traps (Irwin traps) were selected for this study because they are a neutral traps similar to the canopy background color and were designed to specifically collect adult insects that land on a given row crop such as soybeans (Irwin, 1980, 1986). Traps used in this study consisted of a methacrylate container (16.5 × 16.5 × 4.5 cm) and a square ceramic tile (15.5 × 15.5 cm), (Cambridge 815 from Cambridge tile C., PO Box 15071, Cincinnati, OH 46215, USA) which was placed inside the container. This green ceramic tile has an absorbance spectrum similar to the soybean canopy but has been used to monitor aphids landing on several other row crops such as pepper, lettuce and broccoli (Summers et al., 1990; Kirk et al., 1991; Perez et al., 2003; Nebreda et al., 2004). The container was filled with a 50% solution of ethylene glycol in water. The trap was attached to a one-meter vertical metal rod with a two-ended chemistry clamp; the metal rod was

inserted 20 cm into the ground. A second container was placed below the tile in order to avoid losing insect samples after rain. The trap was placed at canopy level; insects captured were collected every two weeks and the solution in the container was changed at the same interval. The trap content was filtered using a funnel, then insects were preserved in ethanol 70% until taxonomic identification. Only insects from the Psylloidea superfamily were identified to species level. One trap per sampling site was used except for the carrot plot in Villena where two traps were used.

Visual inspection

In 2016 twenty plants were visually inspected for eggs and nymphs at each sampling site at two-week intervals from seedling to harvest growth stage in carrot fields. Plants were selected by walking in a zig-zag pattern in a diagonal across the field covering a distance of 100 m. A scale of 0-4 was used to describe nymph and egg density: 0 = 0; 1= 1-4; 2=5-20; 3=21-50; 4=more than 50.

Psyllid identification

Adult psyllids collected by sweep net or green tile traps were identified to species level based on morphological characteristics following taxonomical keys of Burckhardt and Freuler (2000) and Hodkinson (1981). A batch of identified insects and other individuals where morphological identification was not achieved to species level were tested by molecular tools. Molecular identification was performed at Science & Advice for Scottish

Agriculture (SASA) (Edinburgh, United Kingdom). First, psyllid DNA was extracted using the a non destructive method (Percy, 2003). In this protocol, the insect was pierced through the thorax and abdomen using a 0.14 steel pin and the exudate collected and using the DNeasy Blood & Tissue Kit (QUIAGEN), following manufacturer's instructions. New pins were used for each psyllid. Psyllid exoskeletons were preserved in 95% ethanol and 5% glycerol and then stored in plastic vials as voucher specimens. Two regions of psyllid DNA were amplified: Cytochrome oxidase I (COI) region using primers LCO1490 and HCO2198 according to (van de Vossenberg et al., 2013) and the internal transcribed region 2 (ITS2) using primers CAS5p8sFcm and CAS28sB1d according to (Peccoud et al., 2013). Thereafter, amplified regions were sequenced using the Sanger method. Sequences were assembled and then aligned with previously reported ITS2 and COI regions of different psyllid species using the Muscle algorithm (Edgar, 2004). Finally, a neighbor-joining tree was obtained using PAUP 4.0b, in order to identify in which cluster of psyllid species the obtained sequences were located.

Results

Sweep net sampling

B. trigonica was the most abundant species found in all of the carrot fields sampled in 2015 (Table 2) and 2016 (Fig. 1a,b,c) *B. trigonica* was observed since the first sampling dates in Gomezserracín (Segovia) (Late April 2016) (Fig. 1a) and Íscar (Valladolid) (Late May 2016) (Fig 1b). In contrast, *B. trigonica* in Villena (Alicante) was not found until the third sampling date in Mid May 2016 (Fig. 1c). Maximum population peaks of *B. trigonica*

occurred in the first days of August 2016 in Gomezserracín (Segovia) (7 psyllids per sweep) (Fig. 1a), the first days of October 2016 in Íscar (Valladolid) (33 psyllids per sweep) (Fig. 1b) and last days of August 2016 in Villena (Alicante) (1.6 psyllids per sweep) (Fig. 1c). Furthermore, adults of *B. nigricornis* were found on carrots at low levels (Fig. 1a,b,c) (Table 2). Maximum population peaks for *B. nigricornis* in Gomezserracín were observed in early June 2016 (0.3 psyllids per sweep) (Fig. 1 a), Early October (0.62 psyllids per sweep) in Iscar in 2016 (Fig.1b) and mid-July (0.088 psyllids per sweep) in Villena in 2016 (Fig 1c). Occasional catches of other psyllid species in carrot plots are provided in Table 3.

In the potato plot sampled in Gomezserracín in the year 2015, *B. nigricornis* was the dominant psyllid species (Table 2). In the two potato plots sampled by sweep net in 2016, adult psyllid populations were low and number varied significantly between sites. In spite of the low psyllid number of psyllids found, in Aldearrubia (Salamanca) *B. nigricornis* was the most abundant psyllid species showing the highest population peak in early July (0.13 psyllids per sweep) (Fig. 1d). Thereafter, the *B. nigricornis* population progressively declined and no adults were observed in mid and late August when the crop was harvested. Other psyllid species were occasionally found, among them *B. trionica* (Table 3).

Unexpectedly in the potato plot located in Gomezserracín *B. trionica* was the most abundant species, reaching its maximum population peak in mid September 2016 (0.34 psyllids per sweep) (Fig. 1e). Additionally, adults of *B. nigricornis* were also recorded throughout the growing season, reaching its maximum number in mid August 2016 (0.05 psyllids per sweep) (Fig. 1e). Occasional catches of other psyllid species in the potato plots are provided in Table 3.

Horizontal green tile trap sampling

Water tile traps showed *B. trigonica* to be the most dominant psyllid species in all carrot plots sampled. In Gomezserracín the first adults of *B. trigonica* were found in early May, with the population reaching a maximum population peak in mid June (141 psyllids per trap) followed by a decrease in late June and then a gradual increase with similar numbers through July followed by an increase in early August (Fig. 2a). *B. nigricornis* was sporadically detected at very low numbers (Fig. 2a). In the plot located in Íscar, *B. trigonica* was observed at the first sampling followed by a first population peak in early August (47 psyllids per trap) and then by a second population peak in early September (54 psyllid per trap) (Fig. 2b). *B. nigricornis* was only observed at very low numbers from May to mid August (Fig 2b). Both green tile traps located in Villena showed similar trends with very low captures of *B. nigricornis* and maximum population numbers of *B. trigonica* in September (Fig. 2c,d). Very low numbers of other psyllid species were observed in the carrot plots sampled (Table 3).

Water tile traps located in potato plots showed *B. nigricornis* to be the dominant species in both potato plots. In the potato plot located in Aldearrubia, *B. nigricornis* were consistently observed from May to August, showing a maximum peak in mid June (7 psyllids per trap) (Fig. 2e). *B. trigonica* was not observed at this location (Fig. 2e). Other psyllid species were recorded in very low numbers (Table 2). In the potato plot located in Gomezserracín, the maximum peak of *B. nigricornis* occurred in early June (39 psyllids per sweep) (Fig. 2f). *B. trigonica* was also observed in this location from early August to September, showing a maximum peak in mid-August (9 psyllids per trap) (Fig. 2f). The complete list of occasional catches of other psyllid species observed in potato is provided in Table 3.

Visual inspection

The number of eggs and nymphs sampled in carrot plots are shown in Fig. 3. Overall egg and nymph densities increased gradually to reach the highest densities some days before the maximum population peaks shown by adults: early August in Gomezserracín and early September in Íscar and mid to late September in Villena. Due to the low numbers of psyllids present in both potato plots, visual inspection was not performed.

Discussion

Previous studies have shown that only the psyllid species able to colonize a given crop are able to efficiently transmit Lso (Antolínez et al., 2017a; Munyaneza et al., 2016). Accordingly, the knowledge of the population dynamics of psyllids in carrot and potato crops in Europe is important to understanding Lso spread the field and assessing the potential risk of Lso introduction in potato. In this work, we showed that *B. trigonica* and *B. nigricornis* were associated with carrot crops and *B. nigricornis* was consistently associated with potato crops.

In the carrot plots we observed all psyllid life stages (eggs, nymphs and adults) and despite that the identification at species level was performed with adults only, is highly probable that both *B. trigonica* and *B. nigricornis* were colonizing the plots sampled. In fact, different previous studies have confirmed that both species can complete their life cycle in carrot (Hodkinson, 2009 and references therein). Our results are consistent with Teresani et

al (2015) who found *B. trigonica* as the predominant species in carrot and celery followed by low populations of *B. nigricornis*.

On the other hand, our results contrast with a previous report of psyllids collected in carrot fields in Valais Switzerland where *Trioza apicalis*, *B. nigricornis* and *Trioza anthrisci* were found to be the most abundant species (Burckhardt and Freuler, 2000). with *B. nigricornis* the most abundant species in two of the three carrot plots sampled. *B. trigonica* at very low populations. Different environmental conditions might explain differences in psyllid fauna between our study and the one performed by Burckhardt and Freuler. For example, climatic conditions in the Mediterranean region might favour species such as *B. trigonica* over other species such as *B. nigricornis*, *T. apicalis* or *T. anthrisci*. The latter two species, have not been reported in Spain and are commonly associated with Apiaceae plants in cooler and humid regions (Ossiannilsson, 1992; Burckhardt, 1985).

Also in carrots, adults of *B. trigonica* were detected since the first sampling dates, however it is not known if these adults were those that migrated to the production plots or were or were already present. For this species migration behavior has not been studied so far and it is still uncertain if adults overwinter on plants species different to those commonly reported as hosts. Burckhardt and Freuler (2000) suggested that this psyllid overwinters as an adult in conifers or evergreen shrubs (Burckhardt and Freuler, 2000). However, the possibility that in the Mediterranean region adults of this psyllid could be present the whole year in herbaceous plants in carrot producing zones should not be disregarded. Contrary to *B. trigonica*, *B. nigricornis* was absent in the first sampling dates suggesting migration from other hosts or zones to carrot plots. However, further research on the biological cycle of

this psyllid is needed to identify the overwintering hosts or migration habits in Mediterranean conditions.

Regarding to potato, our data consistently associated *B. nigricornis* with all the plots sampled. Despite this study did not provide direct evidence that this psyllid colonizes potatoes because eggs and nymphs were not observed in visual inspections, However, previous reports have confirmed that *B. nigricornis* is able to breed on potato where it can cause severe yield losses (Hodkinson, 1981; Fathi, 2011). Interestingly, to date *B. nigricornis* have not caused any economic damage to potato crops in Spain maybe due to its low populations. Nevertheless, monitoring its populations is highly recommended as a preventive measure to avoid potential outbreaks.

The high numbers of *B. trigonica* in one potato plot were unexpected because previous works have suggested that this species is restricted to Apiaceae plants and does not breed on potatoes (Hodkinson, 2009; Antolínez et al., 2017a). However, this species arrived late to the crop and no immature stages were observed. The arrival dates of *B. trigonica* to this potato plot matches the harvesting dates of carrot plots planted in the same zone. Thus, part of the high populations of *B. trigonica* recorded in carrots might have transiently moved to this potato plot when carrot was not available. Despite not being able to colonize potato, adults of *B. trigonica* might have the ability to survive for some time in non-host plants as has been reported for related psyllid species such as *B. cockerelli* (Munyaneza et al., 2016)

Overall, our results could be analyzed in the context of Lso transmission. Transmission of vector borne pathogens is affected several factors, among them vector abundance and vector efficiency are specially important (Irwin, 1986). High population densities of *B.*

trigonica in carrots reported here and evidence of high Lso transmission rates by *B. trigonica* provided by previous works (Antolínez et al., 2017a,b), could explain the high Lso incidences reported so far in carrot fields in Spain (Teresani et al., 2014). In the case of potato experimental evidence suggests a very low risk of Lso transmission by *B. trigonica* (Antolínez et al., 2017a). On the other hand, it is still unknown if *B. nigricornis* is able to transmit Lso to potato or carrot crops. Nevertheless adults of this insect have tested positive by real time PCR in field surveys (Teresani et al., 2015). This, added to its ability to colonize both potato and carrot suggests a potential for transmission of Lso from carrot to potato crops by *B. nigricornis*. Therefore, further research addressed to assess the transmission ability of Lso by *B. nigricornis* is needed.

The basic knowledge about population density of psyllids associated with carrot and potato crops provided here is important to the design of management practices to control psyllid populations and Lso transmission in the field. Also, information provided here is important to identify potential scenarios for Lso transmission to potato in Europe.

Tables

Table 1.

Table 1. Location and information of carrot and potato plots surveyed by sweep net and water green tile traps during 2016 in Spain.

Crop	Cultivar	Plot location	Province	Latitude	Longitude	Altitude
Carrot	Bangor	Gomezserracín	Segovia	47° 17'24''N	4° 19'32''W	804
	Bangor	Iscar	Valladolid	41°20'05'' N	4° 32'13''W	750
	Soprano	Villena	Alicante	38° 38'47'' N	0° 55'47''W	450
Potato	Monalisa	Aldearrubia	Salamanca	49° 59'11.2''N	5° 29'10.1''W	812
	Monalisa	Gomezserracín	Segovia			

1 **Table 2.**

2 Table 2. Occasional survey of adult psyllids associated to carrots and potato plots in Gomezserracín (Segovia) Spain conducted in July
3 of 2015.

Crop	Location	Sampling method	Psyllid species	Date of sampling	Total number of insects
Potato	Gomezserracín	Sweep net	<i>Bactericera nigricornis</i>	02/07/2015	5
Potato	Gomezserracín	Sweep net	<i>Bactericera tremblayi</i>	02/07/2015	3
Carrot	Gomezserracín	Sweep net	<i>Bactericera trigonica</i>	02/07/2015	20
Carrot	Gomezserracín	Sweep net	<i>Bactericera nigricornis</i>	02/07/2015	1

11 **Table 3.**

12 Table 3. Different psyllid species occasionally found in the carrot and potato plots surveyed in 2016.

Crop	Location	Sampling method	Psyllid species	Date of sampling	Number of insects
Carrot	Gomezterrachín	Sweep net	<i>Bactericera tremblayi</i>	Early July	1
Carrot	Gomezterrachín	Sweep net	<i>Bactericera tremblayi</i>	Mid July	1
Carrot	Iscar	Sweep net	<i>Bactericera tremblayi</i>	Late July	1
Carrot	Iscar	Sweep net	<i>Bactericera tremblayi</i>	Early September	1
Carrot	Villena	Sweep net	<i>Trioza urticae</i>	Mid May	2
Carrot	Villena	Sweep net	<i>Trioza remota</i>	Late October	1
Potato	Aldearrubia	Sweep net	<i>Bactericera trigonica</i>	Early July	1
Potato	Aldearrubia	Water tile trap	<i>Trioza urticae</i>	Early June	1
Potato	Aldearrubia	Water tile trap	<i>Trioza urticae</i>	Mid June	1
Carrot	Gomezterrachín	Water tile trap	<i>Bactericera tremblayi</i>	Mid May	1
Carrot	Gomezterrachín	Water tile trap	<i>Bactericera tremblayi</i>	Mid June	1
Carrot	Iscar	Water tile trap	<i>Bactericera tremblayi</i>	Late May	1
Carrot	Iscar	Water tile trap	<i>Bactericera tremblayi</i>	Early August	1
Carrot	Villena	Water tile trap	<i>Bactericera tremblayi</i>	Late May	1
Carrot	Villena	Water tile trap	<i>Bactericera tremblayi</i>	Early August	1

Figures

Figure 1. Population density of *B. trigonica* (dots and solid line) or *B. nigricornis* (triangles and dotted line) collected by sweep net in carrot plots, (a) Gomezserracín, (b) Íscar, (c) Villena or collected in potato plots, (d) Aldearrubia and (e) Gomezserracín. Y-axis values represent the number of insects per sweep and X-axis represents collecting dates in 2016.

Figure 2. Population density of *B. trigonica* (dots and solid line) or *B. nigricornis* (triangles and dotted line) collected by green tile traps in carrot plots, (a) Gomezserracin, (b) Iscar, (c) and (d) Villena or collected in potato plots in (e) Aldearrubia and (f) Gomezserracin. Y-axis values represent the number of insects per trap and X-axis represents collecting dates in 2016.

Figure 3. Number of eggs (Squares and solid line) or nymphs (diamonds and dotted line) in commercial carrot plots in (a) Gomezserracín, (b) Íscar and (c) Villena. X-axis represents collecting dates of 2016. Y-axis represent scale values used to estimate egg and nymph abundance. Scale for eggs and nymphs was: 0=0, 1=1-4, 2=5-20, 3=21-50, 4=more than 50.

CHAPTER VII. General Discussion

Understanding the epidemiology of vector borne plant diseases requires detailed knowledge of numerous biological factors such as plant, pathogen and vector biology as well as how these organisms interact with each other (Daugherty et al., 2010). While the relationships between pathogenic viruses, plant hosts and vectors such as aphids or whiteflies have been investigated in detail, plant pathogenic bacteria transmitted by vectors have received less attention.

Of the phytopathological problems in Europe, currently one of the most important is psyllid transmission of Lso, affecting species in the family Apiaceae and the potential risk that Lso poses to solanaceous crops particularly potato. The lack of information about European psyllids and the urgent need for effective management practices to reduce crop losses in Apiaceae crops has increased the interest in studying Lso as well as its transmission mediated by psyllids. This study was aimed to fill this gap in knowledge and its results are fundamental to the understanding of the epidemiology of Lso in Europe.

By monitoring the feeding behaviour of *B. trigonica* on carrots (Chapter three) this work clearly showed the feeding behaviour activities related to the transmission of Lso. One key finding was the association of Lso inoculation with the EPG waveform pattern E1 that represents salivation into the phloem sieve elements. This association is consistent with the restricted localization of Lso in phloem tissues as well as with the proposed circulative-propagative mechanism of transmission for this bacterium (Graca, 1991; Ammar et al., 2011a,b, Cicero et al., 2016a,b). Accordingly Lso is inoculated with the watery saliva secreted by the psyllid when the insect reaches the phloem sieve

elements before any sap ingestion (Sandanayaka et al., 2014; Mustafa et al., 2015; Munyaneza et al., 2016). The reason why hemipteran insects salivate into the sieve elements before sap ingestion is not completely understood but previous publications have suggested that components in the secreted saliva help to suppress wound responses triggered by the stylet penetration process into the plant (Tjallingii, 2006; Garzo et al., 2017). It is very likely that this feeding behaviour has favored the evolution of phloem restricted pathogens transmitted in a propagative-circulative manner (Perilla-Henao and Casteel, 2016).

Transmission tests performed in Chapter four confirmed the ability of single psyllids to effectively inoculate Lso in an IAP as short as 8 h. This transmission efficiency was even higher when the number of infected insects per plant increased, even with IAPs lower than 8 h. Thus, just a low number of infected insects feeding for a short time in a susceptible host could be responsible for significant transmission and spread of Lso in the field. Together, these results indicate that *B. trigonica* is a highly efficient vector in carrot. This should be taken into account in order to establish management practices to control Lso spread. While the most effective way to control Lso spread is by controlling vector populations (Gharalari et al., 2009; Munyaneza, 2012), results from this work suggests that only compounds or methods that avoid psyllid contact with phloem tissues would be effective to control Lso spread. Accordingly, management practices such as the use of resistant varieties to psyllid feeding are highly recommended (Buchman et al., 2011). Unfortunately, no resistant varieties have been identified so far for carrot or potato, but recently the use of RNAi technology to control *B. cocklerelli* in Solanaceae plants have been proposed as an alternative strategy (Wuriyanghan and Falk, 2013).

Several studies using different hemipterans insects have correlated the number and duration of phloem salivation with the transmission of different phloem restricted pathogens (Jiang et al., 2000; Moreno-Delafuente et al., 2013; Mustafa et al., 2015; Ning et al., 2015). Our data showed that females of *B. trigonica* significantly reached the phloem more times and tended to salivate longer than males thereby affecting the Lso titre transmitted by males and females. Thus, females of *B. trigonica* with high Lso titres inoculate higher titres than males. This differences may affect field transmission of Lso if the rate of natural populations of *B. trigonica* is higher for females than for males. Although Hodkinson (2009) reported equal sex ratios for males and females in psyllids, other studies suggest higher sex ratios for females than for males (Van der Berg et al., 1991; Chakrabarti and Chakrabarti, 2012). Unfortunately, neither the sex ratio of *B. trigonica* in natural populations and the affect of environmental conditions on these ratios are known. Thus, despite evidence suggesting that female psyllids show characteristics that could be advantageous for transmission such as increased longevity and higher dispersion capacity than males (Hodkinson, 2009; Chakrabarti and Chakrabarti, 2012; Martini et al., 2015; Ouvrard, 2017), further research is still needed to elucidate the real impact of vector sex in pathogen spread.

This work has clearly shown that the ability of a psyllid to colonize a host plant is fundamental for successful transmission of Lso. In chapter Chapter five our data showed that *B. trigonica* was a highly efficient vector of Lso in Apiaceae but failed to transmit Lso to potato plants. This was correlated with the incapacity of *B. trigonica* to breed on or feed from phloem tissues in potato plants. Because Lso is phloem restricted, only organisms able to reach phloem tissues to inoculate the bacteria could serve as vectors. As most psyllids species are commonly reported as highly host specific the

range of plant species naturally infected by species of *Candidatus Liberibacter* is restricted by the host range of the psyllid vector (Haapalainen, 2014).

Apart from feeding behaviour and the ability to colonize a host plant, the colonization of psyllids by Lso is also required for successful transmission. It is well known that *Liberibacter*s are able to colonize and replicate in both plant hosts and its psyllid vector (Haapalainen, 2014; Perilla-Henao and Casteel, 2016). Thus, psyllids can be considered as an alternative host for *Liberibacter* bacteria. This degree of specialization suggests a long-standing evolutionary relationship between the pathogen and its vectors that might also restrict the range of psyllid species able to transmit these pathogens. Accordingly, just psyllid species where Lso is able to circulate and propagate would be able to transmit the disease. Previous studies have suggested that insect anatomical barriers such as the midgut wall and salivary glands constitute significant barriers for bacterial propagation in the psyllid (Ammar et al., 2011a,b; Brown, 2016; Cicero et al., 2016a,b). This might explain why *B. tremblayi* failed to transmit Lso to carrots, despite being able to feed and acquire the bacterium from infected carrot plants as shown in Chapter five.

In addition to all the variables that may affect the natural probability of a vector to inoculating a plant with a pathogen (vector propensity), the number of vectors alighting on a plant (vector activity) is also fundamental to understanding the spread of vector-transmitted pathogens (Irwin & Ruesink 1986). Vector activity is very difficult to measure but it is commonly quantified by determining vector abundance. In Chapter six, vector abundance of *B. trigonica* and *B. nigricornis* associated with carrot and potato crops was described. Whereas *B. trigonica* and *B. nigricornis* can colonize carrot crops, only *B. nigricornis* is able to colonize potato. To date, only the vector capacity of *B. trigonica* has been shown and the vector capacity of *B. nigricornis* is unknown.

Therefore, it is not known if *B. nigricornis* is involved in the spread of Lso to carrots. For the case of *B. trigonica*, the high densities found in carrots in this Thesis are correlated with the high Lso incidences previously reported in carrot fields (Teresani et al., 2014). Even more interesting is the possibility of transmission from carrot to potato by *B. nigricornis* since in Europe this is the only psyllid able to breed on both plants (Burckhardt and Freuler, 2000; Hodkinson, 2009). *B. nigricornis* has been reported as a polyphagous psyllid, feeding from various herbaceous plants including Solanaceae and Apiaceae (Burckhardt and Freuler, 2000; Hodkinson, 2009; Fathi, 2011). Based on all these observations this Thesis suggests that *B. nigricornis* represent a potential threat to both carrot and potato in Europe. However, further research on this psyllid is needed including its capacity to transmit Lso.

CHAPTER VIII. Conclusions

1. Experimental evidence provided in this work supports the proposed mechanism of transmission suggested for Lso. Accordingly, Lso needs to reach the salivary glands of its vector and can be transmitted just when the vector penetrates a sieve element and discharges saliva inside the cells.
2. Lso infection directly influences the feeding behaviour of *B. trigonica*. However, behavioural changes induced at this level do not influence the vector transmission ability of Lso.
3. Differences in the feeding behaviour between males and females of *B. trigonica* are correlated with the titre of Lso in infected plants.
4. *Bactericera trigonica* is a highly efficient vector of Lso in Apiaceae but its vector ability in potato is very low. Accordingly, cross transmission from carrot to potato mediated by *B. trigonica* in the field is unlikely.
5. There is no evidence to support the hypothesis that *B. tremblayi* is an efficient vector of Lso. Further research aimed to compare the colonization process of Lso in *B. tremblayi* and *B. trigonica* could provide key information to understand the mechanism that Lso uses to colonize its vector host.
6. The high Lso incidences reported in field studies in Castilla-Leon and Alicante are consistent with the high abundance of *B. trigonica* observed on carrot fields. Furthermore, it is extremely important to identify if *B. nigricornis* is able to transmit Lso, since it is the only psyllid species able to colonize and reproduce in potato plants. Thus, this psyllid might represent a threat for cross transmission of Lso from carrot to potato and allow secondary spread of the bacteria in potato fields.
7. These findings on the feeding behaviour, transmission and population dynamics of psyllids on Apiaceae and potato crops have important implications on the epidemiology of Lso and provide key information for developing effective disease management strategies.

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