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Departamento de Producción Vegetal

'*Candidatus* Liberibacter solanacearum': detection, characterization, new hosts and epidemiology in Spain

PhD Thesis

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'*Candidatus* Liberibacter solanacearum': detection, characterization, new hosts and epidemiology in Spain

Memoria presentada por Gabriela Ribeiro Teresani
para optar al grado de Doctor Ingeniero Agrónomo

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D. Mariano Cambra Álvarez y Dña. María Milagros López González, Doctores Ingenieros Agrónomos Profesores de Investigación, del Instituto Valenciano de Investigaciones Agrarias, D. Edson Bertolini, Doctor Ingeniero Agrónomo colaborador científico adjunto, del Instituto Valenciano de Investigaciones Agrarias y D. José García Jiménez, catedrático de la Universidad Politécnica de Valencia

CERTIFICAN:

Que Dña. Gabriela Ribeiro Teresani ha realizado bajo su dirección en los laboratorios de Virología e Inmunología y de Bacteriología del Centro de Protección Vegetal y Biotecnología del Instituto Valenciano de Investigaciones Agrarias, el trabajo que con el título "'*Candidatus* Liberibacter solanacearum': detection, caracterization, new hosts and epidemiology in Spain", presenta para optar al grado de Doctor Ingeniero Agrónomo.

Para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 29 de septiembre de 2014.

Dr. Mariano Cambra Álvarez	Dr. María M. López González
Dr. Edson Bertolini	Dr. José García Jiménez

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Abstract

‘*Candidatus* Liberibacter solanacearum’ is a α-Proteo bacterium that is Gram-negative and restricted to the plant phloem and to the hemolymph of psyllids and that acts as vectors. This emerging bacterium has been associated with different diseases in different hosts and associated with carrot (*Daucus carota*) in Spain. Vegetative disorders of unknown etiology have also been observed in celery (*Apium graveolens*) since 2008.

A real-time PCR protocol that was specific to ‘*Ca.* L. solanacearum’ detection, using a TaqMan probe and direct sample preparation methods was developed. This technology was validated in an intra-laboratory study (sensitivity 1, specificity 1 and accuracy 100%) and is available commercially as a complete kit. ‘*Ca.* L. solanacearum’ is associated with the observed syndrome in celery, and a new bacterium haplotype (E) has been identified. With these results, it is concluded that celery is a new host of ‘*Ca.* L. solanacearum’ (Teresani *et al*., 2014a).

Using the newly developed real-time PCR protocol, ‘*Ca.* L. solanacearum’ has been detected in 42.6% of the tested carrot seeds lots and in individual seeds. The number of cells/seeds has been estimated at 4.8±3.3 to 210±6.7, of which only 5% were viable. After 150 days post-germination, 12% of the seedlings showed symptoms and tested positive for ‘*Ca.* L. solanacearum’. Liberibacter-like cells were observed in the phloem sieve elements of the seed coat and in the phloem of the carrot leaf midrib from the

seedlings. These results demonstrate that '*Ca.* L. solanacearum' is transmitted by carrot seeds (Bertolini *et al*., 2014b).

The collected arthropods were classified into families, and the superfamily Psylloidea was identified at the species level, mainly *Bactericera trigonica, B. tremblayi* and *B. nigricornis*. The population dynamics of different psyllids species visiting carrot, celery and potato has been determined, concluding that the largest populations are captured during summer. The bacterium has been detected in the different *Bactericera* species that have been previous cited in addition to *Bactericera* sp. The psyllid species carrying the bacteria can be considered possible vectors of the bacterium (Teresani *et al*. 2014b).

Electrical Penetration Graphs showed that *B. trigonica* was able to feed in the phloem of carrot, celery and potato but not in the phloem of tomato plants. Experimental transmission showed that *B. trigonica* transmitted '*Ca.* L. solanacearum' from carrot to carrot, celery, potato and tomato. More efficient transmission occurred with ten individuals, and the transmission rates were 100% in celery, 80% in carrot and 10% in potato and tomato. The experimental transmission to potatoes threatens this crop (Teresani *et al*., 2014c).

These combined results have built a scientific foundation of the biological and epidemiological aspects of '*Ca.* L. solanacearum' contributing new scientific information that is key in the cultivation of celery and carrot to establish bacterial control strategies. The use

of bacteria-free carrot seed lots will definitely mitigate damage and reduce the risks of transmission to solanaceous crops.

Resumen

‘*Candidatus* Liberibacter solanacearum’ es una α-Proteobacteria, Gram-negativa, limitada al floema de plantas y a la hemolinfa de psílidos que actúan como vectores. Esta bacteria emergente ha sido asociada a diferentes enfermedades en distintos huéspedes y en España a desarreglos vegetativos en zanahoria (*Daucus carota*). Desde 2008 se observaron desarreglos vegetativos en apio (*Apium graveolens*) de etiología desconocida.

Se ha desarrollado un protocolo de PCR en tiempo real con sonda TaqMan y métodos directos de preparación de muestras para la detección específica de ‘*Ca.* L. solanacearum’. La tecnología desarrollada ha sido validada mediante pruebas de comportamiento intralaboratorio (sensibilidad 1, especificidad 1 y precisión 100%) que está disponible comercialmente en forma de kit. Se ha asociado ‘*Ca.* L. solanacearum’ con el síndrome observado en apio y se ha identificado un nuevo haplotipo (E) de la bacteria. Se ha demostrado que el apio es un nuevo huésped de la bacteria. (Teresani *et al*., 2014a).

Mediante el protocolo de PCR en tiempo real desarrollado, también se ha detectado ‘*Ca.* L. solanacearum’ en 42,6% de los lotes comerciales de semillas de zanahoria analizados y en semillas individuales. Se ha estimado en 4.8±3.3 a 210±6.7 el número de células/semilla de zanahoria, de las cuales el 5% resultaron viables. A los 150 días de siembra de semillas de lotes infectados, el 12% de las plántulas presentaron síntomas y resultaron positivas a ‘*Ca.* L. solanacearum’. Se observaron organismos tipo bacteria en el floema

de la cáscara de semillas de lotes infectados y en las plántulas procedentes de las mismas. Estos resultados demuestran que '*Ca.* L. solanacearum' es una bacteria transmitida por semillas de zanahoria (Bertolini *et al*., 2014b).

Se han determinado las familias de artrópodos que visitan los cultivos de zanahoria, apio y patata y la superfamilia Psylloidea ha sido clasificada a nivel de especie, resultando *Bactericera trigonica*, *B. tremblayi* y *B. nigricornis* las especies más capturadas. Se ha determinado la dinámica poblacional de los psílidos, concluyendo que en verano se produce el máximo poblacional. Se ha detectado '*Ca*. L. solanacearum' en las diferentes especies de *Bactericera* citadas anteriormente y en *Bactericera* sp. Estas especies pueden ser consideradas como posibles vectores (Teresani *et al*., 2014b).

Se han efectuado estudios utilizando gráficos de penetración eléctrica que mostraron que *B. trigonica* es capaz de alimentarse en floema de plantas de zanahoria, apio y patata pero no de tomate. Experimentos de transmisión han demostrado que *B. trigonica* transmitió '*Ca*. L. solanacearum' de zanahoria a zanahoria, apio, patata y tomate. Las mayores tasas de transmisión fueron obtenidas cuando se utilizaron 10 individuos, siendo de 100% en apio, 80% en zanahoria y 10% en patata y tomate. La transmisión experimental a patatas representa una amenaza para dicho cultivo (Teresani *et al*., 2014c).

Todos estos resultados han permitido conocer aspectos biológicos y epidemiológicos de '*Ca.* L. solanacearum' que aportan nueva información científica clave para establecer estrategias de

control de la bacteria en los cultivos de apio y zanahoria. El uso de lotes de semillas de zanahoria libres de la bacteria contribuirá definitivamente a mitigar los daños y a reducir riesgos de transmisión a cultivos de solanáceas.

Resum

‘*Candidatus* Liberibacter solanacearum’ és un α-Protobacteri, Gram-negatiu, limitat al floema de plantes i a l’hemolimfa de psíl·lids que actuen com a vectors. Aquest bacteri emergent s’ha associat amb diferents malalties en distints hostes i a desarreglaments vegetatius en safanòria a Espanya. Des de 2008 s’observaren desarreglaments vegetatius en api (*Apium graveolens*) d’etiologia desconeguda.

S’ha desenvolupat un protocol de PCR en temps real amb sonda TaqMan i mètodes directes de preparació de mostres per a la detecció específica de ‘*Ca.* L. solanacearum’. La tecnologia desenvolupada s’ha validat mitjançant proves de comportament intralaboratori (sensibilitat 1, especificitat 1 i precisió 100%) i està disponible comercialment en forma de kit. S’ha demostrat que ‘*Ca.* L. solanacearum’ està associat a la síndrome observada en api i s’ha identificat un nou haplotip (E) del bacteri. Es conclou que l’api és un nou hoste no descrit prèviament (Teresani *et al.*, 2014a).

Mitjançant el protocol de PCR en temps real desenvolupat, també s’ha detectat ‘*Ca.* L. solanacearum’ en el 42.6% dels lots comercials de llavors de safanòria analitzats i en llavors individuals. S’ha estimat en 4.8±3.3 a 210±6.7 el nombre de cèl·lules/llavor de safanòria, de les quals el 5% resultaren viables. Després de 150 dies de sembra de llavors de lots infectats, el 12% de les plàntules presentaven símptomes i resultaren ser positives a ‘*Ca.* L. solanacearum’. S’observaren organismes de tipus bacterià al floema de la corfa de les llavors de lots infectats i a les plàntules procedents

d'elles. Aquestos resultats demostraren que '*Ca.* L. solanacearum' és un bacteri transmissible per llavors de safanòria (Bertolini *et al.*, 2014b).

S'han determinat les famílies d'artròpodes que visiten els cultius de safanòria, api i creïlla, i la superfamília *Psylloidea* s'ha classificat a nivell d'espècie, resultant *Bactericera trigonica*, *B. tremblayi* i *B. nigricornis* les espècies més capturades. S'ha determinat la dinàmica poblacional dels psíl·lids i s'ha conclòs que a l'estiu es produeix el màxim poblacional. S'ha detectat '*Ca.* L. solanacearum' en les diferents espècies de *Bactericera* citades anteriorment i en *Bactericera* sp. Aquestes espècies poden ser considerades com possibles vectors (Teresani *et al.*, 2014b).

S'han efectuat estudis utilitzant gràfiques de penetració elèctrica que mostren que *B. trigonica* és capaç d'alimentar-se del floema de plantes de safanòria, api i creïlla, però no de tomaca. Experiments de transmissió han demostrat que *B. trigonica* va transmetre '*Ca.* L. solanacearum' de safanòria a safanòria, api, creïlla i tomaca. Les majors taxes de transmissió s'obtingueren quan s'utilitzaren 10 individus, variant de 100% en api, 80% en safanòria i 10% en creïlla i tomaca. La transmissió experimental a creïlles representa una amenaça per a aquest cultiu (Teresani *et al.*, 2014c).

Tots aquestos resultats han permés conéixer aspectes biològics i epidemiològics de '*Ca.* Liberibacter solanacearum' que aporten nova informació científica clau per a establir estratègies de control del bacteri en els cultius d'api i safanòria. L'ús de lots de llavors de safanòria lliures de bacteri contribuirà definitivament a

mitigar els danys i a reduir els riscos de transmissió a cultius de solanàcies.

Resumo

‘*Candidatus* Liberibacter solanacearum’ é uma α-Proteobacteria, Gram-negativa, restringida ao floema de plantas e à hemolinfa de psilídeos que atuam como vetores. Esta bactéria emergente tem sido associada com diferentes doenças em distintos hospedeiros e foi associada a desordens vegetativos em cenoura (*Daucus carota*) na Espanha. Desde 2008, observou-se também desordens vegetativos de etiologia desconhecida em aipo (*Apium graveolens*).

Foi desenvolvido um protocolo de PCR em tempo real que utiliza sonda TaqMan e métodos diretos de preparação de amostras para detecção específica de ‘*Ca.* L. solanacearum’. Esta tecnologia foi validada em um estudo intra-laboratório (sensibilidade 1, especificidade 1 e precisão 100%) e está disponível comercialmente em forma de kit. Foi demonstrado que ‘*Ca.* L. solanacearum’ está associada aos sintomas observados em aipo e identificado um novo haplotipo (E) da bactéria. Com estes resultados demonstra-se que o aipo é um novo hospedeiro da bactéria (Teresani *et al*., 2014a).

Utilizando o protocolo de PCR em tempo real desenvolvido, também se detectou ‘*Ca.* L. solanacearum’ em 42,6% dos lotes de sementes de cenoura analisados e em sementes individuais. Se estimou em 4.8±3.3 a 210±6.7 o número de células/sementes de cenoura, das quais somente 5% eram viáveis. Aos 150 dias pós germinação, 12% das plantas apresentavam sintomas e resultaram positivas a ‘*Ca.* L. solanacearum’. Foram observados organismos tipo bactéria no floema da casca de sementes de lotes infectados e

nas plantas provenientes das mesmas. Estes resultados demonstraram que '*Ca.* L. solanacearum' é uma bactéria transmitida por sementes de cenoura (Bertolini *et al*., 2014b).

Foram determinadas as famílias de artrópodes que visitam os cultivos de cenoura, aipo e batata, e a superfamília Psylloidea foi classificada ao nível de espécie, resultando *Bactericera trigonica*, *B. tremblayi* e *B. nigricornis* as espécies mais capturadas. A dinâmica populacional de diferentes espécies de psilídeos foi determinada, sendo possível observar que no verão se capturam as maiores populações do inseto. Identificaram-se novas espécies de psilídeos portadoras da bactéria, e que portanto podem ser consideradas como possíveis vetores (Teresani *et al*., 2014b).

Os estudos realizados utilizando gráficos de penetração elétrica mostraram que *B. trigonica* é capaz de se alimentar de floema de plantas de cenoura, aipo e batata, mas não de tomate. Experimentos de transmissão demonstraram que *B. trigonica* transmitiu '*Ca*. L. solanacearum' de cenoura a cenoura, aipo, batata e tomate. As maiores taxas de transmissão foram obtidas quando se utilizaram 10 indivíduos, sendo de 100% em aipo, 80% em cenoura e 10% em batata e tomate. A transmissão experimental a batata representa uma ameaça para este cultivo (Teresani *et al*., 2014c).

Todos estes resultados permitiram conhecer aspectos biológicos e epidemiológicos de '*Ca.* L. solanacearum' que aportam nova informação científica chave para estabelecer estratégias de controle da bactéria. O uso de lotes de sementes de cenoura livres da bactéria contribuirá definitivamente para acabar com os danos

causados aos cultivos e a reduzir os riscos de transmissão à solanáceas.

Introduction

Introduction

1. '*Candidatus* Liberibacter' genus

'*Ca*. Liberibacter' is a genus of Gram-negative bacteria restricted to the plants phloem and insect hemolymph, alternating its life cycle between the plant host and insect vector species. The term *Candidatus* describes "procaryotic entities for which more than a mere sequence is available but for which characteristics required for description according to the Code are lacking" (Murray & Schleifer, 1994; Murray & Stackebrandt, 1995). Thus, the designation *Candidatus* (*Ca*.) is a category or status, not a taxonomic rank, and is not recognized by the International Code of Nomenclature of Bacteria (the Code) (Lapage *et al*., 1992). The genus names are preceded by the word *Candidatus,* and the entire designation is written between quotation marks. Type strains are not designed as the long-term preservation of strains can be challenging or impossible; however, the identification of 'reference strains' (as infected plants or DNA samples) for comparative purposes is encouraged. The delineation of an individual *Candidatus* relies heavily on sequence data and uses ecological and metabolic information when available. In general, these data are generated through the PCR amplification of the bacterial 16S rRNA gene sequences in the plant host(s), insect vector(s) and other molecular data (Bull *et al*., 2008).

1.1. Taxonomic classification and species

'*Ca*. Liberibacter' is a genus in the family *Rhizobiaceae*. This diverse group of microbes includes either plant pathogens or symbionts with distinctive properties and some human pathogens (Bové, 2006). Phylogenetic analyses of the 16S rRNA gene indicated that the '*Ca*. Liberibacter' genus belongs to the α-Proteobacteria group, and members of the α-2 bacterial subgroup are the closest cultivated relatives to these microbes (Jagoueix *et al*., 1994).

'*Ca*. Liberibacter' species live in intimate association with eukaryotic cells and, in many cases, have acquired the ability to survive and grow within an arthropod vector. Indeed, these bacteria grow in a specialized niche in the eukaryotic plant host, the phloem sieve tubes and are transmitted by arthropod vectors. All species of '*Ca.* Liberibacter' are vectored by psyllids and typically associated and generally recognized as the cause of four serious plant diseases: huanglongbing (HLB), zebra chip, psyllid yellows and yellows decline, which currently threaten and destroy citrus (*Citrus* spp.), potato (*Solanum tuberosum* L.), tomato (*S. lycopersicum* L.) and carrot (*Daucus carota* L.*)* industries, respectively. With the exception of HLB, these diseases have been recently identified in agriculture, thereby inferring a modern evolutionary trajectory; however, there is no overall hypothesis concerning the origins of these diseases (Nelson *et al*., 2013). The host range of the psyllid vector restricts the range of plant species naturally infected by a '*Ca*. Liberibacter' species, which is typically limited a few closely

related plant species (Haapalainen, 2014).

There are five currently recognized '*Ca.* Liberibacter' species, of which four are considered plant-pathogenic species (Bull *et al*., 2010 & 2012). Among the four plant-pathogenic species, three of these bacteria are associated with HLB worldwide, and each species is named based on its presumptive origin: '*Ca*. L. asiaticus' from Asian countries, '*Ca*. L. africanus' from African countries, and '*Ca*. L. americanus' from Brazil (Bové, 2006). HLB is the most serious disease that affects modern citrus industries in Africa, Asia and the Americas, and citrus production in China, Florida (USA) and Brazil is seriously endangered. In addition, the '*Ca*. L. africanus' subspecies "capensis" is symptomless or specifically associated with mild symptoms in the rutaceous Cape chestnut tree *Calodendrum capense* (syn *C. capensis*, *Pallasia capensis*) Christm, and these bacteria have never been detected in commercial citrus species (Garnier *et al.,* 2000; Phahladira *et al.,* 2012). '*Ca.* Liberibacter' HLB-associated species are transmitted by the Asian citrus psyllid, *Diaphorina citri* Kumayama and the African psyllid *Trioza erytreae* Del Guercio (Capoor *et al*., 1967; Yamamoto *et al*., 2006; McLean & Oberholzer, 1965). Recently, *Cacopsylla citrisuga* Yang & Li has been described as a vector in China (Cen *et al*., 2012a). *Diaphorina communis* Mathur, identified in Bhutan (Yamamoto, 2007), has been found to carry '*Ca*. L. asiaticus (Donovan *et al*., 2012). However, the transmission of the bacterium from this insect has not been reported. Recent reviews have provided an in-depth understanding of the biology and

epidemiology of HLB in citrus (Bové, 2006; Da Graça, 1991; Gottwald, 2010; Wang & Trivedi, 2013).

The fourth pathogenic species ('*Ca*. L. solanacearum') has been associated with zebra chip disease in potatoes, psyllid yellows in tomato and yellows decline in carrots. Zebra chip disease has resulted in major economic losses to the North American (USA and Mexico) and New Zealand potato industries, and this disease was first observed on a large scale, affecting potatoes used for manufacturing crisps in Texas (USA) and Mexico. More recent reports have described zebra chip disease in crops in Oregon, Washington and Idaho, representing over 50% of the USA potato production (Nelson *et al*., 2013). Recent reviews (Munyaneza, 2012; Lin & Gudmestad, 2013) have provided an update of the potato pathosystem.

'*Ca*. L. europaeus' (Raddadi *et al.,* 2011), the last '*Ca.* Liberibacter' species was detected in symptomless pear tree plants (*Pyrus communis* L.) in Italy. The species is naturally vectored by the pear psyllid *Cacopsylla pyri* Linnaeus and was subsequently detected in other species of the genus *Cacopsylla* and additional host plants, such as apple (*Malus domestica*), blackthorn (*Prunus spinosa*), and hawthorn (*Crataegus monogyna*) (Camerota *et al*., 2012). '*Ca*. L. europaeus' has also been detected in Scotch broom (*Cytisus scoparius*) in New Zealand crops heavily infested by broom psyllids (*Arytainilla spartiophila*), showing serious disease symptoms (Thompson *et al*., 2013).

The only culturable *Liberibacter* species, designated

Liberibacter crescens, was obtained from a hybrid mountain papaya (*Carica stipulata* x *C. pubescens*) in Puerto Rico (Fagen *et al.,* 2014). Compared with other species in the *Liberibacter* genus, *Liberibacter crescens* has many more biosynthesis genes, which explains why *L. crescens* is culturable, although the other known *Liberibacter* species have not yet been cultured (Duan *et al*., 2009).

Based on 16S rRNA gene sequences, a phylogenetic tree was constructed using the neighbor-joining method, and the results showed that all three huanglongbing (HLB)-associated '*Ca*. Liberibacter' species were clustered along with '*Ca*. L. solanacearum' into a monophyletic group (Lin *et al.,* 2009) (Figure 1).

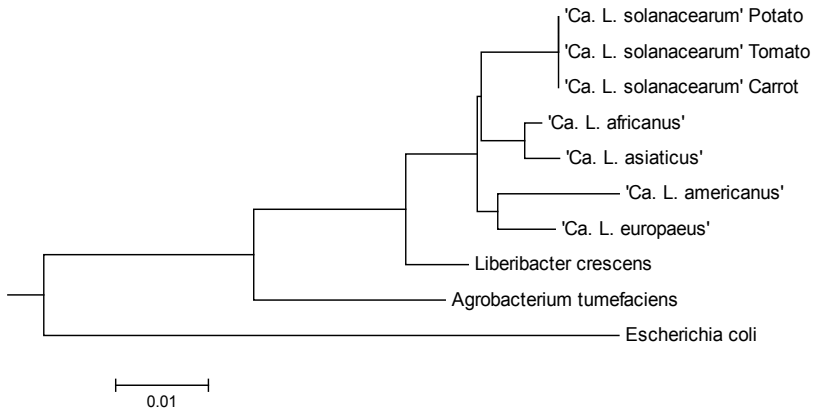

Fig. 1. Phylogenetic tree constructed based on 16S rRNA gene sequences using the neighbor-joining method.

1.2. Morphological characteristics

Electron microscopy showed that the cell wall of the HLB associated bacteria appears as a characteristic, electron-dense layer surrounding the bacterial cell (Garnier & Bové, 1977; Bové, 2006; Tanaka *et al*., 2006). Further microscopic examination using special fixation methods revealed a triple layered ultra-structure membrane (the inner, cytoplasmic and the outer) as part of the Gram-negative cell wall. Similarly, scanning electron microscopy images of '*Ca*. L. solanacearum' in the sieves of infected plants showed that this bacterium has a rod-shaped morphology (Liefting *et al*. 2009a; Secor *et al*., 2009), and this bacterium is approximately 0.2 μm wide and 4 μm long (Liefting *et al*., 2009a) (Figure 2).

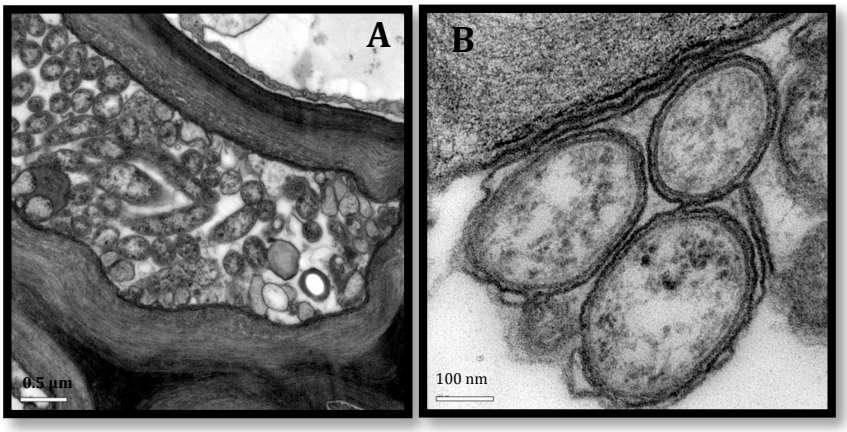

Fig. 2. Transmission electron photomicrographs (TEM). A: Carrot sieve tube elements infected with bacterium-like organisms (BLOs). B: The triple-layered ultrastructure of both the outer cell wall membrane and the inner cytoplasmic membrane suggests the presence of '*Ca*. Liberibacter'-like cells (photograph by Dr. F.A.O. Tanaka, ESALQ-USP, Brazil).

2. '*Ca*. Liberibacter solanacearum'

'*Ca.* L. solanacearum' is a Gram-negative, phloem-limited, insect-vectored prokaryote belonging to the α-Proteobacteria group. Due to the fastidious nature of this bacterium, '*Ca.* L. solanacearum' is unculturable, and the Koch's postulates have not been fulfilled. Consequently, detailed information regarding the general biology, physiology, and pathogenicity of '*Ca*. L. solanacearum' is lacking. Despite these limitations, the whole genome sequence of '*Ca*. L. solanacearum' has been obtained (Lin *et al*., 2011b).

'*Ca*. L. solanacearum' is also known as '*Ca*. L. psyllaurous' (Hansen *et al*., 2008). Because the sequences of '*Ca*. L. solanacearum' and '*Ca*. L. psyllaurous' share > 99% similarity in the 16S rRNA gene, these two bacterial names are considered synonymous (Secor *et al*., 2009; Wen *et al*., 2009). '*Ca*. L. solanacearum' is recommended as the reference name for this bacterium because this name and a description of the bacterium has been published in the International Journal of Systematic and Evolutionary Microbiology (Liefting *et al*., 2009b), the preferred journal for descriptions of uncharacterized bacterial species. The reference strain NZ082226 was obtained from a tomato plant in South Auckland, New Zealand. Hereafter, in this thesis, only the name '*Ca*. L. solanacearum' will be used.

This bacterium has been associated with different plant hosts, including potato, tomato, pepper (*Capsicum annuum* L.), carrot, tamarillo (*Solanum betaceum* Cav.), Cape gooseberry (*Physalis*

peruviana L.), tobacco (*Nicotiana tabacum* L.), aubergine (*Solanum melongena* L.), silverleaf nightshade (*Solanum elaeagnifolium* Cav.), black nightshade (*Solanum ptychanthum* Dun.), wolfberry (*Lycium barbarum* L.) (Haapalainen, 2014) and parsnip (*Pastinaca sativa*).

2.1. Associated diseases

2.1.1. Zebra chip

In 1994, a new potato disease exhibiting symptoms similar to potato purple top and psyllid yellows was described in commercial potato fields in Texas (USA) and Mexico, affecting all cultivars of table-stock and processing potatoes and causing severe losses (Secor & Rivera, 2004). This disease complex was tentatively named zebra chip because of the severe dark and light-striped pattern on both raw tubers and chips made from diseased tubers (Figure 3). This symptom is characteristic of diseases in which the transport of assimilates in phloem vessels is compromised.

At the time zebra chip disease was reported, the causal agent was unknown, and graft experiments were conducted to investigate the transmission of this disease and determine whether the infectious agent/s were transmissible to healthy plants or due to non-biological factors, such as heat stress or toxicity. In addition, research was also conducted to determine whether the symptoms were caused by nutritional deficiencies, toxins or other abiotic factors, or some combination of these factors. Although researchers have not correlated this disease with any specific causes, the results

of the graft- and insect-transmission experiments suggested a vascular plant pathogen, such as a virus, prokaryote or BLO, as a likely transmissible factor (De Boer *et al*., 2007; Munyaneza *et al*., 2007a). Initially, phytoplasmas were suspected as the cause of zebra chip disease symptomology, but the presence of these specialized bacteria could not be consistently confirmed through PCR using universal primers (Secor *et al*., 2005). Subsequently, electron microscopy and molecular characterization suggested that zebra chip might be caused by a BLO (De Boer *et al*., 2007; Li *et al*., 2008; Secor *et al*., 2008).

Fig. 3. Zebra chip symptoms in potato tuber.

Using the primers developed by Liefting *et al*. (2009a), the presence of '*Ca*. L. solanacearum' was first confirmed in potato samples with zebra chip symptoms collected in Texas (USA) (Abad *et al*., 2009). Hansen *et al*. (2008) also reported about the same time the association of '*Ca*. L. psyllaurous' with psyllid yellows in tomatoes and potatoes in the USA. Secor *et al*. (2009) established

the association of '*Ca*. L. solanacearum' with zebra chip disease using electron microscopy and the molecular characterization of DNA fragments specifically amplified from symptomatic potato and tomato plants infected with graft and psyllid transmission from potato plants with zebra chip symptoms. These results, together with transmission electron microscopy evidence (De Boer *et al*., 2007; Secor *et al*., 2009), confirmed the presence and association of a phloem-inhabiting BLO in diseased plants. This pathogen was subsequently associated with potato zebra chip (Liefting *et al*., 2009a). Since the discovery that '*Ca*. L. solanacearum' is responsible for zebra chip disease, many studies have shown consistent evidence that '*Ca*. L. solanacearum' is the putative causal agent of potato zebra chip.

Infected potato plants exhibit a wide range of symptoms similar to potato purple top and psyllid yellows, including chlorosis, twisted stems with a zigzag appearance, swollen nodes, aerial tubers, vascular discoloration, and leaf scorching and wilting (Figure 4).

The primary tuber symptom is extensive brown coloration of the vascular ring and medullary rays throughout the entire length of the tuber. This latter symptom is diagnostic and separates zebra chip disease from other known potato diseases (Secor *et al*., 2009).

Fig. 4. '*Ca*. L. solanacearum'-infected potato plants exhibiting symptoms.

'*Ca*. L. solanacearum' severely disrupts carbohydrate flow in potato plants, leading to zebra chip symptoms in the tubers. Buchman *et al*. (2011a & 2012) showed that the bacterial infection results in overall high glucose and sucrose levels compared with those of uninfected tubers. It has been suggested that the necrotic flecking and streaking of the medullary ray tissue symptoms in infected tubers is associated with the darkening of the resulting processed chips or fries due to elevated sugar levels, which caramelize when the tuber slices are fried ("Maillard Reaction") (Figure 5). Navarre *et al*. (2009) reported high levels of aromatic amino acids and phenolic compounds in the extracts from zebra chip-symptomatic potato tubers. In addition, a greater than 8-fold increase in the tyrosine concentration in symptomatic tubers was also observed, suggesting that the dramatic increase in this amino acid might significantly contribute to the browning observed in infected tubers. Salicylic acid, a key regulator of plant defenses,

was also detected at high concentrations, consistent with the concentration expected from tubers mounting a defense response against a pathogen. Furthermore, Miles *et al*. (2009) showed significant differences in the mineral content between infected and non-infected potato tubers.

Fig. 5. Fried potato chips (A) and French fries processed (B) from '*Ca*. L. solanacearum' infected tubers (photograph by Dr. J.E. Munyaneza, ARS-USDA, USA).

Temperature has a significant effect on the development of '*Ca.* L. solanacearum' in potato plants. Compared with other liberibacters associated with HLB disease, which are typically temperature tolerant (Lopes *et al*., 2009; Munyaneza *et al*., 2012), '*Ca*. L. solanacearum' is heat-sensitive and does not tolerate temperatures above 32°C. Additionally, temperatures at or below 17 °C might significantly slow, but not prevent, '*Ca*. L. solanacearum' development in potato plants. The optimum development of '*Ca*. L. solanacearum' and zebra chip symptoms in potato plants were observed at daily temperature ranging from 27-32°C. At higher daily temperatures ranging from 32-35°C and 35-40°C, no '*Ca*. L.

solanacearum' was detected in potato plants inoculated using insects and no symptom development was observed. In the Americas and New Zealand, the '*Ca.* L. solanacearum' vector *B. cockerelli* exhibits similar temperature preferences (Munyaneza *et al*., 2012).

2.1.2. Psyllid yellows

In January 2008, a tomato disease was observed in three commercial greenhouses in Auckland (New Zealand) and economic losses were reported. The disease symptoms included spiky, chlorotic apical growth, general mottling of the leaves, curling of the midveins, overall stunting of the plants, and fruit deformation in some cultivars. The cultivars varied in severity of symptoms and levels of disease prevalence (Liefting *et al*., 2009a) (Figure 6). Extensive analyses were performed to determine the etiology of this disease, but no pathogenic fungi or culturable bacteria were isolated and generic tests for viruses, including herbaceous indexing, transmission electron microscopy (TEM) and dsRNA purification, generated negative results. Negative results were also obtained from polymerase chain reaction (PCR) analyses for phytoplasmas and viruses (Liefting *et al*., 2009a). The tomato/potato psyllid *Bactericera cockerelli* Sulc. (Hemiptera, Psyllidae) (Burckhardt & Lauterer, 1997) was observed in tomato greenhouses where these symptoms were present. Due to the similarity of symptoms with those reported for psyllid yellows (Wallis, 1955; Arslan *et al*., 1985; Sengoda *et al*., 2010) and the absence of a positive result for other

potential causal organisms, Richards (1928) initially identified this disease as psyllid yellows, likely caused by a toxin associated with psyllid nymphal instar feeding. In April 2008, similar symptoms were observed in glasshouse peppers cultivated on the same property as the infected tomatoes (Figure 6). TEM analyses of thin leaf tissue sections revealed the presence of BLOs in the phloem of symptomatic tomato and pepper plants (Liefting *et al*., 2009a).

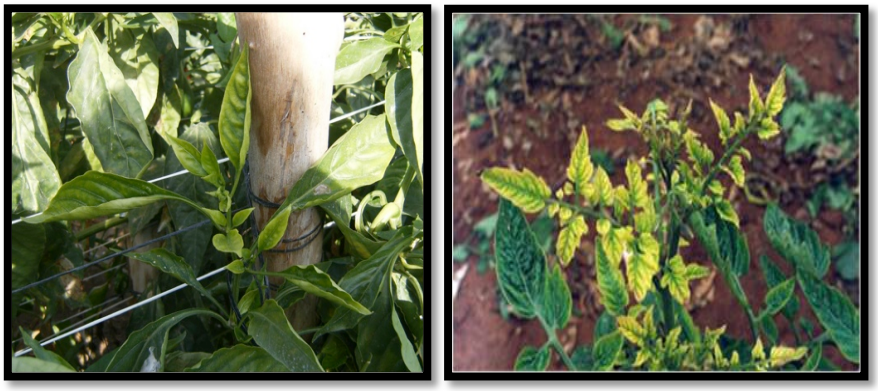

Fig. 6. Symptomatic pepper (left) and tomato (right) plants infected with '*Ca.* L. solanacearum' (photograph by Dr. J.E. Munyaneza, ARS-USDA, USA).

Phylogenetic analysis revealed that the BLOs observed in tomato and pepper plants were '*Ca*. L. solanacearum', a new species of the '*Ca.* Liberibacter' genus. In August 2008, a research group at the University of California-Riverside described another '*Ca.* Liberibacter' species, '*Ca.* L. psyllaurous', implicated in tomato yellow disease resulting from bacterium transmitted through the psyllid *B. cockerelli* (Hansen *et al*., 2008).

2.1.3 Yellow declines

Subsequently after '*Ca*. L. solanacearum' was identified in New Zealand, a new disease associated with '*Ca*. L. solanacearum' in carrot, vectored through the psyllid *Trioza apicalis* Föerster (Hemiptera: Triozidae), was identified in Finland (Munyaneza, *et al*., 2010b). This finding was followed by another report of '*Ca*. L. solanacearum' in carrot fields in Spain (Alfaro-Fernández *et al*., 2012a), France (Loiseau *et al*., 2014) and Morocco (Thazima *et al*., 2014). These findings suggest that the '*Ca*. Liberibacter' species underwent rapid evolutionary divergence, speciation, and adaptation to a wider range of hosts since divergence from their ancestors (Lin & Gudmestad, 2013).

The psyllid carrot pest *T. apicalis* has long been implicated as the causative agent in vegetative disorders in carrot fields, with symptoms resembling those caused by phytoplasmas and *Spiroplasma citri*. However, Nissinen *et al*. (2007) demonstrated that inoculation with one to three psyllids per seedling for three days resulted in the development of severe symptoms in carrots, typically observed one month after insect removal. These authors also suggested that some plant pathogens might be involved in this *T. apicalis*-induced disorder.

Given the recent association of '*Ca*. Liberibacter' species with several crops affected by psyllids, the association of this bacterium with *T. apicalis* was examined. Using conventional PCR primers specific to '*Ca*. L. solanacearum', bacterial amplicons were obtained from field-collected and laboratory-reared psyllids and

field-collected and laboratory-grown petioles from symptomatic carrots. The carrot '*Ca*. Liberibacter' 16S rDNA sequence was 100% identical to an analogous sequence amplified from *T. apicalis,* providing molecular evidence for '*Ca*. Liberibacter' as the causal agent of the observed carrot disorder symptoms. In addition, the sequences from both *T. apicalis* and plants showed 99.9% homology (one single nucleotide polymorphism) with analogous sequences from several solanaceous crops affected with *B. cockerelli.* Phylogenetic analysis of the 16S rDNA sequences from '*Ca*. Liberibacter' indicated that the '*Ca*. Liberibacter' identified in *T. apicalis* and in several solanaceous crops formed a single cluster comprising '*Ca*. L. solanacearum' (Munyaneza *et al*., 2010b).

The identification of '*Ca*. L. solanacearum' in carrot plants in Finland vectored by the psyllid *T. apicalis* is the first report of this bacterium in a non-solanaceous species. Subsequently, this bacterium was also detected in carrots and *T. apicalis* in Sweden and Norway (Munyaneza, 2012). Thereafter, '*Ca*. L. solanacearum' was reported in carrot and celery crops and the psyllid *B. trigonica* in the Canary Islands and mainland Spain (Alfaro-Fernández *et al*., 2012a & b). Damage resulting from '*Ca*. L. solanacearum'-positive psyllids caused up to 100% carrot crop loss in Europe (Alfaro-Fernández *et al*., 2012a & b; Nissinen *et al*., 2012).

The symptoms in carrots infected with '*Ca*. L. solanacearum' include curling and yellowish, bronze and purplish discoloration of the leaves, stunting of the carrot shoots and roots, and proliferation of the secondary roots (Munyaneza *et al*., 2010a; Alfaro-Fernández

et al., 2012a). These symptoms collectively resemble those caused by leafhopper-transmitted phytoplasmas and *S. citri* (Font *et al*., 1999; Lee *et al*., 2006; Cebrián *et al*., 2010; Munyaneza *et al*., 2010a) (Figure 7).

Fig. 7. Symptoms in carrots infected with '*Ca.* L. solanacearum'.

2.2. Molecular characterization

2.2.1. Genome analysis

Using DNA enrichment techniques and next-generation sequencing technology (NGS), the complete genome of '*Ca*. L. solanacearum' from viruliferous *B. cockerelli* has been sequenced and published (Lin *et al*., 2011b), facilitating the development of molecular genetic markers for genotyping and genetic analyses. The circular 1.26 Mbp bacterial genome (GenBank accession number CP002371) encodes a total of 1,192 putative proteins. Approximately 68% of these genes exhibit homology with proteins with known functions, and the remaining proteins have been assigned to hypothetical proteins with unknown functions.

Sequence analyses have identified three copies of complete rRNA operons (16S rRNA, 16S/23S intergenic spacer region or ISR and 50S rRNA genes), 45 genes encoding tRNAs and approximately 35 genes identified as pseudogenes. Currently, no plasmids have been identified in either Liberibacter genome (Lin & Gudmestad, 2013). Compared with the genome of non-phloem-restricted bacterium, the smaller size of the '*Ca*. L. solanacearum' genome reflects the loss of a great number of genes, resulting in a reduction of the genome size associated with adaptation to an obligate lifestyle, in which many of the necessary molecules can be directly acquired from the host (Gil *et al*., 2002).

2.2.2. Haplotypes

To determine the genetic diversity of '*Ca*. L. solanacearum' in agricultural ecosystems, haplotypes were characterized based on single nucleotide polymorphism (SNP) typing of the 16S, 16S/23S and 50S rDNA sequences (Nelson *et al*., 2012). These SNPs are inherited as a package across the three genes.

Four haplotypes (A, B, C and D) of '*Ca*. L. solanacearum' have been described (Nelson *et al*., 2011 & 2012). Haplotypes A and B are associated with diseases in potatoes and other solanaceous crops, whereas haplotypes C and D are associated with carrot diseases. Haplotype A has primarily been detected in crops from Honduras, Guatemala, Western Mexico, USA (Arizona, California, Oregon, Washington and Idaho) and New Zealand, whereas haplotype B has primarily been detected in Mexico and

USA. These two haplotypes show some range of overlap in Texas, Kansas and Nebraska (USA) (EPPO, 2013). Haplotype C has been identified in Finland, Sweden and Norway. Haplotype D has been identified in the Canary Islands, mainland Spain and probably in France. *B. cockerelli* has been described as the vector species for haplotypes A and B in solanaceous crops. *T. apicalis* has been associated with the spread of haplotype C (Munyaneza *et al*., 2010b; Nelson *et al*., 2011), and haplotype D has been associated with the psyllid *B. trigonica* Hodkinson in Spain (Nelson *et al*., 2012). These four haplotypes have not been shown to elicit biological differences in plant or insect hosts. These apparently stable haplotypes suggest separate long-lasting populations of this bacterium.

The recent completion of the '*Ca*. L. solanacearum' genome sequence (Lin *et al*., 2011b) has facilitated the development of molecular genetic markers for genotyping and genetic analyses of '*Ca*. L. solanacearum'. Simple sequence repeat (SSR) markers, also known as variable number tandem repeats or microsatellite markers, are useful for genotyping and the genetic study of bacteria. Lin *et al*. (2012), developed a panel of eight polymorphic SSR markers for the genetic analysis of '*Ca*. L. solanacearum' strains collected from different geographical locations and hosts in Northern America, including USA and Mexico. The multilocus SSR analysis showed a high level of genetic diversity in '*Ca*. L. solanacearum' in North America (USA and Mexico). The analyses indicated that the occurrence of identical haplotypes was not restricted to any

particular geographical area or host cultivar. Overlapping identical haplotypes were observed among different geographical locations, including Texas and Nebraska, and among Texas, Kansas and Mexico. Similarly, no haplotypes were restricted to a particular potato cultivar or solanaceous plant. In contrast to a natural population in agricultural practice, it is expected that the frequent exchange of plant materials facilitate the movement of zebra chip-affected materials, largely reflecting the non-patterned geographical distribution observed among identical haplotypes.

The primary mode of gene transfer among '*Ca*. L. solanacearum' in agricultural ecosystems is not known. However, the large numbers of unique haplotypes identified and the high level of genetic diversity among '*Ca*. L. solanacearum' strains observed in this study suggest that this pathogen has much higher diversity in North America than expected.

2.3. Transmission

2.3.1. Propagative material

The commercialization of '*Ca*. L. solanacearum'-infected potato tubers as a potential source for disease spread is a major concern and could be a threat to the national and international trade of fresh and seed potatoes. In a study conducted in the USA, Henne *et al*. (2010b) showed that approximately 44% of the infected tubers remained viable under greenhouse conditions, but produced hair sprouts and weak plants that died prematurely. Only a low percentage of plants from infected tubers tested positive for '*Ca*. L.

solanacearum'. In the same study, these authors concluded that in areas where zebra chip is currently established, plants produced from '*Ca*. L. solanacearum'-infected seed tubers do not significantly contribute to disease incidence and spread within potato fields. Pitman *et al*. (2011) and Berry *et al*. (2011) conducted studies in New Zealand, and these authors reported a rate as high as 90% for potato plant emergence from tubers testing positive for '*Ca*. L. solanacearum' but not exhibiting typical symptoms. This potato plant emergence resulted in the production of marketable tubers, although plant growth and potato yield were significantly reduced. However, the production of '*Ca*. L. solanacearum'-infected daughter tubers was negligible.

It has been determined that at all plant growth stages, potatoes are susceptible to '*Ca*. L. solanacearum' infection and it takes approximately three weeks after inoculation for symptoms to develop in potato plants and tubers. Plants exposed to '*Ca*. L. solanacearum'-positive psyllids for less than three weeks before harvest typically produce tubers without zebra chip symptoms (Buchman *et al.,* 2012). Infected tubers can develop symptoms in storage, but it is not known yet how extensively or how rapidly these symptoms develop. Rashed *et al*. (2011) reported zebra chip symptom development in the tubers of potato cv. FL1867 after three months in storage at 5°C. The tubers harvested from plants exposed to '*Ca*. L. solanacearum' infected psyllids at two weeks prior to harvest were symptomless. This observation presents a major concern for potatoes produced in the Pacific Northwest of the USA,

where the majority of tubers are stored following harvest and potato fields are susceptible to '*Ca*. L. solanacearum' infection late in the growing season because of the late psyllid migration into potato fields (Munyaneza *et al*., 2009a; Crosslin *et al*., 2012a & b). Notably, plants infected prior to the tuber initiation stage die quickly and fail to produce tubers, thereby reducing the impact of the disease.

In addition, '*Ca*. L. solanacearum' was detected in botanical seeds of chile pepper in Chile (Camacho-Tapia *et al*., 2011) and of carrot in France (Loiseau *et al*., 2014). However, no evidence of seed transmission was available.

Experimental transmission from an infected to a healthy plant could also be obtained using the parasitic plant *Cuscuta campestris* Yunck (dodder), which makes a vascular connection linking the plants through bridging established by the haustoria.

2.3.2. Psyllid vector species

Most obligated plant pathogens depend on vectors for transmission and survival, and insects, mites, nematodes and protists have all been implicated in the transmission of these bacteria. Mechanisms of transmission are best understood by considering the routes of pathogen movement in the insect (circulative *versus* non-circulative) and the sites of retention or target tissues (e.g., stylets, salivary glands). The most fundamental distinction with regard to the mode of transmission is whether ingested particles are circulative or non-circulative in the vector, a

distinction that focuses on the duration and/or site of retention and the route of movement within the insect. Circulative pathogens are taken up into cells, where these bacteria cross multiple membrane barriers, become transported within the vector hemolymph and ultimately exit the saliva of the insect. Circulative pathogens are further classified as circulative non-propagative or circulative propagative depending on whether the acquired pathogen multiplies within the vector. Non-circulative pathogens have a more superficial and transient relationship with the vector and are only associated with the mouthparts and foregut (NG & Perry, 2004).

Psyllids (Hemiptera: Psylloidea) are small 2-3 mm long, four-winged insects that feed on plants, sucking the phloem sap through a stylet. Currently, approximately three thousand species have been identified and classified into 11 families (Hodkinson, 2009; Ouvrard, 2014). The psyllid life cycle starts from mating, followed by oviposition on the host plant leaves, egg hatching and nymphal development through five instar stages, and the emergence of adults after metamorphosis. This life cycle varies depending on the species, environmental conditions, etc. and typically takes approximately one month to complete a cycle. The adult can fly more than 1 km in distance and can also be carried over longer distances with the wind. A mated female typically lays one hundred to one thousand eggs for most of the species studied (Hodkinson, 2009).

Several species of psyllids are important pests of agricultural crops. During feeding, psyllids might cause considerable damage to

their host plants as the injection of toxic salivary secretions into the plants, particularly by the nymphs, might produce serious necrosis, galling, and plant malformations (Munyaneza *et al*., 2010b).

The transmission of '*Ca.* Liberibacter' through the psyllid vector to a plant host occurs via a mechanism similar to that described for persistent insect-transmitted plant viruses (Hogenhout *et al*., 2008). The bacterial cells must pass through the alimentary canal wall, move through the hemolymph, and finally reach the salivary glands, where the bacteria can be passed through salivary secretions into a new host plant during psyllid feeding (Haapalainen, 2014). As described above, several reports have shown that the fastidious bacterium '*Ca.* Liberibacter' can be transmitted by different psyllid species and is associated with newly emerging and economically important diseases of citrus and solanaceous crops. '*Ca*. L. solanacearum' is transmitted by *B. cockerelli* (Munyaneza *et al*., 2010a), *T. apicalis* (Munyaneza *et al*., 2010b) and is associated with *B. trigonica* (Alfaro-Fernández *et al*., 2012b).

2.3.2.1. *Bactericera cockerelli*

B. cockerelli, also known as the tomato/potato psyllid, has been implicated as a vector for '*Ca.* L. solanacearum' on solanaceous crops in North and Central America and New Zealand. This psyllid feeds on the phloem using piercing-sucking mouthparts, and this species is known as a serious and economically important pest of potatoes, tomatoes, and other solanaceous crops in

the western USA, southern Canada, Mexico, Central America and New Zealand (Munyaneza, 2012). Studies have detected this bacterium on plant species in 20 different plant families, and the *B. cockerelli* life cycle is typically completed on some *Solanaceae*, *Convolvulaceae* and *Lamiaceae*. The preferred hosts include aubergine, pepper, tomato and potato (Biosecurity Australia, 2009; Yang & Liu, 2009). Studies conducted in New Zealand following the introduction of *B. cockerelli* (Martin, 2008) have demonstrated a clear host association with aubergine, pepper, tomato, and potato and a poor host status of sweet potato (*Ipomoea batatas* L.), shoo fly plant (*Nicandra physalodes* L.) and other weeds. Nevertheless, this species typically feeds on more plants than it reproduces on, and in the area of origin, *B. cockerelli* overwinters on wild plant species.

In 2007, Munyaneza *et al*., (2007a) provided the first evidence of an association between *B. cockerelli* and zebra chip disease, preceding the identification of '*Ca*. L. solanacearum' as the putative causal agent of the disease. This psyllid species is native to North America and primarily occurs in the Rocky Mountain region of the USA, including Colorado, New Mexico, Arizona, Nevada, Northern Utah, Wyoming, Idaho and Montana, and in Canada, including Alberta and Saskatchewan. *B. cockerelli* has also been identified in Washington and Oregon, where it typically colonizes potato fields in late June and early July. This insect pest is common in the USA in southern and western Texas and has also been documented in Oklahoma, Kansas, Nebraska, South Dakota, North

Dakota, Minnesota and as far west as California and British Columbia (Canada). *B. cockerelli* also occurs in Mexico and Central America, including Guatemala, Honduras and Nicaragua (Munyaneza, 2012). These bacteria were accidentally introduced into New Zealand in the early 2000s (Liefting *et al*. 2009a) and are currently established on both North and South Islands, causing extensive damage to potato, tomato, pepper and tamarillo crops (Teulon *et al*., 2009).

Adult potato psyllids are small, measuring approximately 2.5 mm in length (Wallis, 1955), as shown in Figure 8. The body color ranges from pale green at emergence to dark green or brown within 2-3 days, and these insects eventually turn gray or black thereafter. Prominent white or yellow lines are observed on the head and thorax, and dorsal whitish bands are located on the first and terminal abdominal segments. These white markings are distinguishing characteristics of the psyllid, particularly the broad, transverse white band on the first abdominal segment and the inverted V-shaped white mark on the last abdominal segment (Pletsch, 1947; Wallis, 1955). Adult insects are active in contrast with the largely sedentary nymphal stages. Adult psyllids are good fliers and might readily jump when disturbed. Adult longevity ranges from 20 to 62 days, and females typically live two to three times longer than males, depending on the host plant. Oviposition might last nearly 50 days, with an average fecundity of 300-500 eggs per female over her lifetime (Munyaneza, 2012). The light to dark yellow or orange eggs of *B. cockerelli* are singly deposited on

the lower surface and near the edge of plant leaves. Often, females will lay numerous eggs on a single leaf. The eggs are mounted on short stalks and hatch 3-7 days after oviposition (Pletsch, 1947; Halbert & Munyaneza, 2012). The orange or yellowish-green nymphs are flat with a scale insect-like appearance (Halbert & Munyaneza, 2012; Butler & Trumble, 2012). Potato psyllid nymphs resemble the nymphs of whiteflies, although only the former move when disturbed. The total time of nymphal development depends on temperature and host plant and has been reported to range from 12 to 24 days. Both nymphs and adults produce large quantities of whitish excrement particles (Munyaneza, 2012).

Weather is also an important element governing the biology and damage potential of *B. cockerelli*. This psyllid species adapts to warm, but not hot, temperatures. During migration, cool weather, or at least the absence of elevated temperatures, has been associated with outbreaks of this insect. Optimum psyllid development occurs at approximately 27°C, whereas oviposition, hatching, and survival are reduced at 32°C and cease at 35°C (Munyaneza, 2012). A single generation might be completed in three to five weeks, depending on the temperature. The number of generations varies considerably among regions, typically ranging from three to seven generations. Both adults and nymphs are cold tolerant, with nymphs surviving temporary exposure to temperatures of -15°C, and 50% of adults surviving exposure to -10°C for over 24 h (Henne *et al*., 2010a).

Fig. 8. Adult *Bactericera cockerelli* with characteristic whitish bands on the abdomen and white or yellow lines on the head and thorax (photograph by J.E. Munyaneza).

Outbreaks in Baja California and coastal California (USA) revealed that the potato psyllid in those regions is genetically distinct from the psyllids that overwinter in southern Texas and eastern Mexico, suggesting the existence of two different potato psyllid biotypes, referred to as "western and central" biotypes (Liu *et al*., 2006; Jackson *et al*., 2009). Swisher *et al*. (2012) conducted a genetic study and identified a third biotype (referred to as "northwestern haplotype") that has only been identified in the Pacific Northwest.

Buchman *et al*. (2011b) demonstrated that adult potato psyllids are highly efficient '*Ca*. L. solanacearum' vectors, and nymphs are less efficient than adults. These authors also showed that healthy potato plants exposed to 20 '*Ca*. L. solanacearum'-infected adult potato psyllids for a period as short as one hour developed disease symptoms, whereas a single adult potato psyllid was sufficient to inoculate a potato with '*Ca*. L. solanacearum'

within six hours, leading to the development of zebra chip disease (Buchman *et al*., 2011b); however, the acquisition success is influenced by feeding site and is highest when psyllids have access to the whole plant (Liu & Trumble, 2007). Furthermore, Buchman *et al*. (2011b) discovered that a single infective adult psyllid is as damaging as 25 infective psyllids per plant. It has also been determined that the '*Ca*. L. solanacearum' titer does not differ between males and females, and this bacteria exists at low titers in fifth-instar nymphs and increases with the age of the adult psyllid (Rush *et al*., 2010). Furthermore, it has been shown that '*Ca*. L. solanacearum' negatively affects the *B. cockerelli* population growth rate, at least when the psyllid is reared on tomato (Nachappa *et al*., 2012).

2.3.2.2. *Trioza apicalis*

The psyllid *T. apicalis* is a serious carrot pest in Scandinavia, Finland, and other parts of northern and central Europe, although this insect has been identified in wider areas of Eurasia from Great Britain to Mongolia (Nissinen, 2008). Psyllid-affected carrots exhibit leaf curling, yellowish, bronze and purplish leaf discoloration, shoot and root stunting and secondary root proliferation. These symptoms resemble those caused by leafhopper-transmitted phytoplasmas and *S. citri* in carrots (Font *et al*., 1999; Lee *et al*., 2003; Weintraub & Orenstein, 2004; Lee *et al*., 2006; Duduk *et al*., 2008). Similar to several other economically important species of psyllids, it has long been suggested that *T.*

apicalis affects carrots by injecting toxic saliva into the plants (Markkula *et al*., 1976; Nehlin *et al*., 1994). However, Markkula & Laurema (1971) examined *T. apicalis* salivary gland extractions and salivary secretions, but were unsuccessful in identifying this toxin. Láska (1964) showed that the feeding activity of *T. apicalis* had a systemic effect on the entire carrot plant.

Little is known about the ecology and behavior of the carrot psyllid. The adults pass the winter in diapause on coniferous trees (Rygg, 1977). After leaving hibernation sites in the spring, these insects seek summer hosts, where mating and egg-laying take place. The eggs are singly deposited onto the edges of the carrot leaves, and one female can lay up to 900 eggs (LásKa, 1964). The eggs hatch after 9-14 days, and the nymphs remain sedentary on the underside of the leaves, passing through five instars before becoming adults. The developmental time from egg to imago is approximately seven weeks under laboratory conditions and is slightly longer in the field (Láska, 1964; Rygg, 1977). The oviposition period, during which the plants are injured, lasts for approximately three to six weeks, with one generation per year, and the adults typically die shortly after egg-laying.

The factors that trigger migration are not known. Flight begins at approximately the same time at different latitudes, although the growing season starts later in northern Europe. Both adults and nymphs feed on carrot leaves, and overwintered females cause more severe damage than overwintered males, nymphs, or females of the new generation (Markkula *et al*., 1976). The

symptoms of carrot psyllid infestation include discoloration and curling of the leaves and diminished and distorted root growth. These symptoms result in the qualitative and quantitative reduction of crops (Markkula *et al*., 1976; Rygg, 1977; Burckhardt & Freuler, 2000). After five nymphal instar stages, the new adult generation emerges (Figure 9), which subsequently leaves for winter habitats. Autumn migration might begin in August and continue well into October, depending on the climate and cropping regimes. Thus, this species is univoltine (Kristoffersen & Anderbrant, 2007).

T. apicalis is a less efficient '*Ca.* L. solanacearum' vector compared with *B. cockerelli,* as a high titer of bacteria in the psyllids was required for carrot plant infection and for the disease development (Nissinen *et al*., 2014).

Fig. 9. *Trioza apicalis* on a carrot leaf (photograph by Dr. A. Nissinen, MTT Agrifood Research Finland, Finland).

2.3.2.3. *Bactericera trigonica*

B. trigonica is a psyllid species belonging to the '*Bactericera nigricornis* Förster group' (Hodkinson, 1981), which also contains *B. tremblayi* and *B. nigricornis*. *B. trigonica* have been identified in Algeria, Cyprus, Czech Republic, Egypt, Greece, Hungary, Iran, Israel, Italy, Malta, Portugal Slovakia, Spain, Switzerland and Turkey (Ouvrard, 2014) in different plant host, such as *Ambrosia artemisifolia* L., *Daucus* spp., including carrots.

The three species of this group are morphologically close and are characterized based on the posterior extension of the male proctiger, the short female terminalia, the absence of surface spinules in the forewing, small genal cones and a narrow third antennal segment (Klimaszewski, 1975). *B. trigonica* have primarily been confused with other members of the complex, but these species are easily separated based on the male genitalia and forewing.

The name of the species is derived from the Latin word ¨trigonicus¨, which means triangular, referring to the shape of the male proctiger. *B. trigonica* have a strongly deflexed head that is slightly narrower than the thorax, the genal cones are pyramidal and shorter than half the length of the vertex down the mid-line, directed anterioventrally. The forewing presents typical triazine venation. The fore- and mid-legs are simple, and the hind-legs are saltatorial meracanthi with a well-developed apex of metatibia and 2+1 thick black apical spines; notably, the basal metatarsal spines are absent. The female terminalia is markedly short and

indistinguishable from other members of the complex. The male proctiger is triangular, and the male paramere in lateral view is broadest basally and drawn out into a slender, anteriorly curved apex. In posterior view, the proctiger of *B. trigonica* is more slender and less curved than other members of this group, and the apex of the penis is short and relatively broader than that of other members of this group (Figure 10).

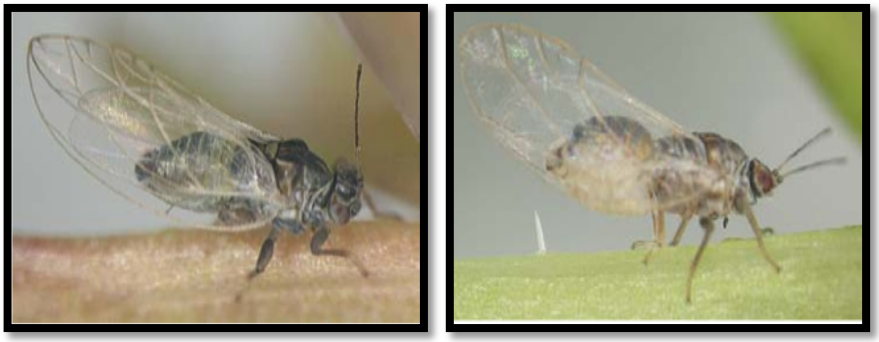

Fig. 10. Male (left) and female (right) *Bactericera trigonica* adults (photograph by Dr. F. Siverio, ICIA, Canary Island).

Although the biology of psyllid species, such as *B. cockerelli* (Pletsch, 1947; Butler & Trumble, 2012) and *B. tremblayi* Wagner (Hossein & Jafarloo, 2008), has been well described, little is known about *B. trigonica* biology. This species has two or three generations per year, feeds on carrots and related plants, and the adults overwinter in evergreen shrubs (Hodkinson, 2009).

In 2012, '*Ca*. L. solanacearum' was identified in carrots in Spain and associated with *B. trigonica* (Alfaro-Fernández *et al*., 2012a & b), but transmission assays were not performed.

2.4. '*Ca.* L. solanacearum' diagnosis and detection

The prevention of infection is essential to avoid the dissemination of the pathogens through different vehicles, such as contaminated propagative plant material, vectors, irrigation water, soil, etc., and the implementation of preventive measures requires highly sensitive, specific and reliable detection methods. The accurate detection of phytopathogenic organisms is crucial for all aspects of plant pathology, from basic research on the biology of pathogens to the control of the diseases associated with these microbes (López *et al*., 2009).

Traditionally, the available techniques for the detection and diagnosis of plant pathogenic bacteria have been microscopic observations, isolation, biochemical characterization, bioassays and pathogenicity assays, serology (ELISA) and molecular amplification tests. However, biological indexing, electron microscopy, some biochemical and staining tests and the conventional amplification of nucleic acids have been used for testing pathogens of the genus *Spiroplasma* and phytoplasmas. Over time, different methods, including biological indexing (Roistacher, 1991), chemifluorescence (Schwarz, 1968), TEM (Saglio *et al*., 1971), ELISA and immunofluorescence (Garnier *et al*., 1987) and dot-blot DNA hybridization (Villechanoux *et al*., 1992), have been developed for the detection of HLB-related '*Ca.* Liberibacter' species. For example, for the last 20 years, the detection of HLB bacteria through TEM has been the only reliable technique to undoubtedly confirm this disease and has been used to establish its

presence in many countries of Africa and Asia (Garnier & Bové, 1996).

Nucleic acid-based methods are sensitive, specific and facilitate the determination of genetic relationships. Currently, the most appropriate techniques for the detection of non-culturable '*Ca*. Liberibacter' pathogens are those based on polymerase chain reaction (PCR). Compared with conventional methods, PCR offers several advantages, as organisms do not need to be cultured before their detection, and this method is also rapid and versatile.

Conventional PCR has demonstrated sensitivity and specificity under optimized and controlled conditions; however, this method does not provide information about the amount of the pathogen in the sample. Moreover, conventional PCR also requires agarose gel electrophoresis, hybridization or colorimetric detection. Conventional PCR, a simple, quick and sensitive technique, was first adapted for the detection of '*Ca*. L. asiaticus' and '*Ca*. L. africanus' in the 1990s, based on the amplification of 16S rRNA (Jagoueix *et al*., 1994) or β operon (Hocquellet *et al*., 1999) sequences. Loop-mediated isothermal amplification (LAMP) (Okuda *et al*., 2005) and FISH assays (Fujikawa *et al*., 2013) have also been developed for '*Ca*. L. asiaticus' detection.

Real-time PCR facilitates the monitoring of a reaction as it progresses, eliminating the need to manipulate amplicons post-reaction, reducing the high risk of contamination. It also uses fewer reagents and less staff-time and provides additional data during the analysis. Real-time PCR has been demonstrated as an indispensable

tool widely used in a range of bacterial detection protocols and several real-time PCR-based protocols for the detection of '*Ca.* Liberibacter' species from citrus (Li *et al*., 2006; Teixeira *et al*., 2008; Coletta-Filho *et al*., 2010; Morgan *et al*., 2012; Fujikawa *et al*., 2013; Bertolini *et al*., 2014a), and these techniques have been made commercially available as complete kits. For the effective use of this method in the analysis of plant or environmental samples, efficient RNA and/or DNA extraction protocols are typically required, but the nucleic acid purification step can be totally circumvented using direct sample preparation methods, such as dilution or spot immobilization on membranes (Capote *et al*., 2009; De Boer & López, 2012) or partially circumvented using so-called "direct PCR" methods (Fujikawa *et al*., 2013). Tissue-print and/or squash systems on membranes (Bertolini *et al*., 2008; Vidal *et al*., 2012b) are real direct methods of sample preparation in which neither extract preparation nor nucleic acid purification are necessary. However, a major drawback of systems based on target immobilization is the small amount of sample that can be loaded onto the support. This limitation could be avoided by coupling these easy sample preparation methods with highly sensitive techniques, such as real-time PCR. Squash real-time PCR is a useful tool for the detection of nucleic acid targets in insect vectors and has been successfully used to detect '*Ca*. L. americanus' and '*Ca*. L. asiaticus' in *D. citri* specimens (Bertolini *et al*., 2014a).

In the last five years, several detection methods for '*Ca*. L. solanacearum' have been developed, including both conventional

and quantitative real-time PCR (Crosslin *et al*., 2011; Crosslin & Munyaneza 2009; Hansen *et al*., 2008; Li *et al*., 2009; Liefting *et al*., 2008 & 2009a; Lin *et al*., 2009; Lin *et al*., 2011a; Munyaneza *et al*., 2009b; Pitman *et al*., 2011; Ravindran *et al*., 2011a & b; Wen *et al*., 2009 & 2011). In addition, the detection of the bacterium in psyllids using PCR is relatively straightforward (Crosslin *et al*., 2011; Munyaneza *et al*., 2009b). The uneven distribution and variation in the '*Ca*. L. solanacearum' titer in different parts of infected potato plants has been observed, making the detection of this bacterium inconsistent using conventional PCR (Buchman *et al*., 2011b; Crosslin & Munyaneza, 2009; Li *et al*., 2009; Levy *et al*., 2011). '*Ca*. L. solanacearum' can reach a titer in tomato plants greater than 300 times that observed in potato plants (Sengoda *et al*., 2013).

Two multiplex PCR methods have been developed to detect '*Ca*. L. solanacearum' in plants, one utilizing a potato β-tubulin gene sequence (Wen *et al*., 2009) in a conventional PCR format and the other using a plant mitochondrial cytochrome oxidase gene sequence in a real-time PCR format (Lin *et al*., 2009). In addition to PCR primers specific for 16S and 23S rDNA genes, primers utilizing genomic DNA, such as a conserved bacterial housekeeping gene, adenylate kinase (Ravindran, *et al*., 2011a), have also been used. Amplification using these primers has been demonstrated to have a detection limit of approximately 0.65 ng. Genomic-based PCR primers have also been developed to facilitate the detection of '*Ca*. L. solanacearum' in the potato psyllid, and these primers have

been valuable in determining the frequency of the bacterium in vector species.

Few studies have investigated the efficiency of detecting '*Ca*. L. solanacearum' in infected potato plants. A recent study showed that the overall efficiency of the current PCR technology associated with the detection of true positives was as low as 60% (Wen *et al*., 2009). Detection efficiencies have generally been determined to be higher for the belowground portions of plants, such as stolons, roots, and tubers, as opposed to leaves, leaf petioles, and aboveground stems (Li *et al*., 2009; Wen *et al*., 2009). However, these results have been contradicted by recent studies showing '*Ca*. L. solanacearum' higher detection frequencies in the upper- and middle-tier leaves than in the lower aboveground tissues, although the belowground tissues were not assayed (Levy *et al*., 2011). The presence of PCR inhibitors in tuber tissues is problematic, and caution should be exercised when using tuber tissue to detect '*Ca*. L. solanacearum' infections (Lin & Gudmestad, 2013).

There has been interest in quantifying the development of '*Ca*. L. solanacearum' in hosts after infection. To this end, the expected amplicon for the PCR reaction was cloned in a plasmid, and the number of DNA copies was estimated. This method was useful for determining that 20 copies of the target 16S rDNA of '*Ca*. L. solanacearum' could be detected in the DNA extract of a zebra chip-affected plant using a quantitative PCR format. The application of this methodology for the analysis of naturally infected potato plants from the field showed that the majority of

plants harbored a '*Ca*. L. solanacearum' population of 10^5 to 10^6 cells/g of tissue (Li *et al*., 2009), and 92% of all field samples tested positive for '*Ca*. L. solanacearum' using real-time PCR, whereas only 40 to 46% tested positive using conventional PCR, depending on the primers used.

Several conventional and real-time PCR protocols have also been described for '*Ca*. L. solanacearum' detection in insect vectors. Using conventional PCR '*Ca*. L. solanaceraum' was detected in eggs, different nymph instars stages and in adults of *B. cockerelli* (Hansen *et al*., 2008). In addition, this bacterium was also detected using conventional PCR in field-collected and laboratory-reared *T. apicalis* in southern Finland (Munyaneza *et al*., 2010b). There are reports of '*Ca*. L. solanacearum' in unidentified psyllids species in the genera *Acizzia* and *Trioza* collected from *Acacia* and *Pittosporum* species and in foliage samples from these plant species in New Zealand (Scott *et al*., 2009). Furthermore, '*Ca*. L. solanacearum' has been detected in one specimen of the psyllid *T. chenopodii* collected from Russia; however, further testing of other specimens failed to confirm the presence of this bacterium (Kölber *et al*., 2010).

Despite recent improvements in PCR detection technology, there is still a need for improvement and validation. For example, the development of a multiplex PCR format to distinguish '*Ca*. L. solanacearum' haplotypes (Lin *et al*., 2012) and simultaneously determine potato psyllid biotypes (Jackson *et al*., 2009; Swisher *et al*., 2012) could be useful in epidemiological studies. Further

improvements in real-time PCR could also facilitate epidemiological studies of diseases caused by '*Ca*. L. solanacearum', as demonstrated with HLB-associated '*Ca*. Liberibacter' species in citrus (Hung *et al*., 2004; Li *et al*., 2006; Teixeira *et al*., 2008).

2.5. Control strategies

In the absence of other effective disease management strategies for phloem-limited bacteria, the exclusive use of plant material certified as free of the bacterium and the application of insecticides targeted against potato and carrot psyllids are the only means to effectively manage diseases associated with '*Ca*. L. solanacearum'. Current assays have focused on the efficacy of various insecticides on the life stages of the potato psyllid. For example, thiamethoxam and abamectin were demonstrated as effective in significantly reducing adult populations of *B. cockerelli,* although the residual effects on adult mortality were relatively short lived (approximately 24h) (Gharalari *et al*., 2009). Insecticides, such as spiromesifen, were demonstrated as effective against nymphs (Goolsby *et al*., 2007). Unfortunately, there is a lack of evidence that the use of insecticides to manage psyllid populations will effectively reduce the incidence and severity of zebra chip or other '*Ca*. L. solanacearum'-associated diseases under conditions where potato psyllid populations harbor high frequencies of '*Ca*. L. solanacearum' and a high disease prevalence is present on the crop. Thus, few studies involving the potato/tomato psyllid have

investigated the potential to improve host resistance and insecticides to effectively manage both the vector and the disease (Liu *et al*., 2006).

Current pest management practices rely on the use of insecticides to increase psyllid mortality, but these methods could also be used to effectively manage potato psyllid populations by impacting each life stage in ways that do not affect mortality. For example, some insecticides, such as imidacloprid, abamectin, and pymetrozine, have been shown to reduce probing durations while concomitantly increasing the amount of time adult psyllids spend off the leaf surface, suggesting that these insecticides also act as repellents. Perhaps more importantly, two of the insecticides used to manage psyllid populations, imidacloprid and abamectin, also significantly lowered the transmission of '*Ca*. L. solanacearum', which might reduce subsequent infections (Butler *et al*., 2011). However, the development of insecticide resistance compromises the ability of chemical treatments to effectively limit the rate of disease development, and some populations of potato psyllid with resistance to imidacloprid have been reported (Lin & Gudmestad, 2013).

Other disease management tactics, including the development of traps that attract adult psyllids through the use of volatile chemical attractants, have also been investigated (Guedot *et al*., 2010). Entomopathogenic fungi might also provide additional components for integrated pest management (Lacey *et al*., 2011), in addition to the use of kaolin particle films, horticultural mineral oil

treatments (Vidal *et al*., 2010) and other biorational insecticides, which have been demonstrated as useful in repelling all developmental stages of the psyllid (Peng *et al*., 2011; Yang *et al*., 2010b) or interfering with the transmission process. Biological control could also be an alternative. *B. cockerelli* has various natural enemies, including chrysopid larvae, coccinellids, geocorids, anthocorids, mirids, nabids, syrphid larvae, and parasitoids. (Munyazeza, 2012). Other parasitoids of psyllid species, including *Tamarixia radiata* and *Diaphorencyrtus aligharensis*, which attack *D. citri* (Bové, 2006) and *Tamarixia triozae,* and *Metaphycus psyllidis,* which attack *B. cockerelli* (Munyazeza, 2012), have also been identified. These potential alternative methods of management are a priority and have been extensively investigated (Lin & Gudmestad, 2013). Cultivation at insect-proof facilities is another option when the crop could support the cost of this strategy.

The future for disease management likely lies with disease-resistant cultivars. The development of zebra chip-resistant germplasm is an ongoing priority (Lin & Gudmestad, 2013). In addition, the European and Mediterranean Plant Pathogen Organization (EPPO) recommends that vegetative material for propagation and production should come from areas free of the psyllid vectors and bacteria. The propagating material could also be produced at insect-proof facilities. Seeds and ware potatoes should come from areas free of zebra chip disease. Alternatively high-grade seed potatoes might be imported under post-entry quarantine,

and ware potatoes might be imported only for industrial processing purposes (EPPO, 2013). However, there are no recommendations for the other bacterial hosts.

The successful management of new '*Ca*. L. solanacearum'-associated diseases requires the use of integrated protocols adapted to each specific condition, crop and vector for the different diseases associated with this recently discovered pathogen. Consequently, the development of strategies for different countries and hosts should be based on scientific knowledge and modified according to the available information.

Objectives

Objectives

'*Candidatus* Liberibacter solanacearum' is an emerging bacterium that is associated with several economically important diseases in horticultural crops. Little is known about its biological and epidemiological aspects in crops other than potato and tomato. Therefore, the general objective of this thesis was to evaluate the risks of transmission of '*Ca*. L. solanacearum' from carrot to carrot, celery, potato and tomato and to study the etiology of vegetative disorders of celery, their seed transmission and the insect vector species that are involved in the natural spread of the bacterium. Therefore, the following specific objectives were proposed:

1. To develop a real-time PCR method for '*Ca*. L. solanacearum' detection and use it as a tool to investigate whether vegetative disorders on celery are associated with bacterium detection.
   1.1. To develop a specific real-time PCR protocol and direct sample preparation methods for bacterium detection. Validate, by intra-laboratory performance studies, a complete kit for '*Ca*. L. solanacearum' detection;
   1.2. To investigate the presence of '*Ca*. Liberibacter'-like cells in celery using electron microscopy and assess whether the detection of the bacterium is associated with vegetative disorders in this species; and

1.3. To determine the '*Ca*. L. solanacearum' haplotype and to perform phylogenetic analysis to establish the relationships with other available sequences.

2. To investigate whether '*Ca*. L. solanacearum' is a carrot seedborne pathogen.
   2.1. To setup a real-time PCR method to detect the bacterium in carrot seed lots;
   2.2. To localize '*Ca*. Liberibacter'-like cells in carrot seeds using electron microscopy;
   2.3. To quantify the total bacterial cells, to determine the viability of the method and to perform experimental transmission studies; and
   2.4. To determine the '*Ca*. L. solanacearum' haplotype in carrot seeds and seedlings that emerge from infected seeds and to perform a phylogenetic analysis.
3. To search for potential vectors of '*Ca*. L. solanacearum' in different hosts.
   3.1. To monitor the arthropods that are visiting/landing on carrot, celery and potato;
   3.2. To identify the psyllid species that are collected in the fields; and
   3.3. To determine the different psyllid species that acquire the bacterium.
4. Assessment of '*Ca*. L. solanacearum' transmission by *Bactericera trigonica.*

4.1. To monitor, using Electrical Penetration Graphs, the ability of *B. trigonica* to feed continuously in the phloem of different host species; and

4.2. To evaluate the *B. trigonica* efficiency to experimentally transmit '*Ca*. L. solanacearum' from carrot to carrot, celery, potato and tomato crops.

Association of '*Candidatus* Liberibacter solanacearum' with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection

Teresani, G.R., Bertolini, E., Alfaro-Fernández, A., Martínez, C., Tanaka, F.A.O., Kitajima, E.W., Roselló, M., Sanjuán, S., Ferrándiz, J.C., López, M.M., Cambra, M., Font, M.I.

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Association of '*Candidatus* Liberibacter solanacearum' with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection

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Abstract

A new symptomatology was observed in celery (*Apium graveolens* L.) in Villena, Spain in 2008. Symptomatology included an abnormal amount of shoots per plant and curled stems. These vegetative disorders were associated with '*Candidatus* Liberibacter solanacearum' and not with phytoplasmas. Samples from plant sap were immobilized on membranes based on the spot procedure and tested using a newly developed real-time PCR assay to detect '*Candidatus* L. solanacearum'. Then, a test kit was developed and validated by intra-laboratory assays with an accuracy of 100 %. Bacterial-like cells with typical morphology of '*Ca*. Liberibacter' were observed using electron microscopy in celery plant tissues. A fifth haplotype of '*Ca*. L. solanacearum', named E, was identified in celery and in carrot after analyzing partial sequences of 16S and 50S rRNA genes. From our results, celery (Fam. *Apiaceae*) can be listed as a new natural host of this emerging bacterium.

Introduction

Celery (*Apium graveolens* L., Fam. *Apiaceae*) cultivation is of increasing interest in European countries. In Spain, there are about 1,640 ha of celery crops, mainly in the Mediterranean regions, which account for an annual production of 80,000 t. Celery is affected by fungal, viral and bacterial pathogens causing various well-known diseases (Schneider *et al*., 2013). In 2008 in Villena, Alicante, Spain, celery plants showed symptoms that had not been observed before i.e. abnormal amount of shoots, curling of stems and yellowing (Figure 1 A, B, C, D). These vegetative disorders were observed in the crop in the three overlapping cultivation cycles (early, medium and late) from March to November, in cvs. 'Loretta', 'Monterrey' and 'Imperial' of var. dulce (Mill.). Only severe stunting appeared in cv. Brillant of var. rapaceum (Mill). Consequently, there was a relevant yield reduction and economic losses from 2008 to 2009 in Villena, followed by other Spanish celery growing regions. Celery was grown in these areas together with carrot (*Daucus carota,* Fam. *Apiaceae*) in plots that exhibited a high prevalence of '*Candidatus* L. solanacearum' infected plants (EPPO, 2013; Munyaneza *et al*., 2010a).

'*Ca.* L. solanacearum', also named as '*Ca.* L. psyllaurous' (Hansen *et al.,* 2008) is a Gram-negative bacterium. It cannot be cultured *in vitro* yet. It is restricted to the plant's phloem, transmitted through vegetative propagation and naturally by several psyllid species (Munyaneza, 2012). '*Ca.* L. solanacearum' causes a

disease affecting potato (zebra chip) (Secor *et al*., 2009). Besides causing disease in potato, '*Ca.* L. solanacearum' can cause serious damage and economic losses in tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), eggplant (*S. melongena*), tamarillo (*S. betaceum*), tomatillo (*Physalis peruviana),* tobacco (*Nicotiana tabacum),* carrot and weeds in the *Solanaceae* family (EPPO, 2013; Munyaneza, 2012).

The bacterium is transmitted in a persistent (transovarial) way by the psyllid *Bactericera cockerelli* (Munyaneza *et al*., 2007a). It has also been detected in the psyllids *Trioza apicalis* (Munyaneza *et al*., 2010b), *B. trigonica* (Alfaro-Fernández *et al*., 2012b), other *Trioza* species and in *Accizia* species (Scott *et al*., 2009).

Conventional and real-time PCR methods have been developed to detect and/or identify '*Ca.* L. solanacearum' in plant material and insect vectors (Crosslin *et al*., 2010; Hansen *et al*., 2008; Li *et al*., 2009; Liefting *et al*., 2009b; Lin *et al*., 2009; Munyaneza *et al*., 2010b; Ravindran *et al*., 2011a; Ravindran *et al*., 2011b; Wen *et al*., 2011). In most bacterial models, real-time PCR has advantages over conventional PCR since it is more sensitive and reliable (De Boer & López, 2012; Li *et al*., 2009). The PCR templates require extract preparation and nucleic acid purification, which is laborious, time consuming and increases the risk of contamination (Olmos *et al*., 1996). Nucleic acid purification step can be overcome using direct sample preparation methods such as tissue-print and/or squash or spot immobilization on membranes (De Boer & López, 2012). The main drawback of target immobilization

is the small amount of target nucleic acid available on the support. This limitation can be offset by coupling these preparation methods with highly sensitive techniques such as real-time PCR (Bertolini *et al*., 2008).

Fig. 1. Severe symptoms in celery plants associated with '*Candidatus* Liberibacter solanacearum'. Abnormal amount of shoots and curling of stems (left) compared with a symptomless plant (right) (A). Proliferation, abnormal amount of shoots (B). Curling of stems (left) compared with a normal stem (right) (C). Mild symptoms in marketable plants (D).

In this paper, we present the first formal scientific report of the presence of '*Ca.* L. solanacearum' in celery in Spain and describe its haplotype and the symptoms that it causes in celery. A detection method for '*Ca.* L. solanacearum' using sample immobilization on membranes and a complete kit for accurate detection of the bacterium based on universal '*Ca.* Liberibacter' primers and a specific probe for real-time PCR are developed and validated, as well.

Materials and Methods

Plant material and prevalence of symptoms

About 37 ha of celery crops were annually inspected by technical staff from Agrícola Villena cooperative (Villena Coop. V., which produces 20% of the fresh celery and carrots in Spain). Inspections were conducted on the field and in the packing house, from 2008 to 2012 at Villena, Alicante, Spain to estimate the percentage of field plants showing symptoms and the number of packing house discards. During this period, 2,655 celery plants were randomly collected from experimental plots to estimate the prevalence of the disorder and/or '*Ca.* L. solanacearum'.

In September 2010, at harvest, 502 plants of the celery cvs. 'Loretta', 'Imperial' and 'Monterrey' from experimental plots were classified into three categories according to the severity of the symptoms. The classification was as follows: i) 174 plants as '+++' with severe symptoms making the celery unmarketable (Figure 1 A

and B), ii) 150 plants as '+' with mild symptoms that could be marketed, and iii) 178 symptomless and marketable plants. Each sample was tested for '*Ca.* L. solanacearum' and phytoplasmas to investigate the etiology of the disorders. Healthy and diseased carrot plants of cvs. Maestro and Bangor, growing together with celery, that tested negative and positive, respectively, against '*Ca.* L. solanacearum' were collected in April 2010. The plants were transplanted to pots and grown in a greenhouse (P2 level of biological containment) and used as controls.

Sample preparation

Leaf samples (about 1g/plant) were collected from the middle part of celery and carrot plants into separate plastic bags and stored at 4ºC for up to one week until use. Extracts were prepared using a Homex 6 (Bioreba, Switzerland) homogenizer, grinding the plant material 1:5-10 (w/v) in PBS extraction buffer (NaCl, 8 g/l; $NaH_2PO_4.2H_2O$, 0.4 g/l; $Na_2HPO_4.12H_2O$, 2.7 g/l; pH 7.2). One ml of extract from each plant was stored at -20^{o}C until use.

Direct sample preparation without DNA purification (spot procedure)

Freshly prepared or frozen plant extracts were immobilized on membranes (Olmos *et al*., 1996) by spotting 5 µl of crude plant extract onto pieces (approximately 0.5 cm^2) of positively charged nylon membrane (Roche, Mannheim, Germany) or Whatman 3MM filter paper (GE Healthcare Europe GmbH, Freiburg, Germany), in

Eppendorf tubes (Capote *et al*., 2009). Spotted plant extracts were left to dry for 5 min and then stored at room temperature in the dark until required. The DNA was released from each membrane by adding 100 µl of distilled water per tube (Bertolini *et al*., 2010). Each tube was then vortexed and placed on ice. Then, 3 µl from this preparation was used as the template for real-time PCR.

DNA purification

Total DNA was purified from 200 µl of crude plant extract by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol or the CTAB (cetyl trimethyl ammonium bromide) protocol (Murray & Thompson, 1980). Purified DNA was stored at -20 ºC until use. These DNA templates were considered as the "gold-standard" when comparing the conventional method using purified DNA and the direct methods of sample preparation without DNA purification.

Comparison of sample preparation methods using naturally infected celery plants: DNA extraction *vs*. spot

The 502 plants of the celery cvs. 'Loretta', 'Imperial' and 'Monterrey' from experimental plots (described above) were tested using two sample preparation methods: DNA extraction (considered as the gold standard technique) (Murray & Thompson, 1980) and the spot procedure (Olmos *et al*., 1996). Cohen's Kappa (κ) coefficient and the Bias-adjusted Kappa (BAK) index were used to calculate the coincidental results between methods (Byrt *et al.,*

1993). Both sample preparation methods were compared using the same plant extract and the newly developed real-time PCR protocol (see below).

Probe design

Nucleotide sequences internal to patented universal '*Ca.* Liberibacter' species forward CaLsppF (5'-GCAGGCCTAACACATGCAAGT-3'), and reverse CaLsppR 5'-(GCACACGTTTCCATGCGTTAT-3') (Bertolini *et al.,* 2010) primers were selected to design a '*Ca.* L. solanacearum' specific probe. Alignment of nucleotides based on the 16S rDNA sequences of '*Ca.* L. solanacearum' recovered from GenBank, was performed. Primer Express software (Applied Biosystems, Foster City, CA, USA) was used to design the specific CaLsolP probe. The probe was labeled with fluorescent dyes 5`FAM-AGCGCTTATTTTTAATA GGAGCGGCAGACG-3` TAMRA.

Real-time PCR

Real-time PCR using CaLsppF and CaLsppR primers and the newly designed (CaLsolP) TaqMan probe was carried out using two real-time PCR systems: StepOne Plus (Applied Biosystems) and Light Cycler 480 (Roche). Various concentrations of primers (from 0.3 to 1.0 μM) and probe (from 80 to 300 nM) were used to tested the optimum amplification conditions. The optimum reaction mix consisted of 1 x Path-IDTM qPCR master mix (Ambion, Grand Island, NY, USA), 0.5 μM of each CaLsppF and CaLsppR primers

150 nM of CaLsolP TaqMan probe and 3 µl of purified DNA or DNA from the spotted samples, in a final volume of 12 µl. Purified DNA and spots of crude extracts prepared from symptomatic carrot and/or celery plants infected with '*Ca.* L. solanacearum' were used as positive controls in each PCR reaction. Purified DNA and spots of crude extracts from healthy carrot and/or celery plants, DNAse free distilled water and non-spotted pieces of membrane were used as negative controls in each PCR assay. The real-time PCR amplification protocol consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data acquisition and analysis were performed with the thermal cycler's software. The default threshold set by the machine was slightly adjusted above the noise in the linear part of the growth curve. This new real-time assay was compared with the procedure of Li *et al*. (2009).

Specificity and sensitivity

One hundred and ten reference '*Ca*. L. solanacearum' DNA samples from 10 different origins, 17 DNA samples of '*Ca*. Liberibacter' species other than '*Ca*. L. solanacearum', 16 strains of bacterial species that affect solanaceous and other crops and 81 unidentified bacterial isolates from celery, carrot and potato microbiota (Table 1), were used to test specificity of the new real-time assay. Sensitivity was tested using serial dilutions of a '*Ca*. L. solanacearum' positive celery extract cv. 'Loretta' (prepared by homogenizing 1:10 (w/v) plant material in PBS buffer pH 7.2) in a healthy celery extract of the same cultivar (negative to '*Ca*. L.

solanacearum' by PCR). A sample of each dilution was spotted on Whatman 3MM paper and/or used for DNA purification. Spotted extracts and purified DNA were used to compare four conventional (see below) and two real-time PCR protocols.

Table 1. '*Candidatus* Liberibacter' species used for detection in various plant hosts using real-time PCR.

Bacterial isolates and strains	Host – year	N	Origin (reference)	PCR
'*Ca.* Liberibacter solanacearum'	*Apium graveolens - 2010*	10	Villena-Spain (IVIA[a]-A10.1-10)	+
'*Ca.* Liberibacter solanacearum'	*A. graveolens - 2011*	10	Villena-Spain (IVIA[a]-A11.1-10)	+
'*Ca*. Liberibacter solanacearum'	*A. graveolens - 2012*	15	Villena-Spain (IVIA[a]-A12.1-15)	+
'*Ca*. Liberibacter solanacearum'	*Bactericera trigonica - 2010*	10	Canary Islands-Spain (ICIA[b]-Bt10.1-10)	+
'*Ca*. Liberibacter solanacearum'	*B. trigonica – 2011*	10	Canary Islands-Spain (IVIA[a]-Bt11.1-10)	+
'*Ca*. Liberibacter solanacearum'	*B. nigricornis - 2012*	10	La Rioja-Spain (IVIA[a]-Bn 12.1-10)	+
'*Ca*. Liberibacter solanacearum'	*Daucus carota - 2010*	15	Villena-Spain (IVIA[a]-Z10.1-15)	+
'*Ca*. Liberibacter solanacearum'	*D. carota - 2010*	10	Canary Islands-Spain (ICIA-Z10.1-10)	+
'*Ca*. Liberibacter solanacearum'	*D. carota - 2011*	10	Villena-Spain (IVIA[a]-Z11.1-10)	+
'*Ca*. Liberibacter solanacearum'	*D. carota - 2012*	10	Canary Islands-Spain (ICIA[b]-Z12.1-10)	+
'Ca. Liberibacter americanus'	*Catharanthus roseus*	1	Brazil*	-
'Ca. Liberibacter americanus'	*Citrus* sp.	1	Brazil*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Brazil	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	China*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Philippines*	-

'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Philippines (PH 70)*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Florida-USA*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	India (Poona 70)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	India (Poona 70)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Indonesia-Bali*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Malaysia (95-1)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Mauritius Island (95-11)*	-
'Ca. Liberibacter africanus'	*Citrus* sp.	1	Reunion Island-France (USA-7)*	-
'Ca. Liberibacter africanus'	*C. roseus*	1	South Africa (AFS 84)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Taiwan (LK 70)*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Thailand (TH 88)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Vietnam (95-35)*	-
Agrobacterium tumefaciens	*Prunus* sp.	1	USA (C 58)	-
Clavibacter michiganensis subsp. *michiganensis*	*Solanum lycopersicum*	1	Spain (IVIA 873-6)	-
C. michiganensis subsp. *sepedonicus*	*S. tuberosum*	1	USA (NCPPB 2140)	-
Dickeya sp.	*S. tuberosum*	1	Spain (IVIA[a] 1374-13)	-
Dickeya sp.	*S. tuberosum*	1	Spain (IVIA[a] 2688-1-2)	-
Pectobacterium atrosepticum	*S. tuberosum*	1	United Kingdom (SCRI 1001)	-
Pectobacterium carotovorum	*S. tuberosum*	1	United Kingdom (SCRI 194)	-
Pseudomonas cichorii	*Lactuca sativa*	1	Spain (IVIA[a] 593)	-

Pseudomonas corrugata	*S. lycopersicum*	1	UK (NCPPB 2445)	-
Pseudomonas mediterranea	*S. lycopersicum*	1	Spain (IVIA[a] 592-4-4)	-
Pseudomonas syringae pv. *syringae*	*Pyrus communis*	1	Spain (IVIA[a] 773-1)	-
Pseudomonas syringae pv. *tomato*	*S. lycopersicum*	1	Spain (IVIA[a] 1001-1a)	-
Ralstonia solanacearum	*S. tuberosum*	1	Spain (IVIA[a] 1602)	-
Rhodococcus fascians	*Dahlia sp.*	1	France (CFBP 41)	-
Rhodococcus sp.	*Nicotiana tabaccum*	1	Spain (IVIA[a] 4264)	-
Xanthomonas vesicatoria	*S. lycopersicum*	1	Spain (IVIA[a] 3617)	-
Microbiota strains	*S. tuberosum*	50	Spain (IVIA[a] 1F to 50F)	-
Microbiota strains	*D. carota*	21	Spain (IVIA[a] 3925-1 to 3925-21)	-
Microbiota strains	*A. graveolens*	10	Spain (IVIA[a] AM-1 to AM-10)	-

*From INRA-Bordeaux, France, provided by Prof. J.M. Bové
[a)] IVIA - Instituto Valenciano de Investigaciones Agrarias
[b)] ICIA - Instituto Canario de Investigaciones Agrarias
[c)] NCPPB – National Collection of Plant Pathogenic Bacteria
[d)] SCRI – Scottish Crop Research Instituto

Conventional PCR protocols

Four previously described conventional PCR protocols using different primers were compared: OA2 and OI2c (Liefting *et al.,* 2009a); Lso TX F and Lso TX R; Lso adk F and Lso adk R (Ravindran *et al.,* 2011a); LsoF and OI2c (Li *et al.,* 2009). Amplifications were performed as described by these authors. Purified DNA and spots of crude extracts prepared from symptomatic carrot and/or celery plants infected with '*Ca.* L.

solanacearum' were used as positive controls in each PCR reaction. Purified DNA and spots of crude extracts from healthy carrot and/or celery plants, DNAse free distilled water and non-spotted pieces of membrane were used as negative controls in each PCR assay.

Intra-laboratory validation of a complete kit

A complete kit (Ref. CaLsol/100; Plant Print Diagnòstics SL, Valencia, Spain) for accurate detection of the bacterium was developed based on the new real-time assay and primers (Bertolini *et al.,* 2010). The kit used lyophilized master mix and was based on a direct method of sample preparation. The kit was tested in three different laboratories at Instituto Valenciano de Investigaciones Agrarias (IVIA), using three different real-time PCR systems (StepOne Plus and LightCycler 480 described earlier and SmartCycler-Cepheid). Ten blind samples immobilized on Whatman 3MM filter paper were used: 5 positive for '*Ca.* L. solanacearum' and 5 negative. Template preparation was performed according to the kit manufacturer's instructions. Positive samples were '*Ca.* L. solanacearum' infected extracts from carrot and celery plants. Negative samples were extracts from healthy carrot, celery, potato, *Nicotiana benthamiana* and periwinkle. The amplification conditions for the kit were 4°C higher than those described previously for real-time amplification using fresh (non-lyophilized) master mix. Six replicate reactions were performed on each sample. Diagnostic parameters (sensitivity, specificity and accuracy) were calculated according to https://www.antonio-olmos.com/parameters/

online/calculator.html. Positive or negative results and Ct values were recorded for each sample.

Association of '*Ca.* L. solanacearum' and/or phytoplasmas with symptoms

The 502 plants of celery from experimental plots (described above) were tested for '*Ca*. L. solanacearum' using the real-time PCR kit described in this paper and for phytoplasmas using de procedure of Hren *et al*., (2007). Results were compared using multinomial regression analysis ('Program R' www.r-project.org). Phytoplasmas were included in this study because they can cause symptoms similar to those caused by '*Ca.* L. solanacearum' on some plant species.

Electron microscopy

Leaf midribs from symptomatic plants that had tested real-time PCR positive for '*Ca*. L. solanacearum' from commercial celery fields were prepared for transmission (TEM) and scanning (SEM) electron microscopy. For TEM analyses, midribs were first fixed in 'Karnovisky' solution for 24 h, then fixed with osmium tetroxide in 1% cacodylate buffer for 1 h and contrasted with uranyl acetate 0.5% overnight. Samples were then dehydrated using increasing concentrations of acetone (30-100%). Infiltration and embedding was performed in 1:1 acetone (100%): 'Spurr' epoxy resin for at least 5 h and then in pure resin 'Spurr' overnight or longer, depending on the infiltration capacity of the samples.

Polymerization was carried out at 70°C for three days. The resin blocks were cut into 70 nm sections (using an ultramicrotome with diamond knife), which were placed on copper´s screens, contrasted with uranyl acetate 3% and lead citrate (Reynolds *et al.,* 1963). Samples were examined using a JEOL JEM 1011 transmission electron microscope and images were captured using a digital camera.

For SEM analyses, midribs were fixed with 'Karnovisky' solution for 24 h, infiltrated with glycerol, immersed in liquid nitrogen and fractured with a scalpel. Fractured pieces were post-fixed in 0.1% OsO_4 for 1 h, dehydrated in acetone, dried at the critical point, gold coated by sputtering and examined in a LEO 435 VP scanning electron microscope (Tanaka *et al.,* 2006).

Phylogenetic analyses

The Phylogenetic analyses were performed to determine haplotypes and to establish the relationship with other available sequences of '*Ca.* L. solanacearum' in different regions and hosts. '*Ca.* L. solanacearum' PCR products from celery and carrots, amplified with 16S rDNA OA2/OI2c primers (1,168 pb) and with 50S rDNA rplj CL514F/CL514R primers (669pb) (Munyaneza *et al.,* 2009b), were purified using High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and sequenced**.** Sequences were compared with 23 sequences from other hosts in GenBank (NCBI). Nucleotide sequences were aligned using Clustalw software implemented in Geneious program. Phylogenetic trees were inferred

using MEGA 5.1, and the neighbor-joining algorithm (Kumar *et al.,* 2004), with 1,000 bootstrap replicates.

Six '*Ca.* L. solanacearum' sequences from celery and two from carrot based on 16S rRNA, 16S/23S ISR and 50S rRNA genes obtained in this work and four sequences from the previously described haplotypes (A, B, C and D), were aligned within the respective gene regions using Geneious program. The SNPs were visually identified and annotated. A phylogenetic tree was constructed with MEGA 5.1, using the UPGMA algorithm.

Results

Prevalence of symptoms and '*Ca.* L. solanacearum' detection

The prevalence of symptoms in celery cultivars in Villena was: 50% (2008), 60% (2009), 32% (2010), 0.8% (2011) and 0.1% (2012). The cv. 'Loretta' was the most susceptible, showing symptoms in up to 70% of the plants in 2009 and 2010. The cv. 'Imperial' followed by the cv. 'Monterrey' were less susceptible. The number of positive plants for '*Ca*. L. solanacearum' out of the total number of randomly tested plants was 32/52 (62%) in 2009, 252/503 (50%) in 2010, 82/900 (9%) in 2011 and 6/1200 (0.5%) in 2012.

Real-time PCR assay, specificity and sensitivity

The new real-time PCR assay using CaLsolP probe and CaLsppF/CaLsppR primers was specific for '*Ca.* L. solanacearum´ targets with a PCR product of 111 bp. All reference '*Ca.* L. solanacearum' isolates (Table 1) were amplified. No amplification occurred with 17 '*Ca.* Liberibacter' isolates causing citrus huanglongbing (HLB) disease, 16 bacterial species that affect tomato, potato, pepper and other crops, or from 81 microbiota isolates from celery, carrot and potato.

Using purified DNA (from infected celery) only two out of the four conventional PCR protocols detected '*Ca.* L. solanacearum' (Table 2). The protocol of Ravindran *et al.* (2011a) that uses Lso TX F and Lso TX R primers was the most sensitive, amplifying down to a 10^{-2} dilution. However, the sensitivity of the real-time PCR assays (i.e. the new assay described in this paper and the assay of Li *et al.* (2009) was higher than with conventional PCR, amplifying down to a 10^{-3} dilution. Using spotted samples, no amplification was obtained with conventional PCR assays and only real-time PCR assays detected '*Ca.* L. solanacearum' down to a 10^{-2} dilution.

Table 2. Comparison of the sensitivity of conventional and real-time PCR-based protocols using purified DNA and the spot procedure to detect '*Candidatus* Liberibacter solanacearum' in infected celery extract.

	PCR protocol	DNA extracted from crude extract serial dilutions							Direct spot from crude extract serial dilutions						
		1	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	1	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Conventional	Liefting *et al*., 2009	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	Li *et al*., 2009 (Lsof/OI2c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ravindran *et al.* 2011(adk)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ravindran *et al.2011* (TX)	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Real-time	Li *et al*., 2009 (Lsof/HLBr)	22.8	25.7	28.4	33.7	34.5	-	-	28.8	32.5	34.5	-	-	-	-
	This paper	23.6	26.5	28.3	31.6	32.5	-	-	28.8	28.2	34.0	-	-	-	-

* Positive sample (Ct)

Comparison of sample preparation methods using naturally infected celery plants: DNA extraction *vs.* spot

Of 502 samples tested using the new real-time PCR assay, 151 samples were positive using both DNA extraction and spot procedure. One hundred samples were positive using only purified DNA and one sample was positive using only spot procedure. The remaining samples were negative independent from the sample preparation method used. The coincidence between methods was 80%. The estimated prevalence (positive by both methods) was

30%. The Cohen's kappa coefficient (κ) was 0.60 ± 0.040 and the Bias-adjusted Kappa (BAK) index was 0.58 ± 0.040.

The Ct values from spotted samples on nylon or paper membranes were similar (data not shown). In addition, no differences were found between DNeasy or CTAB (data not shown).

Intra-laboratory validation of a complete kit

'*Ca.* L. solanacearum' was detected only in the "blind" positive samples at Ct values ranging from 29.8 ± 2.2 to 37.2 ± 1.5 in all three laboratories (data not shown). Similar Ct values were obtained with the different thermal cyclers. The calculated diagnostic parameters (sensitivity and specificity) were 1.0 indicating a 100 % accuracy for the kit.

Association of '*Ca.* L. solanacearum' and/or phytoplasmas with symptoms

The percentage of celery plants positive for '*Ca*. L. solanacearum' in each symptom classification group were: 86% in plants with severe symptoms (+++), 42% in plants with mild symptoms (+) and 21% in symptomless plants (Table 3). Multinomial logistic regression analysis showed that the only significant independent variable ($Pr < 2^{-16}$) was the presence of '*Ca.* L. solanacearum' whereas the presence of phytoplasmas (Pr = 0.84) did not affect the model.

Table 3. Association of symptom intensity in celery plants grown in experimental plots and detection of '*Ca.* Liberibacter solanacearum' and/or phytoplasmas by real-time PCR

Symptoms	Real-time PCR			
	'*Ca.* L. solanacearum'		Phytoplasmas	
	Positive	Negative	Positive	Negative
+++[a]	150[b]	24	28	146
+	63	87	25	125
-	38	140	26	152
Total	251	251	79	365

[a] Celery plants with severe symptoms (unmarketable) (+++), with mild symptoms (+) and without symptoms (-).
[b] Number of plants that were positive or negative for '*Candidatus* Liberibacter solanacearum' or phytoplasmas by real-time PCR.

Electron microscopy observations

Bacteria-like organisms (BLOs) were observed using SEM in the phloem sieve tubes of celery samples that had tested real-time PCR positive for '*Ca.* L. solanacearum' (Figure 2 A and B). The BLOs were neither observed in the associated companion or mesophyll cells, nor in symptomless celery plants testing negative for '*Ca.* L. solanacearum'. Using TEM, observed BLO cells were pleomorphic and surrounded by an electron dense cell wall separate from the cytoplasmic membrane, which was slightly rippled, wrinkled or uneven (Figure 2 C and D). The BLO cells were triple-layered membrane (i.e. outer cell wall membrane and the inner cytoplasmic) suggesting the presence of '*Ca.* Liberibacter' cells

(Figure 2 D) rather than phytoplasma cells, which do not have a cell wall.

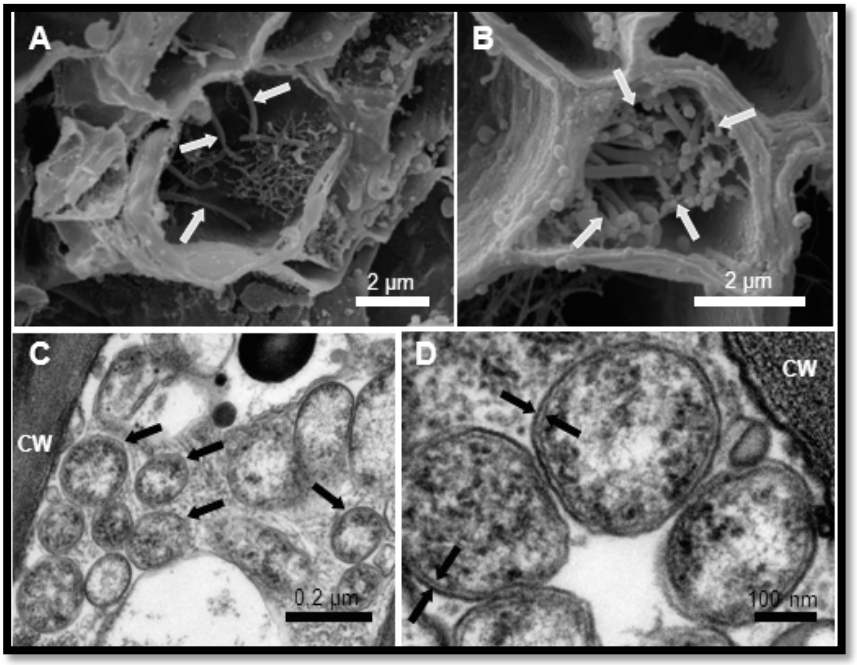

Fig. 2. Scanning (SEM) and transmission (TEM) electron microscopy photomicrographs of celery tissue samples. Presence of bacteria-like organisms (BLOs) (white arrows) in the phloem sieve tubes analyzed by SEM (A and B). TEM studies shows BLO (black arrows) individual cells pleomorphic in shape and surrounded by an electron dense cell wall separate from the cytoplasmic membrane, slightly rippled and wrinkled (C and D). Cells showing a triple-layered ultrastructure of both the outer cell wall membrane and the inner cytoplasmic membrane (between two black arrows) suggesting the presence of '*Candidatus* Liberibacter'-like cells (D). CW- plant cell wall.

Phylogenetic analyses

Based on bootstrap consensus phylogenetic trees of the 16S and 50S rRNA genes, celery strains grouped into two clusters for 16S rRNA (data not shown). The first cluster contained the Spanish celery isolates collected in 2007, 2009 and 2010, the carrot isolates from Finland and Spain, and one Spanish isolate recovered from *B. trigonica*. The second cluster contained the celery and carrot isolates collected in Spain in 2011. These clusters belonged to a broad cluster which was separated from another cluster that grouped all the tomato isolates from USA. For 50S rDNA, celery isolates were grouped into the same clusters as for 16S rDNA (data not shown). Table 4 is based on a previous report (Nelson *et al.,* 2012) with the addition of descriptions of SNPs for the new haplotype numbered as 5 and named E. Today, this haplotype is represented by isolates from celery and carrot plants grown in Spain in 2011.

Table 4. SNP differences between haplotypes. The reference sequence for the 16S and 23S rRNA genes is EU 812559.1 and for 50S rRNA gene is EU 834131.1. Nucleotide numbers count from the beginning of the reference sequence, haplotypes A, B, C and D as previously described by Nelson *et al*. 2011 and 2012.

Description	Gene region	Haplotypes				
		A	B	C	D	E
116 C>T	**16S rRNA**	C	C	C	T	C
151 A>G		A	A	A	A	G
212 T>G		T	C	T	T	T
581 T>C		T	C	T	T	T
959 C>T		C	C	C	C	T
1039 A>G		A	A	G	G	A

1073 G>A		G	G	G	A	G
1620 A>G	**ISR-23S**	A	A	A	A	G
1632 G>A		A	A	A	A	G
1648 G>A		G	G	G	G	A
1742 A>G		A	A	A	G	A
1748 C>T		C	C	C	T	C
1858_1859insG		-	G	G	-	-
1859_1860insT		-	T	-	-	-
1860_1861delT		T	T	T	-	T
1873 A>G		A	A	A	A	G
1920 T>C		T	T	C	T	T
583 G>C	**50S rRNA**	G	G	C	G	G
622 A>G		A	A	A	G	A
640 C>T		C	C	T	C	C
669 G>C		G	C	G	G	G
689 C>T		C	C	C	T	T
691 G>T		G	T	T	G	G
700 A>G		A	A	A	G	A
712 G>T		G	T	G	G	G
722 G>A		G	G	G	G	A
749 C>A		C	C	C	A	C
780_781insA		-	-	A	A	A
785 G>A		G	A	G	G	G
849 T>C		T	T	T	C	C
909 T>C		T	C	C	C	C
920 T>C		T	C	C	C	T
922_923insTGT		-	-	TGT	-	-
955 G>T		G	G	T	G	G
987 T>G		T	G	G	G	G
993 A>G		A	A	G	A	A
1041 G>A		G	A	A	G	G
1049 A>G		A	G	A	A	A
1107 G>A		G	A	G	G	G
1110_1111insC		-	-	C	-	-
1122 G>A		G	A	A	A	A
1143 G>A		G	A	G	G	G

[a] The reference sequence for the 16S and 23S ribosomal RNA (rRNA) genes is EU 812559.1 and for 50S rRNA gene is EU 834131.1. Nucleotide numbers count from the beginning of the reference sequence (haplotypes A, B, C, and D), as previously described by Nelson et al. (2011 & 2012).

A cladogram (Figure 3) on the 16S rRNA gene shows the divergence of the different haplotypes described up to now. GenBank accession numbers were KF737346 (Celery, Spain, 16S rDNA) and KF737348 (Carrot, Spain, 16S rDNA).

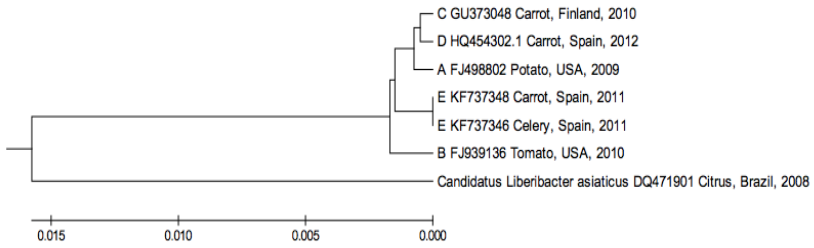

Fig. 3. Cladogram of '*Candidatus* Liberibacter solanacearum' haplotypes on the 16S rRNA gene segment (1011 bp) with '*Candidatus* Liberibacter asiaticus' as the outgroup using the UPGMA algorithm. GenBank accession number and origin are indicated.

Discussion

Species of '*Ca*. Liberibacter' are emerging plant pathogens associated with economically important diseases (Bové, 2006; Munyaneza, 2012) but their causes are still not clear. Although, '*Ca.* L. solanacearum' is only pathogenic to *Solanaceae* (Liefting *et al.,* 2009b), it has also been associated with diseases in other botanical families such as *Apiaceae* in different geographical areas (Munyaneza, 2012). The emergence of the disease in celery crops may be linked to the proximity of infected carrots with '*Ca.* L. solanacearum', to the presence of *B. trigonica* populations (Alfaro-Fernández *et al.,* 2012a & b, Villaescusa *et al.,* 2011) and probably

to the existence of other psyllid vector species. Infection of carrots with '*Ca.* L. solanacearum' in different geographical areas (i.e. Finland, France, Spain, and Sweden) suggests that carrot seeds might be the first source of inoculum in these countries, despite the fact that seed transmission of '*Ca.* L. solanacearum' has not been reported yet (Munyaneza, 2012). The bacterium '*Ca.* L. solanacearum' was consistently detected in symptomatic celery plants using conventional PCR and confirmed by sequencing. Phytoplasmas belonging to Aster yellows and Stolbur groups and *Celery mosaic virus* (Font *et al*., 2010) were sporadically detected (data not shown).

During the study period, the prevalence of symptoms in celery decreased markedly from >30% plants in 2008-2010 to <1% plants in 2011-2012. These figures corresponded to a decrease in '*Ca.* L. solanacearum' detection from 62-50% in 2009-2010 to 9-0.5% in 2011-2012. The summer of 2008 was the coldest within the 2008-2012 period, showing only a few peaks above 30ºC. These temperatures probably favored the multiplication of '*Ca.* L. solanacearum' in plants as well as in psyllid vectors. In the following years, temperatures above 30ºC were frequent. These high temperatures may have resulted in a decrease in the prevalence of the bacterium. The fact that '*Ca.* L. solanacearum' was detected in greenhouse plants grown at 15-25ºC but not in the same plants after one month at about 30ºC (data not shown), indeed suggests that high temperature may affect prevalence of the pathogen (Lopes *et al*.,

2009; Munyaneza *et al*., 2012) and is worthy of further investigation.

The designed TaqMan probe was specific to '*Ca.* L. solanacearum'. The new real-time PCR protocol and the one previously described (Li *et al*., 2009) showed similar sensitivity; being higher than the most sensitive conventional PCR protocol (Ravindran *et al*., 2011a). Using DNA extraction, the detection level was the 10^{-4} dilution of the celery extract. Detection levels decreased to 10^{-2} dilution when spots of crude plant extracts were used as template. Real-time PCR is currently considered the most accurate and reliable method for detection and diagnosis of plant pathogenic bacteria (De Boer & López, 2012; Li *et al*., 2009; López *et al*., 2009). The differences in sensitivity observed between conventional and real-time PCR in this work are related to the different levels of detection of the utilized protocols. Our results are in agreement with previously described comparative assays for '*Ca.* L. solanacearum' detection in which real-time PCR was 10 to 100 fold more sensitive than conventional PCR using different primers and protocols (Li *et al*., 2009).

The use of friendly direct methods of sample preparation prior real-time PCR is highly recommended for large-scale use (De Boer & López, 2012). In this case, we used a spot procedure as in other validated methods (Bertolini *et al*., 2008; Byrt *et al*., 1993; Olmos *et al*., 1996; Vidal *et al*., 2012b) for '*Ca*. L. solanacearum' detection. The sensitivity and specificity of the conventional preparation of celery samples based on DNA extraction was equal to 1.0 and it was

considered as a gold standard, independent from the false positives inherent to high sensitivity (López *et al*., 2009). Nevertheless, spot's sensitivity (0.60) and specificity (0.99) guarantee a low number of false positives. The calculated Cohen's kappa coefficient and the BAK index showed a moderate concordance between methods as defined by Landis and Koch (Landis & Koch, 1977). The agreement between spotted samples and the gold standard, the simplicity in the preparation and the considerably lower cost compared with DNA purification, confirm the appropriateness of the spot method for detection of '*Ca*. L. solanacearum' and for large-scale screening purposes.

The efficacy of a commercially available complete kit was demonstrated through intra-laboratory performance studies, showing an excellent sensitivity and specificity. The recommended kit's real-time protocol differs from the protocol that uses fresh master mix in four degrees (64ºC instead of 60ºC) for the amplification reaction. In fact, in previous assays lyophilization was shown to alter the specificity of the TaqMan probes.

In carrot plants, mixed infections of '*Ca.* L. solanacearum' and phytoplasmas are frequently detected (Alfaro-Fernández *et al*., 2012a) and it was not possible to associate either pathogen with the symptoms observed. In celery plants mixed infections were less frequent and in multinomial logistic regression analysis the presence of '*Ca.* L. solanacearum' was the only significant independent variable. Although these results may be considered as false negatives due to the possible presence of PCR inhibitors, it is very

likely that the symptoms in these plants were caused by other pathogens, such as phytoplasmas, since visual description of symptoms is not fully specific to '*Ca.* L. solanacearum'. In addition, only typical '*Ca*. Liberibacter'-like cells with an electron dense layer cell wall with a triple-layered ultrastructure (Bové, 2006) were observed in microscopic examinations by TEM and SEM (Figure 2).

Four '*Ca.* L. solanacearum' haplotypes (designated A, B, C and D) have been described affecting several crops worldwide. Haplotypes are described from single nucleotide polymorphisms (SNPs) that are inherited as a package in three gene regions, 16S rRNA, 16S/23S intergenic spacer region (ISR) and 50s rRNA (Nelson *et al*., 2011; Ravindran *et al*., 2011a). Haplotype A has been found from Central to North America and New Zealand whereas haplotype B has been found in Mexico and North America. Both haplotypes are present in solanaceous crops and are transmitted by the vector *B. cockerelli* (Nelson *et al*., 2011). Haplotype C is present in Finland and was first described in carrot in association with the carrot psyllid *T. apicalis* (Nelson *et al*., 2011). Haplotype D was described in mainland Spain and the Canary Islands, associated with carrot and *B. trigonica* (Munyaneza, 2012; Nelson *et al*., 2012). The new haplotype E, was characterized by five nucleotide changes in the 16S rDNA, seven in the ISR-23S and five in the 50S rDNA regions. The spatial and temporal coexistence of the same haplotypes in carrot and celery suggests the natural transmission between both plant species.

B. cockerelli, the vector of the zebra chip disease, has never been detected in Spain. Haplotypes D and E detected in carrot and celery plants could be associated with their natural spread by *B. trigonica* and maybe by other psyllid species. The almost permanent presence of '*Ca*. L. solanacearum' along the year in carrot and celery crops, even in symptomless plants, could also contribute to the spread of the bacterium.

In conclusion, we have demonstrated that the bacterium '*Ca*. L. solanacearum' is directly associated with a vegetative disorder in celery in Spain. The developed real-time PCR detection system and the newly discovered haplotype of this bacterium expand our knowledge of '*Ca*. L. solanacearum' and will improve our ability to develop appropriate control strategies.

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*The references of this paper (chapter) are included in the list of "Literature cited"

Transmission of '*Candidatus* Liberibacter solanacearum' in carrot seeds

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Transmission of '*Candidatus* Liberibacter solanacearum' in carrot seeds

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Abstract

A protocol for the specific detection and quantification of '*Candidatus* Liberibacter solanacearum' in carrot seeds using real-time PCR was developed. The bacterium was detected in 23 out of 54 carrot seed lots from 2010 to 2014, including seeds collected from diseased mother plants. The total average number of '*Ca*. L. solanacearum' cells in individual seeds ranged from 4.8±3.3 to 210±6.7 cells/seed from three seed lots, but using propidium monoazide to target live cells, 95% of the cells in one seed lot were found to be dead. Liberibacter-like cells were observed in the phloem sieve tubes of the seed coat and in the phloem of carrot leaf midrib from seedlings. The bacterium was detected as early as 30 days post-germination, but more consistently after 90 days, in seedlings grown from PCR positive seed lots in an insect-proof P2 level containment greenhouse. Between 12% and 42% of the seedlings from positive seed lots tested positive for '*Ca*. L. solanacearum'. After 150 days, symptoms of proliferation were observed in 12% of seedlings of cv. Maestro. '*Ca*. L. solanacearum' haplotype E was identified in the seeds and seedlings of cv. Maestro. No phytoplasmas were detected in symptomatic seedlings

using a real-time assay for universal detection of phytoplasmas. Our results show that to prevent the entry and establishment of the bacterium in new areas and its potential spread to other crops, control of '*Ca*. L. solanacearum' in seed lots is required.

Introduction

'*Candidatus* Liberibacter solanacearum' is a Gram-negative bacterium restricted to the plant's phloem. This emerging bacterium cannot be cultured *in vitro* yet (Munyaneza, 2012). Moreover, it is associated with economically important diseases such as: zebra chip in potato (*Solanum tuberosum* L.) (Secor *et al*., 2009), psyllid yellows in tomato (*S. lycopersicum* L.) (EPPO, 2013), yellows decline and vegetative disorders in carrots (*Daucus carota* L.) (Munyaneza *et al*., 2010a) and vegetative disorders in celery (*Apium graveolens* L.) (Teresani *et al*., 2014a). Besides this, the bacterium can infect and cause serious damage and economic losses in pepper (*Capsicum annuum* L.), eggplant (*S. melongena* L.), tamarillo (*S. betaceum* Cav), tomatillo (*Physalis peruviana* L.*),* tobacco (*Nicotiana tabacum* L.). It also affects weeds in the family *Solanaceae* (Munyaneza, 2012; EPPO, 2013).

In potato '*Ca.* L. solanacearum' can be transmitted from mother tubers to growing plants and to progeny tubers (Pitman *et al*., 2011). Nevertheless, the main pathway in potato is transmission in a persistent (transovarial) way by the psyllid *Bactericera cockerelli* (Munyaneza *et al*., 2007a). '*Ca.* L. solanacearum' has also been detected in the psyllid species *Trioza apicalis* (Munyaneza

et al., 2010b), *B. trigonica* (Alfaro-Fernández *et al*., 2012b), other *Trioza* and in *Accizia* species (Scott *et al*., 2009).

Outbreaks of yellows decline and vegetative disorders in carrot crops have been reported recently in geographically distant areas and countries in Europe (EPPO, 2013). This suggests seed transmission could be involved in the natural spread of the bacterium (Teresani *et al*., 2014a). Vegetative disorders associated with '*Ca.* L. solanacearum' were detected for the first time in 2008 in Villena, Spain, an important carrot-producing area in Alicante province (Alfaro-Fernández *et al*., 2012a). The disease has caused severe economic losses in carrot production for the fresh market. The bacterium was also detected in 2010 in Finland, in 2012 in Sweden and Norway, and in carrot fields used for seed production in France (EPPO, 2013; Loiseau *et al*., 2014).

Five '*Ca.* L. solanacearum' haplotypes (designated A, B, C, D and E) have been described affecting several crops worldwide. Haplotype A has been found from Central to North America whereas haplotype B has been found in Mexico, North America and New Zealand. Both haplotypes are present in solanaceous crops and are transmitted by the vector *B. cockerelli*. Haplotype C is present in Finland and was first described in carrot in association with the carrot psyllid *T. apicalis* (Nelson *et al*., 2011). Haplotypes D and E were described in Spain, associated with carrot and celery crops and are transmitted by *B. trigonica* (Teresani *et al*., 2014a).

Real-time PCR methods can detect and quantify bacterial genomes but do not differentiate between living (or viable) and dead

cells. Recently, however, DNA-intercalating dyes such as etidium monoazide (EMA) (Nocker & Camper, 2006; Trivedi *et al*., 2009) or propidium monoazide (PMA) (Nocker *et al*., 2007; Temple *et al*., 2013) have been successfully used to detect and quantify DNA from only live cells. We have therefore, used these dyes for the analyses of '*Ca*. L. solanacearum' cell viability.

Although '*Ca*. Liberibacter' species have already been detected in seeds of pepper (*Capsicum annuum* L.) (Camacho-Tapia *et al*., 2011) and citrus (Albrecht & Bowman, 2009; Hartung *et al*., 2010; Hilf *et al*., 2013). However no evidence of seed transmission was presented.

The main objectives of this work were i) to detect, quantify and assess the viability of '*Ca.* L. solanacearum' in carrot seeds, ii) to detect the bacterium and observe symptoms in carrot seedlings grown from infected seeds and iii) to prove that the bacterium is seedborne and seed transmitted and associated with economically important vegetative disorders in carrot. The epidemiological repercussions of these findings are discussed.

Material and Methods

Plant material

Fifty-four commercial carrot seed lots were sampled officially from the Agricola Villena Coop. V. (producers of 30% of the total marketed carrots in Spain) from 2010 to 2014. Additionally, seeds from infected carrot plants were collected in three fields used for seed production in France ("Region Centre",

Orléans) (Table 1).

Extract preparation from carrot seeds

Both bulk and individual seed samples were prepared and analysed. For bulk seeds, 1 g (approx. 450 seeds) were washed by shaking for 30 min in a 50 ml Falcon tube containing 50 ml of washing buffer (distilled water + 0.5% Triton X-100) in an attempt to remove fungicide treatment. After washing, the seed was placed in a heavy duty plastic bag (Plant Print Diagnòstics, Valencia, Spain) with 10 ml of PBS extraction buffer (NaCl, 8 g/l; $NaH_2PO_4.2H_2O$, 0.4 g/l; $Na_2HPO_4.12H_2O$, 2.7 g/l; pH 7.2) with 0.2% DIECA (sodium diethyl dithiocarbamate) and 2.0% PVP-10 and crushed with the aid of a hammer. One ml of the extract from each seed lot was stored at -20°C until use. For individual seeds, seeds from 16 seed lots (Table 1) were washed and then squashed with the aid of a rounded end of a pipette tip in Eppendorf tubes containing 200 µl extraction buffer.

Table 1. Carrot seed lots tested by real-time PCR against '*Candidatus* Liberibacter solanacearum' using bulk (1g) or individual seeds with two sample preparation methods spot and DNA purification.

Seed lot code	Cultivar	Seeds			
		1 gram (450 seeds) Ct[a]		Individual seeds (x/y)[b]	
		Spot	DNA purif.	Spot	DNA purif.
1/2010	**Bangor**	Undet.[c]	Undet.	na[d]	na
2/2011	**Bangor**	Undet.	Undet.	na	na

3/2011	**Bangor**	Undet.	Undet.	na	na
4/2012	**Bangor**	Undet.	Undet.	na	na
5/2012	**Bangor**	Undet.	Undet.	na	na
6/2013	**Bangor**	Undet.	36.5	15/50	29/50
7/2013	**Bangor**	Undet.	36.8	na	na
8/2014	**Bengala**	Undet.	Undet.	na	na
9/2013	**Bolero**	34.3	27.7	7/50	10/50
10/2012	**CAC 3075** [e]	37.1	32.5	3/30	28/30
11/2012	**Amsterdam** [e]	28.2	23.9	9/30	14/30
12/2012	**CAC 3075** [e]	29.1	24.6	21/30	22/30
13/2012	**Carboli**	30.9	24.4	10/50	42/50
14/2012	**Carboli**	Undet.	Undet.	na	na
15/2013	**Dordogne**	Undet.	Undet.	na	na
16/2013	**Elegance**	34.3	28.9	10/50	42/50
17/2013	**Exelso**	Undet.	Undet.	na	na
18/2013	**Exelso**	Undet.	Undet.	na	na
19/2013	**Exelso**	Undet.	Undet.	na	na
20/2013	**Exelso**	Undet.	38.3	2/50	12/50
21/2014	**Exelso**	Undet.	Undet.	na	na
22/2013	**Laguna**	Undet.	Undet.	na	na
23/2010	**Maestro**	29.2	24.5	14/50	21/50
24/2010	**Maestro**	Undet.	Undet.	na	na
25/2010	**Maestro**	31.4	24.6	na	na
26/2010	**Maestro**	Undet.	31.7	na	na
27/2011	**Maestro**	Undet.	Undet.	na	na
28/2011	**Maestro**	Undet.	Undet.	na	na
29/2011	**Maestro**	30.2	24.8	na	na
30/2012	**Maestro**	31.8	24.4	4/100	16/100
31/2012	**Maestro**	Undet.	32.2	na	na
32/2013	**Maestro**	30.6	24.2	na	na
33/2013	**Maestro**	Undet.	Undet.	na	na
34/2013	**Maestro**	31.0	25.6	24/50	43/50
35/2013	**Maestro**	29.7	25.3	12/50	30/50
36/2013	**Maestro**	Undet.	Undet.	na	na
37/2013	**Musico**	28.9	22.7	28/50	48/50
38/2013	**Musico**	Undet.	35.1	na	na
39/2013	**Musico**	Undet.	Undet.	na	na
40/2013	**Musico**	Undet.	Undet.	na	na
41/2013	**Namur**	Undet.	Undet.	na	na
42/2013	**Naval**	Undet.	Undet.	na	na
43/2013	**Newhall**	Undet.	32.8	13/50	38/50
44/2014	**Niagara**	Undet.	Undet.	na	na
45/2014	**Niagara**	Undet.	Undet.	na	na
46/2011	**Romance**	Undet.	Undet.	na	na
47/2013	**Romance**	Undet.	Undet.	na	na
48/2013	**Romance**	Undet.	Undet.	na	na

49/2012	**Soprano**	Undet.	Undet.	na	na
50/2012	**Soprano**	Undet.	Undet.	na	na
51/2013	**Soprano**	Undet.	Undet.	na	na
52/2013	**Soprano**	Undet.	Undet.	na	na
52/2013	**Soprano**	31.5	27.7	28/50	29/50
54/2013	**Yaya**	Undet.	33.7	9/50	21/50

[a] : Ct= Cycle threshold of real-time PCR.
[b]: x/y= number of seeds in which '*Candidatus* Liberibacter solanacearum' was detected / total number of seeds analysed.
[c]: Undet.= Undetermined Ct after 40 cycles.
[d]: na= Not analysed.
[e]: French origin.

DNA purification

Total DNA from 200 µl of seed extracts (from 1 g or from individual seeds) was purified using the CTAB (cetyl trimethyl ammonium bromide) protocol (Murray & Thompson 1980). Purified DNA was stored at -20 ºC until use.

Direct sample preparation without DNA purification (spot procedure)

Freshly prepared or frozen seed extracts were immobilized on membranes by loading 5 µl of crude seed extract onto pieces of Whatman 3MM filter paper (GE Healthcare Europe GmbH, Freiburg, Germany), which had been previously introduced into Eppendorf tubes. The DNA targets were then extracted using 100 µl distilled water (Bertolini *et al*., 2014a; Teresani *et al*., 2014a), vortexed, placed on ice and 3 µl used as the template for real-time PCR assays.

Real-time PCR and haplotype determination

Real-time PCR assays were performed using CaLsppF (5'-GCAGGCCTAACACATGCAAGT-3') and CaLsppR (5'-GCACACGTTTCCATGCGTTAT-3') universal primers for liberibacters (Bertolini *et al*., 2014a) and a '*Ca.* L. solanacearum' specific CaLsolP (5` FAM-AGCGCTTATTTTTAATAGGAGCGG CAGACG-3` TAMRA) TaqMan probe (Teresani *et al*. 2014a), using a StepOne Plus thermal cycler (Applied Biosystems, Foster City, CA, USA) or Light Cycler 480 (Roche, Mannheim, Germany). The reaction mix consisted of 1 x Path-ID™ qPCR master mix (Ambion, Grand Island, NY, USA), 0.5 µM of each CaLsppF and CaLsppR primers, 150 nM of CaLsolP TaqMan probe and 3 µl of the template (purified DNA or direct extraction from the spot) in a final volume of 12 µl. Positive and negative controls were used in each PCR reaction. The real-time PCR amplification protocol consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data acquisition and analysis were performed with the thermal cycler's software. The default threshold (baseline) set by the machine was slightly adjusted above the noise in the linear part of the growth curve. Alternatively, real-time amplification was done using the commercially available CaLsol/100 kit (Plant Print Diagnòstics, Valencia, Spain) according to manufacturer's instructions and also the real-time PCR protocol described by Li *et al*. (2009). Symptomatic carrot seedlings were also tested for phytoplasmas (universal detection) using the real-time PCR procedure of Hren *et al*. (2007).

The '*Ca*. L. solanacearum' haplotype was determined according to the method described by Nelson *et al*. (2011).

Transmission electron microscopy observations

Seeds cv. Maestro (lot 30/2012) and leaf midribs from symptomatic carrot seedlings from the same seed lot were prepared for transmission electron microscopy (TEM). Seed coats and leaf midribs were fixed in 'Karnovsky' solution for 24 h, then fixed with osmium tetroxide in 1% cacodylate buffer for 1 h and contrasted with uranyl acetate 0.5% overnight. Samples were then dehydrated using increasing concentrations of acetone (30-100%). Infiltration and embedding was performed in 1:1 acetone (100%): 'Spurr' epoxy resin for at least 5 h and then in pure resin 'Spurr' overnight or longer, depending on the sample's infiltration capacity. Polymerization was carried out at 70°C for three days. The resin blocks were cut into 70 nm sections (using an ultramicrotome with diamond knife), placed on copper screens and contrasted with uranyl acetate 3% and lead citrate. Samples were examined using a JEOL JEM 1011 (JEOL, Tokyo, Japan) TEM and images were captured using a digital camera.

Quantification of total '*Ca*. L. solanacearum' cells in individual carrot seeds

For generation of a standard curve, amplified products were obtained through conventional PCR using the designed primers CaLsolF (5'- AGTTTGATCATGGCTCAGAAC-3') / CaLsolR (5'- ACGCGGG CTCATCTCT-3'), based on the EU980389 sequence of '*Ca.* L. solanacearum' from the 16S rRNA gene, showing an amplification product of 210 bp. The products were inserted into pGem-T easy vector (Promega Corp., Madison, Wisconsin, USA) and cloned in *Escherichia coli* JM-109. Plasmid DNA was extracted with the Wizard Miniprep kit (Promega Corp.). Avogadro's constant was used to estimate the number of plasmid DNA copies. Ten-fold serial dilutions of plasmid DNA copies were prepared from 1.38 x 10^{10} to 1.38. Serial dilutions were then divided into aliquots and stored at 4°C until used to generate the standard curve. The average of three repetitions of the same 10-fold serial dilution were performed to determine the theoretical sensitivity and the reliability of the qPCR assay. We have assumed that the 16S rRNA gene is present in one copy in the '*Ca*. L. solanacearum' genome, therefore one plasmid copy is equivalent to one cell of '*Ca*. L. solanacearum'. Cell numbers were transformed to log cell numbers.

For quantification '*Ca*. L. solanacearum' in individual carrot seeds, 30 seeds from each of three seed lots (10/2012, 11/2012 and 12/2012, Table 1), that tested positive for the bacterium, were squashed as described previously and spotted onto Whatman 3MM

filter paper and the DNA eluted or the DNA was purified, for real-time PCR analysis.

Quantification of live (viable) '*Ca*. L. solanacearum' cells in 1 g bulk samples of carrot seed

One gram of seed from seed lot 12/2012 (Table 1), collected from '*Ca*. L. solanacearum' infected carrot plants, was comminuted in 10 ml of extraction buffer. Samples were analysed with PMA (live bacteria) and without PMA (total bacteria). Twenty aliquots of 0.2 ml were used in each treatment. To quantify the genomes from live bacteria, 0.2 ml aliquots from PMA-treated samples were used in a final concentration of 100 μM. Tubes were incubated with occasional inversion to allow the dye to intercalate with DNA at 30°C in the dark for 10 min. After incubation, tubes were vortexed for 5 s and exposed to 100% light intensity for 15 min in a PhAST Blue apparatus (Geniul, Barcelona, Spain). After this treatment, conditions were maintained the same for both (with and without PMA). DNA was extracted using the CTAB method and samples were analysed by real-time PCR. The quantification of total and live '*Ca.* L. solanacearum' cells was performed using cycle threshols (Ct) values from these assays to estimate cell numbers from the standard curve. Cell numbers (log) with and without PMA treatments were compared using analysis of variance (ANOVA) and STATGRAPHICS 5.1 software (StatPoint Technologies Inc., Warrenton, VA, USA).

Assessment of transmission of '*Ca.* L. solanacearum' from seeds to seedlings

Six hundred carrot seeds cv. Maestro (lot 30/2012) that tested positive for '*Ca*. L. solanacearum', and 600 seeds from lots of cv. Amsterdam (lot 12/2012) and cv. CAC 3075 (lots 10/2012 and 11/2012) harvested from diseased carrot seed mother plants that tested positive for the bacterium, were germinated. In addition, 2,000 carrot seeds cv. Maestro (lot 27/2011), that tested negative for the bacterium were also germinated for negative controls. Seedlings were transplanted and grown in a P2 level containment greenhouse (insect-proof) at 15-26ºC for 150 days. Every 30 days, up to 100 plants per lot were collected (200 plants for the negative control) and tested by real-time PCR for '*Ca*. L. solanacearum'. From greenhouse grown material, after 90 days, the remaining seedlings were transplanted to 3 litre-pots using 1-3 seedlings/pot. From these seedlings 1 g of leaf tissue per plant was collected every 30 days into separate plastic bags (Bioreba, Reinach, Switzerland). The extract was then prepared, the DNA extracted and 3 µL of purified DNA used as the template for real-time PCR analysis. All greenhouse and field plants (see below) were visually inspected weekly for symptoms, starting 30 days post-germination and during the 150/180-day cultivation period.

An experimental carrot plot (19,000 seeds) of cv. Maestro from lot 30/2012 was also established in an open field in Villena, Spain. Conventional cultivation was conducted during six months from the beginning of June to the end of November, 2012. Carrot

plants were weekly visually inspected for symptoms and 100 plants were collected at random and analysed by real-time PCR for '*Ca.* L. solanacearum' after three and six month's cultivation.

Experimental transmission of '*Ca.* L. solanacearum' by dodder from positive carrot seedlings

Cuscuta campestris Yunck (dodder) was used to establish a vascular connection between a carrot symptomatic seedling cv. Maestro and two healthy carrot seedlings of the same cultivar in a P2 level containment greenhouse at 15-26 ºC. Dodder was also grown on healthy carrot plants as the negative control. After 60 days leaf samples were collected, extracts were prepared as previously described and DNA purified and analyzed by real-time PCR, as above.

Results

Detection of '*Ca*. L. solanacearum' in carrot seeds

For bulk seeds (1g of seed), 23 out of the 54 commercial seed lots tested positive for the bacterium by real-time PCR with 15 testing positive when either spot or purified DNA was used, and a further 8 testing positive when purified DNA was used (Table 1). The remaining seed lots were negative for both preparation methods. For individual seeds, '*Ca*. L. solanacearum' was detected in seeds from the same seed lot irrespective of the preparation method used, but with greater numbers of individual seeds (16%-96%) testing

positive when purified DNA was used as the template compared with eluted DNA from the direct sample preparation method.

Haplotype E was found in cv. Maestro carrot seeds (lot 30/2012). No specific haplotype was identified in the other positive seed lots due to the lack of amplification by conventional PCR of the ISR-23S intergenic region except in lot 30/2012. However 16S rRNA and 50S rRNA genes were amplified in some positive seed lots suggesting a mixture of haplotypes D and E sequences.

Transmission electron microscopy observations

Bacteria-like organisms (BLOs) were observed using TEM in the phloem sieve tubes of the seed coats, as well as in the phloem of carrot midrib seedlings originating from '*Ca*. L. solanacearum' positive seed lot 30/2012 (Figure 1).

The BLO cells were pleomorphic and surrounded by an electron dense cell wall separate from the cytoplasmic membrane, which was slightly rippled, wrinkled or uneven. The BLO cells were triple-layered (i.e. including an outer cell wall membrane and an inner cytoplasmic membrane) (Figure 1), a feature consistent with '*Ca.* Liberibacter' cells.

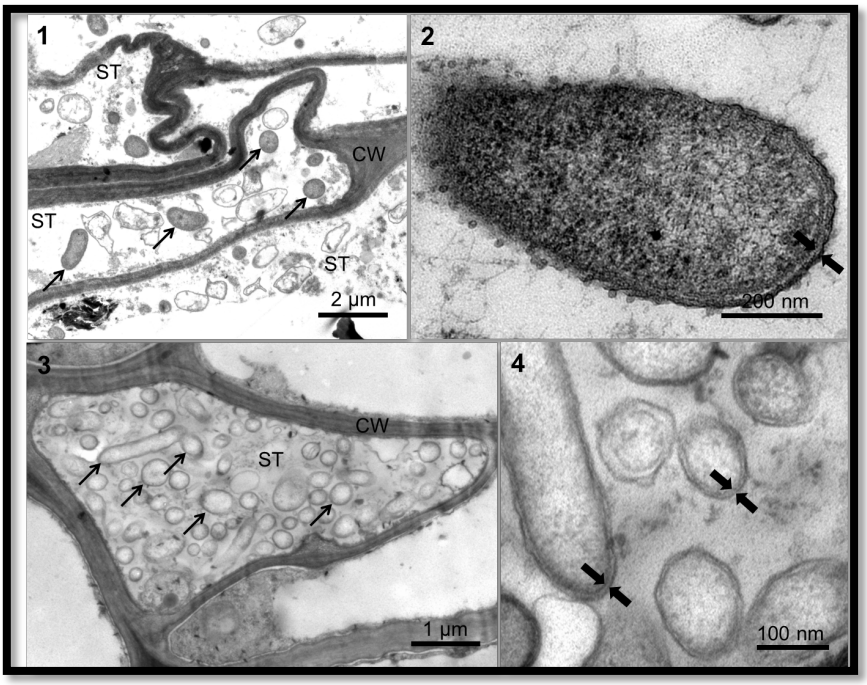

Fig. 1. Transmission electron microscopy (TEM) photomicrographs of carrot seed coat and tissue of carrot seedling. 1) Presence of bacteria-like organisms (BLOs) (black arrows) in the phloem sieve tubes (ST) of seed coat; 2) individual bacterial cell pleomorphic in shape and surrounded by an electron dense cell wall separate from the cytoplasmic membrane, slightly rippled and wrinkled; 3) presence of BLOs (black arrows) in the leaf phloem sieve tubes of carrot seedlings; 4) bacterial cells showing a triple-layered ultrastructure of both the outer cell wall membrane and the inner cytoplasmic membrane (between two black arrows) suggesting the presence of '*Ca.* Liberibacter'-like cells. CW= plant cell wall.

Quantification of total '*Ca*. L. solanacearum' cells in individual carrot seeds

A standard curve of Ct value and the log of the cell number from 1.38×10^8 to 1.38 showed a R^2= 0.999 (Figure 2). The log of the average number of '*Ca*. L. solanacearum' cells in individual seeds from three infected seed lots ranged from 0.69 ± 0.52 to 2.32 ± 0.82, corresponding to 4.8 ± 3.3 to 210 ± 6.7 cells/seed (Table 2).

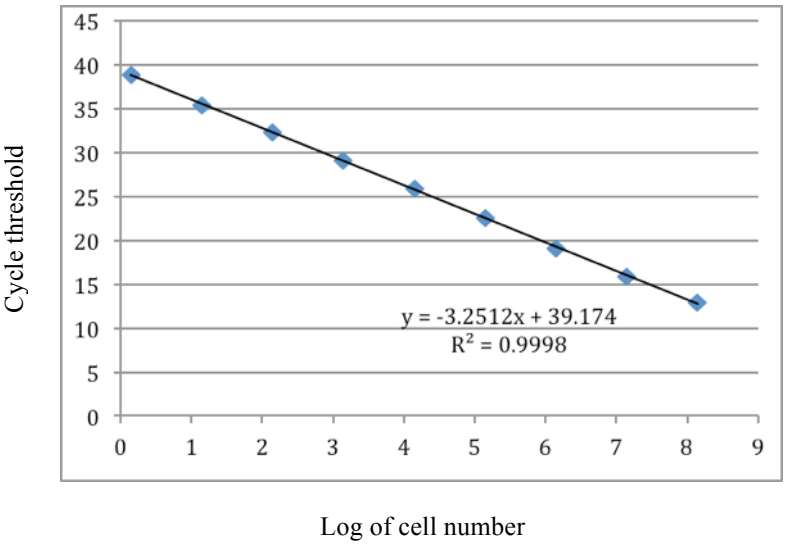

Fig. 2. Standard curve obtained with the average of three repetitions of 10-fold serial dilutions from 1.38 x 10^8 to 1.38 of '*Candidatus* Liberibacter solanacearum' cells.

Lot 10/2012 had the lowest number of '*Ca*. L. solanacearum' cells/seed with an average of 4.8 ± 3.3, but the highest number of seeds infected (93%). Lot 12/2012 had the highest number of '*Ca*. L. solanacearum' cells/seed with an average of 210 ± 6.7 but with 73% of the seeds infected. The highest number of '*Ca*. L. solanacearum' cells detected in an individual carrot seed was 3.6 x 10^4 (log of the number of cells/seed = 4.55) (Table 2).

Table 2. Real-time PCR analysis and quantitation of '*Candidatus* Liberibacter solanacearum' in individual seeds from three different seed lots.

Seed lot code	Nº of positive seeds/Nº of analysed seeds	Estimated % of positive seeds	Log ('*Ca.* L. solanacearum' cells/carrot seed) range	Log average ± SD (Estimate nº of cells per seed)
11/2012	14/30	47	0.04 to 3.70	1.68 ± 1.15 (48 ± 14.4)
12/2012	22/30	73	0.59 to 4.55	2.32 ±0.82 (210 ± 6.7)
10/2012	28/30	93	0.02 to 1.73	0.69 ±0.52(4.8 ± 3.3)

Quantification of live (viable) '*Ca*. L. solanacearum' cells in 1 g bulk samples of carrot seed

Positive real-time PCR amplifications were observed using DNA extracted from all suspensions of PMA-treated and non-PMA treated seed extracts. When PMA was used, there was a significant reduction ($P < 0.05$) in the Ct values from 27.94 to 22.88 and in the log number of '*Ca*. L. solanacearum' cells/g seed which ranged from 4.15 ± 0.28 to 2.88 ± 0.32, corresponding to 1.4×10^4 and 7.6×10^2 cells/g seed (Table 3).

Table 3. Real-time PCR analysis and quantification of '*Candidatus* Liberibacter solanacearum' cells in carrot seeds with propidium monoazide (live cells) and without propidium monoazide treatment (dead and live cells).

Treatment	Ct range	Log ('*Ca.* L. solanacearum' cells/g of seeds) range	Log average ± SD (Estimate n° of cells per g of seeds)
With PMA	26.0 to 31.1	2.06 to 3.37	2.88 ±0.32* (760 ± 2.1)
Without PMA	20.9 to 25.5	3.50 to 4.64	4.15 ±0.28 (14,400 ± 1.9)

*Average log cells ± standard error from 20 repetitions. Treatments are significantly different ($P < 0.05$ using ANOVA).

Assessment of transmission of '*Ca.* L. solanacearum' from seeds to seedlings

The bacterium was first detected in seedlings of lots 11/2012 and 30/2012 respectively after 30 and 60 days post-germination when grown in a P2 level containment greenhouse (Table 4). The bacterium was frequently detected in seedlings aged 90 days or above but was detected mostly in 150-day seedlings with the percentage detection ranging between 12% and 42% for different seed lots (Table 4). '*Ca*. L. solanacearum' was detected in a 5% of seedlings cv. Maestro (lot 30/2012) after three months reaching 12% after 150 days cultivation. In the open field plot the same plants of the same cultivar were 2% infected after 90 days and 96% infected after 180 days. Over the whole 150 days of sampling '*Ca*. L. solanacearum' was detected in 4.2%, 6.8% and 10.1% seedlings of respectively cv. Amsterdam (lot 11/2012) and cv. CAC 3075 (lots 10/2012 and 12/2012). In these lots 15%-42% seedlings were infected after 150 days (Table 4). None of the 1,000 seedlings of cv.

Maestro (lot 27/2011) tested positive for the bacterium over the 150 days cultivation period.

Table 4. Detection of '*Candidatus* Liberibacter solanacearum' by real-time PCR in carrot seedlings grown from positive seed lots in a P2 level containment greenhouse.

Seed lot code	Cultivar	Seeds	Seedlings					
			Days					
			30	60	90	120	150	Total
10/2012	**CAC 3075**	**9/10**	0/100	0/100	6/78	7/52	13/49	26/379
11/2012	**Amsterdam**	**5/10**	1/100	0/100	5/100	0/95	15/100	21/495
12/2012	**CAC 3075**	**9/10**	0/100	0/100	0/63	5/100	42/100	47/463
30/2012	**Maestro**	**16/100**	0/100	2/100	5/100	5/100	12/100*	24/500
27/2011	**Maestro**	**0/100**	0/200	0/200	0/200	0/200	0/200	0/1,000

* Symptoms observed.

Symptoms of proliferation of dwarfed shoots and a dense hairy growth of secondary roots (Figure 3) were observed in 12% of cv. Maestro (lot 30/2012) after 150 days of cultivation in the P2 level containment greenhouse. No symptoms were observed in the seedlings of the cvs. Amsterdam and CAC 3075. None of the 1,000 analysed seedlings from seeds of cv. Maestro (lot 27/2011) that tested negative for the bacterium showed symptoms. No phytoplasmas were detected in symptomatic or symptomless carrot seedlings grown under greenhouse conditions. In the open field, symptoms of proliferation were observed in cv. Maestro plants after three months cultivation and after six months almost 100% of the plants were affected.

Haplotype E was found in symptomatic carrot seedlings of the cv. Maestro (lot 30/2012). However, in carrot seedlings of cv.

Amsterdam (lot 11/2012) and cv. CAC 3075 (lots 10/2012 and 12/2012) no specific haplotype was identified due to a lack of amplification by conventional PCR of the ISR-23S intergenic spacer region. Nevertheless, sequence analysis of 16S rRNA and 50S rRNA genes suggesting a mixture of haplotypes D and E sequences.

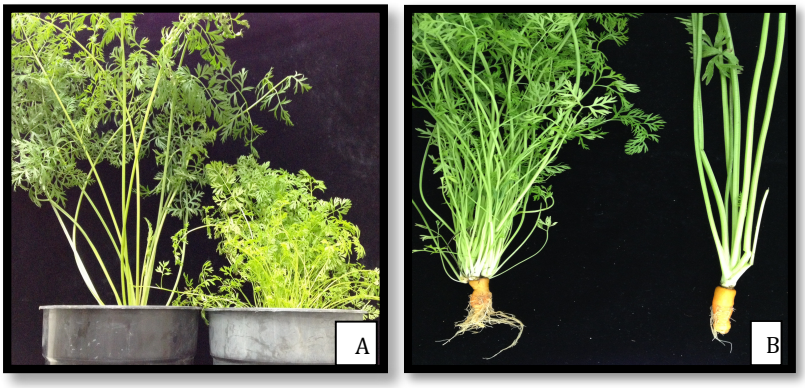

Fig. 3. Symptoms of '*Candidatus* Liberibacter solanacearum' infection in carrot seedlings cv. Maestro (30/2012 lot) grown in a P2 level containment greenhouse. A) Proliferation of dwarfed shoots of a seedling (left) and symptomless carrot plant (right). B) Dense hairy growth of secondary roots (left) and symptomless carrot plant (right).

Experimental transmission of '*Ca.* L. solanacearum' by dodder from positive carrot seedlings

Dodder plants established vascular connections between donor and receptor seedlings. '*Ca.* L. solanacearum' was detected in the two receptor carrot plants after 60 days. The bacterium was not detected in the control plants.

Discussion

Seedborne phytopathogenic bacteria act as primary inoculum for many important plant diseases (Dutta *et al*., 2013). Our results show that '*Ca.* L. solanacearum' is a seedborne pathogen in carrot with persistent infection and symptoms in carrot seedlings. To our knowledge, this is the first report of seed transmission of a '*Ca.* Liberibacter' species.

Specific and sensitive real-time PCR protocols are useful tools for detection of bacterial (Schaad & Frederick, 2002; López *et al*., 2009). In our work, detection of '*Ca.* L. solanacearum' in carrot seed lots and in individual carrot seeds was confirmed using the real-time PCR protocols described by Teresani *et al*. (2014a) and Li *et al*. (2009) or the CaLsol/100 kit, using direct sample preparation or DNA purification methods. The use of purified DNA as template showed a higher sensitivity than the direct methods for the detection of '*Ca.* L. solanacearum', as reported by Teresani *et al*. (2014a) and also Bertolini *et al*. (2014a) for the detection of '*Ca.* Liberibacter' species in citrus plants.

Total bacterial (live and dead) cells were quantified in individual carrot seeds by real-time PCR. Temple *et al*. (2013) had previously quantified the seedborne carrot bacterium *Xanthomonas hortorum* pv. *carotae* using several grams of carrot seeds instead of individual seeds. Three copies of the 16S rRNA gene were reported in '*Ca*. L. asiaticus' (Kim & Wang, 2009) and '*Ca*. L. europaeus' (Raddadi *et al*., 2011). Although the sequence of '*Ca*. L. solanacearum' genome was available (Lin *et al*., 2011b) the copy

number of the 16S rRNA gene has not been determined yet. Assuming that one copy is present, the number of estimated cells of '*Ca*. L. solanacearum' in individual seeds was found to be highly variable within the seed lot and between seed lots. The average number of cells/seed in the lot with the highest estimated bulk bacterial concentration (lot 12/2012) was 210±6.7 cells/seed. However, in individual seeds from the same lot, a high variability among the estimated number of cells/seed was observed with estimated log values ranging from 0.59 to 4.55, corresponding to 4 to 36,000 '*Ca*. L. solanacearum' cells/seed. Similar variability has been reported for other phytopathogenic bacteria in the number of colony forming units per seed (Dutta *et al*., 2013). The seed lot (lot 10/2012) with the highest percentage of positive seeds had the lowest estimated average number of bacterial cells/seed.

The PMA treatment allowed the quantification of live '*Ca*. L. solanacearum' cells in carrot seeds and this showed that the majority (about 95%) of the seed bacterial population was dead. Nevertheless, the remaining live cells (5%) were enough to cause infection in carrot seedlings germinated from infected seeds. These results are similar to those reported for '*Ca*. L. asiaticus' (83% dead cells), after treatment with ethidium monoazide (Trivedi *et al*., 2009).

Using TEM '*Ca.* Liberibacter'-like cells were seen (Figure 1) in the phloem of the carrot seed coat. It seems likely therefore that the bacterium may penetrate undifferentiated cells of the seedling radicle in the very early stages of seed germination eventually

reaching the phloem of young seedlings to cause a persistent infection and disease symptoms. Similarly, '*Ca*. L. asiaticus' DNA has been frequently detected in the seed coat of sweet orange and grapefruit collected from huanglongbing-infected trees, although seedling infection was not be shown (Albrecht & Bowman, 2009; Hilf *et al.,* 2013). Further research is necessary to assess the mechanism of seed transmission of phloem-limited bacteria.

We have demonstrated a persistent infection of '*Ca*. L. solanacearum' in seedlings from positive seed lots grown in a P2 level containment (insect proof) greenhouse. The first detection was after 30 days (only in one seedling) with the bacterium more consistently detected in all seed lots by real-time PCR, 90-120 days post-germination. However, the bacterium was only consistently detected in all 4 seed lots tested after 150 days cultivation in the greenhouse. This lack of consistent detection might explain why the bacterium had not been previously detected in seedlings and consequently had not been considered a seedborne pathogen. Positive samples were not only detected in seeds from diseased plants (Amsterdam and CAC 3075) where 15% - 42% of the seedlings were infected after 150 days but also in the commercial seed lot 30/2012 cv. Maestro with 12% infection.

The only seedlings showing symptoms were in cv. Maestro (lot 30/2012) after 150 days post-germination when all 12% of the infected plants showed symptoms when grown in a P2 level containment greenhouse. The same lot planted in open field showed 96% diseased plants. This plot was not harvested for consumption

due to the severe symptoms. It is therefore important to use '*Ca.* L. solanacearum'-free seed in areas where psyllid vector species can efficiently spread the initial inoculum. Seedlings from cv. Maestro (27/2011 negative lot) grown in a P2 level containment greenhouse did not show symptoms nor was the bacterium detected during the cultivation cycle. No phytoplasmas were detected by PCR in the analysed symptomatic carrot seedlings grown under greenhouse conditions. The bacterium was transmitted by dodder from a positive carrot seedling to the receptor carrot plants used, demonstrating the transmissibility of the seedborne bacterium.

Haplotypes are defined based on single nucleotide polymorphisms (SNPs) that are inherited as a package in three gene regions, 16S rRNA, 16S/23S intergenic spacer region (ISR) and 50S rRNA (Nelson *et al*., 2011). No amplification occurred in the ISR region except in lot 30/2012 in which the haplotype E was identified both in seeds and seedlings. However, amplification of the 16S rRNA and 50S rRNA genes, revealed the presence of haplotypes D and E sequences suggesting a mixture of both haplotypes or a new, no yet described, haplotype. Haplotype identification should be considered as helpful in order to pursue the possible source and origin of infected seed lots. However, previous reports (Lin *et al*., 2012) using 8 simple sequence repeat (SSR) markers described 33 different haplotypes indicating that a non-clear genetic structure exists among '*Ca*. L. solanacearum' haplotypes based on geographical proximity or host. In addition, the current commercial carrot seed lots contain a mixture of seeds from different origins and

production years, impairing the identification of consistent relationships between haplotype and seed lot source and origin. Nevertheless, it should be established whether the currently identified haplotypes have any biological significance in terms of vectors, hosts, transmission or even geographical origin.

Overall, our results indicate high infestation levels of '*Ca*. L. solanacearum' in carrot seed lots from 11 of the most frequently grown cultivars in Spain. The highest number of lots analysed was from cv. Maestro (the prevalent cv. grown at Villena area), where 64.3% of the lots were positive for '*Ca.* L. solanacearum'. Unfortunately no information is current available about the origin of the seed lots and the location of the seed mother plants used in this work, except from France, where the bacterium was detected in seed production fields (Loiseau *et al.,* 2014) in the cvs. Amsterdam and CAC 3075. The origin of '*Ca*. L. solanacearum' in Europe is unknown, but since the bacterium has not yet been detected in any naturally occurring plant species in Europe we speculate that it may have been introduced into Europe with carrot seeds, in spite of the Nelson *et al*. (2013) hypothesis that assume that carrot haplotype D is natural to Europe. However so far '*Ca*. L. solanacearum' has been only reported in carrots in France, Finland, Norway and Spain (Munyaneza *et al*., 2010a; Alfaro-Fernández *et al*., 2012a; EPPO, 2013; Loiseau *et al*., 2014). It was also detected in celery grown next to carrot in Spain, suggesting that carrot was the most probable source of inoculum (Teresani *et al*., 2014a).

The relationship between the levels of seed contamination with bacteria and the prevalence and severity of disease in the field has been studied by several authors (Schaad *et al*., 1980; Gitaitis & Nilakhe, 1982; Dutta *et al*., 2013). Umesh *et al*. (1998) reported that seed contamination levels were positively correlated with *Xanthomonas campestris* pv. *carotae* populations in leaves and with the prevalence and severity of carrot bacterial blight. Potato plants produced with zebra chip affected "seed tubers" do not significantly contribute to zebra chip prevalence and spread in potato fields (Henne *et al*., 2010b; Pitman *et al*., 2011). However, there is a lack of available data concerning phloem restricted bacteria in true seeds in which disease development and epidemics will be highly dependent on the primary inoculum supplied by seeds, the presence of efficient vector species and environmental factors.

The presence of psyllid vectors are essential for the spread of '*Ca*. L. solanacearum'. In the present work the presence of psyllids vector species, including *Bactericera trigonica*, in the field increased the prevalence of the bacterium from 2% (5% in greenhouse conditions) to practically 100% after the six months cultivation. Previous experience with emerging diseases in the citrus and potato industries indicates psyllid species, feeding briefly outside their normal plant host range, could introduce a pathogen to another crop (Nelson *et al.*, 2013). This might present a serious risk for other economically important crops, such as potato, tomato and eggplant that could naturally be infected by other haplotypes than A or B.

In order to prevent the introduction of the bacterium to new areas, to reduce the inoculum in carrot fields and to mitigate spread to other potential hosts, control of the bacterium in seed lots that are sold in Europe and word-wide is required. Therefore, the production of carrot seed in pest free areas for '*Ca*. L. solanacearum' or under insect-proof facilities to produce carrot seed free from '*Ca*. L. solanacearum' need to be investigated, as do seed treatments that could inactivate the bacterium from infected seed. In addition, strategies are required to reduce the populations of psyllid vector species and the spread of '*Ca*. L. solanacearum' to other crops.

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*The references of this paper (chapter) are included in the list of "Literature cited"

The search for potential vectors of '*Candidatus* Liberibacter solanacearum': population dynamics in host crops

Teresani, G.R., Hernández, E., Bertolini, E., Siverio, F., Marroquín, C., Molina, J., Hermoso de Mendoza, A., Cambra, M.

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Abstract

'*Candidatus* Liberibacter solanacearum' has recently been reported to be associated with vegetative disorders and economic losses in carrot and celery crops in Spain. The bacterium has been described as a carrot seedborne pathogen and it is transmitted by psyllid vector species. From 2011 to 2014 seasonal and occasional surveys in carrot, celery and potato plots were performed. The sticky plant method was used to monitor the arthropods that visited the plants. The collected arthropods were classified into Aphididae and Cicadellidae, and the superfamily Psylloidea was identified to the species level. The superfamily Psylloidea represented 35.45% of the total arthropods captured on celery in Villena and 99.1% on carrot in Tenerife (Canary Islands). The maximum flight of psyllid species was in summer, both in mainland Spain and the Canary Islands, reaching a peak of 570 specimens in August in Villena and 6,063 in July in Tenerife. The main identified psyllid species were as follows: *Bactericera trigonica* Hodkinson, *B. tremblayi* Wagner and *B. nigricornis* Förster. *B. trigonica* represented more than 99% of the psyllids captured in the Canary Islands and 75% and 38% in 2011 and 2012 in Villena, respectively. In addition, *Trioza urticae*

Linnaeus*, Bactericera* sp.*, Ctenarytaina* sp., *Cacopsylla* sp., *Trioza* sp. and *Psylla* sp. were captured. '*Ca.* L. solanacearum' targets were detected by squash real-time PCR in 19.5% of the psyllids belonging to the different *Bactericera* species. This paper reports at least three new psyllid species that carry the bacterium and can be considered as potential vectors.

Introduction

'*Candidatus* Liberibacter solanacearum' (Liefting *et al*., 2009a), which is also known as '*Ca*. Liberibacter psyllaurous' (Hansen *et al*., 2008) (Bacteria: Proteobacteria: Alphaproteo bacteria: Rhizobiales: *Rhizobiaceae*), is a Gram-negative bacterium restricted to plant phloem and insect hemolymph that cannot be cultured *in vitro* today (Liefting *et al*., 2009b). Five '*Ca*. L. solanacearum' haplotypes (designated A, B, C, D and E) have been described to affect several crops worldwide. Haplotype A has been found from Central to North America and New Zealand, haplotype B has been found in Mexico and the United States of America, haplotype C is present in Finland, Sweden and Norway, haplotype D is present in the Canary Islands, mainland Spain, Morocco and likely in France, and haplotype E is present in mainland Spain, France and Morocco (Nelson *et al*., 2011; Nelson *et al*., 2012; Teresani *et al*., 2014a; Thazima *et al*., 2014).

'*Ca*. L. solanacearum' is associated with zebra chip disease in potato (Secor *et al*., 2009) and is also associated with serious vegetative disorders and important losses in tomato (*Solanum*

lycopersicum L.), pepper (*Capsicum annuum* L.), aubergine (*S. melongena* L.), tamarillo (*S. betaceum* Cav.), tomatillo (*Physalis peruviana* L.), tobacco (*Nicotiana tabacum* L.), carrot (*Daucus carota* L.), celery (*Apium graveolens* L.) and weeds in the Solanaceae family (EPPO, 2013; Teresani *et al*., 2014a). The bacterium have been recorded in Finland (Munyaneza *et al*., 2010a) and Spain (Alfaro-Fernández *et al.*, 2012a) to be associated with vegetative disorders in carrot causing leaf curling, yellow and purple discoloration of leaves, stunted growth of shoots and roots and the proliferation of secondary roots. More recently, the bacterium was also associated in celery with symptoms such as an abnormal amount of shoots, curling of stems and yellowing (Teresani *et al*., 2014a), producing relevant yield reductions and important economic losses to the carrot and celery industries.

The bacterium is primary transmitted by carrot seeds (Bertolini *et al*., 2014b) and is afterwards transmitted by different psyllid species in a persistent way. *Bactericera cockerelli* Sulc is the vector of haplotypes A and B in solanaceous crops (Nelson *et al*., 2011). *Trioza apicalis* Förster was first described in carrot in Finland (Munyaneza *et al*., 2010b) and transmitting the haplotype C (Nelson *et al*., 2011) and *B. trigonica* Hodkinson is associated with the transmission of '*Ca*. L solanacearum' haplotype D in carrot and likely with haplotype E in carrot and celery in Spain (Alfaro-Fernández *et al*., 2012b; Nelson *et al*., 2012; Teresani *et al*., 2014a). In another '*Ca*. Liberibacter' associated disease, *Diaphorina citri* Kumayama*, Trioza erytreae* Del Guercio and *Cacopsylla citrisuga*

Yang & Li have also been described as '*Ca.* Liberibacter' spp. vectors of huanglongbing (HLB), an important citrus disease (McLean & Oberholzer, 1965; Capoor *et al*., 1967; Bové, 2006; Cen *et al*., 2012a). In addition, there are reports of '*Ca*. L. solanacearum' detection in non-identified species of psyllids in the genera *Acizzia* and *Trioza* collected in New Zealand (Munyaneza, 2012).

Several trapping methods have been used in surveys to determine the arthropod species that are present on crops or visit crops to establish the population dynamics. These methods include the observation of established colonies, suction, water and sticky fishing-line traps as well as the sticky plant or shoot method (Cambra *et al.*, 2006). The sticky shoot method, which uses glue-covered bait leaves, shoots or the entire plant, has been extensively used to determine the arthropod species that visit crops and monitor aphid species in adult trees (Cambra *et al*., 2000; Marroquín *et al*., 2004; Vidal *et al.*, 2012a). This method is the most efficient for estimating the numbers of insects landing on the plants (Hermoso de Mendoza *et al*., 1998). In Spain, arthropods occurring in carrot and celery crops have been previously studied using Moericke´s yellow traps (Villaescusa *et al*., 2011), but the captured arthropods were not identified at the species level. No data of psyllid species that visit potato crops have been available until now.

Conventional and real-time polymerase chain reaction (PCR) protocols have been described for '*Ca*. L. solanacearum' detection in plant material and insect vectors. Using conventional PCR '*Ca*.

L. solanacearum' was detected in eggs, different nymph instars stages and adults of *B. cockerelli* (Hansen *et al.,* 2008). The bacterium was also detected by conventional PCR in field-collected and laboratory-reared *T. apicalis* in southern Finland (Munyaneza *et al.*, 2010b). Squash real-time PCR is a useful tool for the detection of nucleic acid targets in insect vectors and was successfully used to detect '*Ca*. L. americanus' and '*Ca.* L. asiaticus' in *D. citri* specimens (Bertolini *et al*., 2014a). The squashing of individual psyllids on membranes is a direct method of sample preparation in which neither extract preparation nor nucleic acid purification is necessary. The main drawback of these systems based on target immobilisation is the small amount of sample that can be loaded onto the support. This limitation is overcome by coupling these preparation methods with highly sensitive techniques such as real-time PCR (De Boer & López, 2012). In addition, the immobilisation of targets on paper is simpler and much faster than deoxyribonucleic acid (DNA) extractions and can be used with quarantine pathogens without risks. The presence of DNA targets in individual psyllids can be accessed from fresh and previously captured individuals stored in alcohol and/or squashed on paper (Marroquín *et al*., 2004).

The knowledge of arthropod species that visit crops and the seasonal fluctuations of their populations is basic for the identification of putative vector species and the development of control strategies (Cambra *et al*., 2006). Thus, the main goal of this study was to evaluate the psyllid species that landed on carrot,

celery and potato crops, with high prevalence of '*Ca*. L. solanacearum', grown in different Spanish regions to determine the population dynamics. We also investigated whether the bacterium was associated to any of the different captured psyllid species.

Materials and Methods

Hosts and monitoring sites

Seasonal surveys were carried out in carrot and celery crops at Villena (Alicante) and Tenerife (Canary Islands) during different growing seasons between 2011 and 2012. Occasionally, carrot crops were also surveyed in the La Rioja region (Santo Domingo de la Calzada), and these surveys were extended to potato in Tenerife and Valencia from 2012 to 2014. The cvs. and monitored crops were as follows: Loretta and Golden var. *dulce* (Mill.) Pers. in celery plots grown in Villena; cv. Maestro in Villena and cv. Bangor in La Rioja in carrot plots; cv. Vivaldi in Valencia in a potato plot; cv. Bangor in carrot plots and cv. Slaney in Tenerife in a potato plot (Table 1).

A total of 16 commercial plots located in different regions where '*Ca*. L. solanacearum' prevalence was high were selected since 2011 to 2014. For the seasonal monitoring of arthropods that visit celery plants in 2011, three plots of approximately 1 ha were selected in Villena, representing each celery cycle of cultivation. In 2012, another six celery fields of 1 ha were also seasonally monitored in Villena, one during the early cycle, two in the middle cycle and three in the late cycle in an attempt to cover the possible

differences between plots over time. Two fields (1.3 and 0.4 ha, respectively) were also selected in 2012 in Tenerife for seasonal insect monitoring on carrot, one from each carrot cycle of cultivation, as well as, one potato field of approximately 0.2 ha. Finally, one carrot plot in La Rioja and one in Villena in 2012, one carrot plot in La Rioja in 2013, and one potato plot in Valencia in 2014 were selected to extend insect catches for identification purposes and to test '*Ca*. L. solanacearum' presence in the insect (Table 1).

Table 1. Information on the experimental plots of celery, carrot and potato seasonally or occasionally monitored in mainland Spain and the Canary Islands from 2011 to 2014.

	Crop	Year	Cultivar	Plot				Cycle	Beginning[a]	End[a]
				Reference	Location	Latitude	Longitude			
Seasonal	Celery	2011	Loretta	01010111	Villena	38º35'59" N	0º52'30' 'W	Early	03/11	06/14
			Loretta	08010111	Villena	38º36'29" N	0º53'1" W	Middle	06/03	09/12
			Loretta	26010111	Villena	38º35'46" N	0º52'34" W	Late	08/01	11/10
		2012	Loretta	36010112	Villena	38º40'11" N	0º54'45" W	Early	03/14	06/13
			Loretta	23010112	Villena	38º37'23" N	0º53'13" W	Middle	06/21	09/02
			Loretta	31010112	Villena	38º37'33" N	0º55'29" W		06/26	09/13
			Loretta	11010212	Villena	38º35'25" N	0º52'18" W		07/12	10/22
			Loretta	08010212	Villena	38º36'70" N	0º52'37" W	Late	07/27	11/15
			Golden	40010112	Villena	38º36'31" N	0º52'2" W		08/02	11/12
	Carrot		Bangor	20120001	Tenerife	28º30'13" N	16º21'53"W	Middle	05/08	07/17
			Bangor	20120002	Tenerife	28º30'23" N	16º20'57"W	Late	08/17	10/25
Occasional	Potato		Slaney	20120003	Tenerife	28º30'13" N	16º21'55"W	Middle	05/17	07/12
	Carrot		Maestro	18100312	Villena	38º37'81" N	0º52'34" W	Late	08/10	02/13
			Bangor	20120004	La Rioja	42º32'34" N	2º53'43" W	Late	05/23	12/19
		2013	Bangor	20130001	La Rioja	42º34´32" N	2º53´44" W	Late	05/06	12/27
	Potato	2014	Vivaldi	20140001	Valencia	39º31´49" N	0º22´47" W	Early	01/15	05/15

[a] Month/day of the beginning and end of the crop.

Monitoring of arthropods

Arthropods monitoring was focused on Hemiptera belonging to Cicadellidae and Aphididae and the superfamily Psylloidea. The sampling for the seasonal surveys was performed weekly since the

emergence of the plants until harvest during all the cycles of cultivation. It was done sporadically during occasional surveys. Monitoring was conducted sporadically during the occasional surveys.

For the seasonal surveys, the same 20 celery or 50 carrot plants were randomly selected in the plot and non-destructively sampled at weekly intervals. The whole plant was initially sprayed with glue (Souverode aerosol, Scotts, France) however, as the plants grew larger (4 weeks) only 1-2 fully developed leaves were sprayed. The sprayed leaves were detached after a week, and the new sticky leaves were prepared. The removed sticky leaves with arthropods stuck on the surface were placed in turpentine to dissolve the glue, and collected specimens were washed in soapy water to remove the solvent (Marroquín *et al*., 2004). The collected arthropods were kept in 70% alcohol for later counting and identification.

For the occasional surveys, a sampling site was randomly selected within the field, and 10 consecutive plants were monitored. One to two fully developed leaves from each plant were sprayed every 10 days. The attached arthropods were treated as previously described. The carrot surveys in La Rioja (2012 and 2013) and Villena (2012) were performed in autumn (September to November), whereas the potato survey in Valencia was performed in spring (March to May) and in Tenerife in spring-summer (May to July).

Identification of arthropods

Aphids, leafhoppers and psyllids were kept in alcohol, and the other arthropods were discarded. The selected families were counted by date of capture and then identified. Only the superfamily Psylloidea was identified to the species level due to the important role they may play in the transmission of '*Ca*. L. solanacearum'.

The identification was based on morphological characteristics using classification keys (Ribout 1936, Ribout 1952; Ramírez (1955, 1956, 1959); Shaposhnikov & Davletshina, 1967; Hodkinson *et al*., 1981; Hermoso de Mendoza, 1982; Ossiannilsson *et al.,* 1992; Ouvrard & Burckhardt, 2012). Some specimens were mounted on slides following the method of Hodkinson & White (1979) and photographed.

Detection of '*Ca*. L. solanacearum' targets in psyllid species

The identified individual psyllid specimens were squashed on Whatman 3 MM membranes (GE Healthcare Europe) using the round bottom of an Eppendorf tube until the complete disruption of the insect (Olmos *et al*., 1996; Bertolini *et al*., 2014a). The membrane containing squashed psyllids was carefully cut around the sample (approximately 0.5 cm^2) and inserted into Eppendorf tubes. DNA was released from the piece of the membrane by adding 100 µl of distilled sterile water and vortexed. Three µl were analysed by specific '*Ca*. L. solanacearum' real-time PCR according to Teresani *et al*. (2014a) using a CaLsol/100 kit (Plant Print Diagnòstics, Valencia, Spain). Positive and negative controls

(5 µl of crude extract of infected and healthy plant material spotted on a piece of membrane, respectively) and PCR reagents were used. Psyllids were considered positive when an exponential amplification curve occurred and the Ct value was below 45.

Results

Arthropod monitoring

A total of 18,751 arthropods were captured during the seasonal surveys. From this total, 2,695 arthropods were caught on celery in 2011 in Villena. The superfamily Psylloidea (1,373 individuals) and the family Aphididae (762) were the most frequently found, followed by Cicadellidae (560) (Table 2). In 2011, the higher numbers of captured arthropods were observed in the middle cycle of cultivation (June 3rd to September 12th). A total of 1,533 specimens were caught in 2012 on celery. Cicadellidae was the most frequently found family in the sticky plants (924 specimens), followed by Aphididae (483) and Psylloidea (126) (Table 2). In 2012, a low number of psyllid species were caught in comparison with 2011, and no remarkable peaks of the populations of any the species were observed.

A total of 14,523 arthropods were caught on carrot crops in Tenerife. The superfamily Psylloidea was the predominant (14,401 specimens) followed by Aphididae (62) and Cicadellidae (60) (Table 2).

Table 2. Total numbers and percentage of arthropods collected in seasonal surveys on celery plants in Villena in 2011 and 2012 and carrot plants in Tenerife in 2012 using the sticky plant method.

Crop	Location	Year	Cycle of cultivation	Aphididae	Cicadellidae	Psylloidea	Total
Celery	Villena	2011	Early	144	24	47	215
			Middle	568	346	792	1,706
			Late	50	190	534	774
			Total (%)	762 (28.3)	560 (20.8)	1,373 (50.9)	**2,695**
		2012	Early	269	101	12	382
			Middle[a]	36	352	16	845
				50	353	38	
			Late[b]	19	35	4	308
				50	55	44	
				59	28	12	
			Total (%)	483 (31.5)	924 (60.3)	126 (8.2)	**1,533**
Carrot	Tenerife	2012	Middle	52	37	14,262	14,351
			Late	10	23	139	172
			Total (%)	62 (0.4)	60 (0.4)	14,401 (99.1)	**14,523**

[a] Two experimental plots by cultivation cycle of celery.
[b] Three experimental plots by cultivation cycle of celery.

Psyllid species composition

The overall numbers of captured psyllid species (15,900 individuals) in the seasonal surveys are shown in Table 3. The population dynamics of psyllid species captured in celery plots in Villena (2011) are presented in Figure 1. A total of 1,499 psyllids were caught on celery in Villena, 1,373 in 2011 and 126 in 2012. In 2011, the predominant species were as follows: *B. trigonica* (1,085 specimens) followed by *B. tremblayii* Wagner (225) and *B. nigricornis* Förster (2).

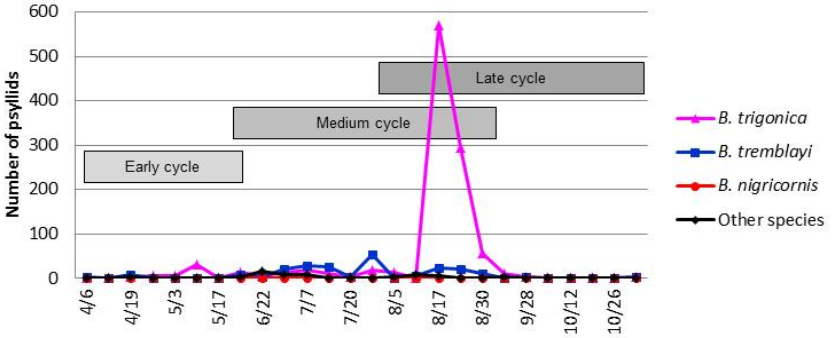

Fig. 1. Population dynamics of psyllid species monitored using the sticky plant method on celery plants in Villena in 2011 at the different cycles of cultivation.

Sixty-one specimens from other psyllid species (*Bactericera* sp., *Trioza* sp. and *Psylla* sp.) were also caught in 2011. Two maximum population peaks of *B. trigonica* were observed during the summer of 2011. The most important peak occurred from August 10th to 17th, with 570 specimens captured. The second peak occurred from August 24th to 30th, with 293 specimens captured (Figure 1).

The population dynamics of psyllid species captured on celery plots in Villena (2012) are presented in Figure 2. The two species found in the second year of seasonal surveys were *B. trigonica* (48) and *B. tremblayi* (46). Thirty-two specimens from other non-identified psyllid species (most likely *Bactericera* sp.) were also captured. *B. nigricornis* was not found on celery plants in the Villena area in 2012.

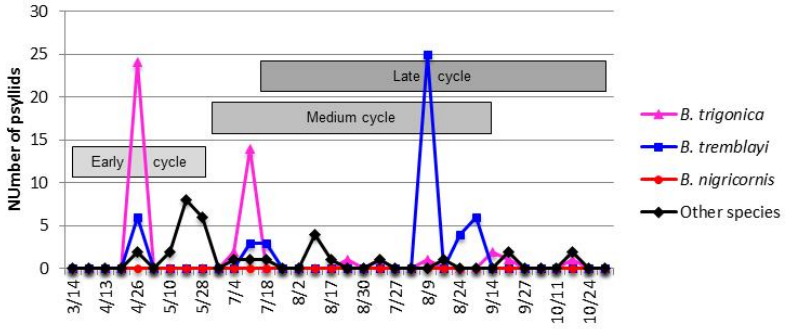

Fig. 2. Population dynamics of psyllid species monitored using the sticky plant method on celery plants in Villena in 2012 at the different cycles of cultivation. Combined data for the middle and late cycles.

A total of 14,401 psyllids were caught on carrot fields in Tenerife, with *B. trigonica* as the dominant psyllid species, followed by *Bactericera* sp. and *Trioza urticae* Linnaeus (Figure 3).

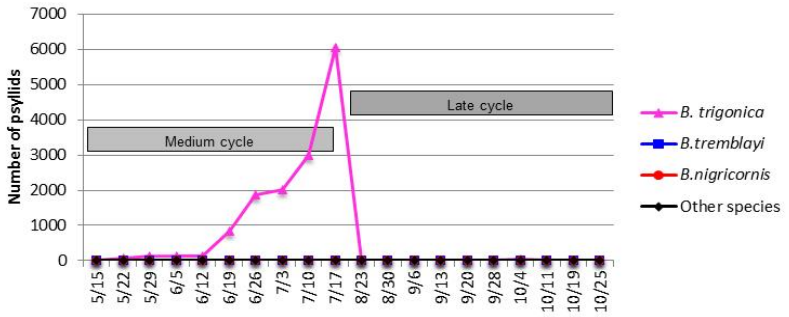

Fig. 3. Population dynamics of psyllid species monitored by the sticky plant method on carrot plants in Tenerife in 2012 at medium and late cycles of cultivation.

In the middle cycle of cultivation, 14,255 *B. trigonica* and 7 specimens from other psyllid species (*Bactericera* sp.) were caught. In the late cycle, a lower number of psyllids than in the previous

cycle was found: 119 *B. trigonica* and 20 specimens of *T. urticae*, *Ctenarytaina* sp. and *Cacopsylla* sp. (other species in Table 3) were also identified in Tenerife (Figure 3).

Table 3. Different psyllid species captured in seasonal surveys on sticky celery and carrot plants in 2011 and 2012. Estimation of the number of specimens carrying the bacterium was determined by real-time polymerase chain reaction (PCR).

	Location	Year	Cycle of cultivation	*B. trigonica*	*B. tremblayi*	*B. nigricornis*	Other[c]	Total
Celery	Villena	2011	Early	0+/36[a]	3+/9	0+/0	0+/2	3+/47
			Middle	43+/566	10+/180	0+/1	0+/45	53+/792
			Late	0+/483	0+/36	0+/1	0+/14	0+/534
			Total	**43+/1,085**	**13+/225**	**0+/2**	**0+/61**	**56+/1,373**
		2012	Early	0+/5	0+/2	0+/0	0+/5	0+/12
			Middle	0+/4	1+/2	0+/0	0+/10	2+/55
				1+/27	0+/3	0+/0	0+/9	
			Late	0+/4	0+/0	0+/0	0+/0	0+/59
				0+/4	0+/39	0+/0	0+/0	
				0+/4	0+/0	0+/0	0+/8	
			Total	**1+/48**	**1+/46**	**0**	**0+/32**	**2+/126**
Carrot	Tenerife	2012	Middle	31+/95 (14,255)[b]	0+/0	0+/0	3+/7[d]	34+/102 (14,262)
			Late	38+/119	0+/0	0+/0	0+/20	38+/139
			Total	**50+/214**	**0+/0**	**0+/0**	**3+/27**	**53+/241 (14,401)**

[a] '*Candidatus* Liberibacter solanacearum' positive psyllid specimens/total of analysed psyllid specimens.
[b] Thirty-one positive psyllids out of 95 analysed from a total of 14,255 captured.
[c] Other species: *T. urticae*, *Bactericera* sp., *Cacopsylla* sp., *Ctenarytaina* sp., *Psylla* sp. and *Trioza* sp.
[d] The three positive specimens belonging to *Bactericera* sp.

A total of 811 psyllids were caught in the occasional surveys on carrot and potato from 2012 to 2014 (Table 4). *B. trigonica, B. nigricornis* and *B. tremblayi* were identified in the carrot surveys performed in 2012 and 2013 in La Rioja and Villena. *B. nigricornis* (38 specimens) and *B. tremblayi* (25) were the only species captured in La Rioja in 2012, and *B. trigonica* (26) and *B. tremblayi* (2) were the species captured in Villena in 2012. *B. trigonica* (476

specimens), *B. tremblayi* (44) and other psyllid species (70) were captured in La Rioja in 2013. *B. trigonica* (7 specimens), *B. nigricornis* (2) and other non-identified psyllid species (2) were the only species captured in the potato surveys in 2014 in Valencia, whereas *B. trigonica* (102) and *Bactericera* sp. (17) were caught on potato in Tenerife.

Table 4. Different psyllid species collected in occasional surveys on carrot by sticky leaves in the La Rioja region and Villena and on potato in Tenerife and Valencia. Estimation of the number of specimens carrying the bacterium was determined by real-time polymerase chain reaction (PCR).

Psyllid species	Carrot			Potato	
	2012		2013	2012	2014
	La Rioja	Villena	La Rioja	Tenerife	Valencia
B. trigonica	0+/0[a]	0+/26	210+/476	35+/102	0+/7
B. tremblayi	24+/25	0+/2	33+/44	0+/0	0+/0
B. nigricornis	36+/38	0+/0	0+/0	0+/0	0+/2
Other	0+/0	0+/0	50+/70[b]	0+/17	0+/2
Total	60+/63	0+/28	293+/590	35+/119	0+/11

[a] '*Candidatus* Liberibacter solanacearum' positive psyllid specimens/total of analyzed psyllid specimens.
[b] Other indicates non-identified specimens, most likely *B. trigonica,* that had key morphological characteristics damage that was caused during the process of recovery from the sticky plant.

Detection of '*Ca.* L. solanacearum' DNA targets in individual psyllids

'*Ca.* L. solanacearum' targets were amplified by squash real-time PCR from *B. trigonica*, *B. tremblayi*, *B. nigricornis* and other non-identified psyllid species (Table 3 & Table 4). Targets of the bacterium were amplified in 43 out of 1,085 *B. trigonica* and in 13 out of 225 *B. tremblayi* individuals caught on celery crops located

at Villena in 2011. '*Ca.* L. solanacearum' targets were only detected in one out of 48 *B. trigonica* tested and in one out of 46 *B. tremblayi* analysed in 2012. The bacterium was not found in *B. nigricornis* or the other non-identified psyllid species in Villena during both years. Two hundred forty one out of the 14,401 captured psyllids in the seasonal surveys on carrot in Tenerife were analysed (Table 3). The *B. trigonica* and *Bactericera* sp. collected on carrot in Tenerife in both years tested positive against '*Ca.* L. solanacearum'. In the middle cycle of cultivation, approximately 1% of the total *B. trigonica* captured were analysed; and in 31 specimens out of 95 *B. trigonica* and in 3 specimens out of 7 *Bactericera* sp*.,* positive amplification was observed. In the late cycle of carrot cultivation, 38 out of 119 *B. trigonica* were positive against the bacterium. None of the 20 specimens from the other psyllid species tested positive (Table 3).

'*Ca.* L. solanacearum' targets were amplified in 24 out of 25 *B. tremblayi* and in 36 out of 38 *B. nigricornis* in occasional carrot surveys in La Rioja in 2012. In 2013, bacterial targets were amplified in 210 out of 476 *B. trigonica*, in 33 out of 44 *B. tremblayi* and in 50 out of 70 non-identified psyllid species. Thirty-five out of 102 *B. trigonica* collected in occasional potato surveys in Tenerife in 2013 tested positive to *'Ca.* L. solanacearum' by real-time PCR. The bacteria were not detected in the other psyllid species collected in this crop or in the crop itself. '*Ca.* L. solanacearum' targets were not amplified from psyllids captured in the potato crops in Valencia in 2014 (Table 4).

Discussion

The knowledge of the seasonal dynamics and abundance of arthropods in crops is key to determine the species responsible for the natural spread of '*Ca.* L. solanacearum'. In adition, due to little or no available information on the arthropod species that lands on economically important crops, is necessary to identify the species that visit the potential hosts of the bacterium in different ecological areas in Spain. This is a basic foundation that is necessary to design strategies to mitigate the natural spread of the bacterium.

During the seasonal arthropod surveys performed in celery crops in Villena in 2011 and 2012, 35.4% of the catches belonged to the superfamily Psylloidea and important differences were observed between both years in the number of specimens and the prevalent families captured. In 2011, psyllids were the most frequently identified arthropods. The summer period was the season with most captured insects, corresponding to the middle cycle and the beginning of the late cycle of celery cultivation. The most prevalent species in this period was *B. trigonica,* which was previously associated with '*Ca*. L. solanacearum' transmission in Spain (Alfaro-Fernández *et al*., 2012b). In 19.5% of the psyllid species tested, '*Ca.* L. solanacearum' targets were amplified, suggesting the high prevalence of the bacterium in the celery plants grown in the monitored areas. This fact could justify the higher prevalence of symptoms and the important crop losses in the middle and late cycles of cultivation. In 2012, the most frequent visitors were included in the families Cicadellidae, followed by Aphididae

and the superfamily Psylloidea. In this year, although there were two celery plots sampled in the middle cycle and three in the late cycle of cultivation, the prevalence of arthropods was very low compared with the previous year. Consequently, a lower prevalence of symptoms in celery was observed, whereas the presence of symptomatic carrots was similar to the previous years. This was likely, due to the primary infection caused by carrot seed transmission that was recently demonstrated (Bertolini *et al*., 2014b). In both of the survey years, the arthropods collected on sticky celery plants showed the same psyllid species structure: *B. trigonica* was always the prevalent species and *B. nigricornis* was the less frequently observed species, with only two caught specimens in 2011. The same psyllid species are visiting early potato crops in Valencia, representing a threat for the crop if non-solanaceaous haplotypes are able to colonise potato plants. In addition, non-identified *Trioza* sp*., Psylla* sp. and *Bactericera* sp. (but not *B. cockerelli* and *T. apicalis*), were found on carrot, celery and potato in mainland Spain.

In total, 99.1% of the 14,401 captured insects on carrot during seasonal surveys performed in Tenerife in 2012 were psyllids. This high number is typically found in the middle cycle of cultivation, with a maximum peak of 6,063 specimens caught in summer. Almost all the psyllid species captured were *B. trigonica*, ranging from 99.9% in the middle cycle to 85% in the late cycle of carrot cultivation. *T. urticae*, *Bactericera* sp., *Cacopsylla* sp. and *Ctenarytaina* sp. were the other psyllid species captured in the

monitored plots, showing a high population number and diversity of species in the Canary Islands. The population dynamics of psyllids in the potato plots in Tenerife were in agreement with the dynamics observed in carrots. The comparison of the number of psyllid species caught on celery in mainland Spain and on carrot in the Canary Islands suggests that carrot is the preferential host for the species found in the monitored areas. In fact, in Villena, where carrot and celery are grown in the vicinity, a higher prevalence of psyllid species was found in carrot than in celery (data not shown).

The Spanish mainland climate varies across the peninsula among the three main climatic zones, which can be distinguished according to the geographical location and orographic conditions. The typical Mediterranean climate is represented by Valencia, the continental Mediterranean climate by Villena and the last zone, with some oceanic characteristics is represented by the climate of La Rioja. The subtropical climate is the predominant climate in the surveyed carrot and potato areas in the Canary Islands. Dixon *et al*. (1985) reported that the population dynamics of aphid species can vary depending on the year and other factors, such as whether conditions, natural enemies, abundance in previous years or human actions. Some of these factors could explain the variation in the number of arthropod species caught among the different years and cycles of cultivation in different areas. In fact, the rainfall in 2012 (352.4 mm) was approximately twice that in 2011 (181.7 mm) in Villena. In Tenerife, the late cycle of cultivation occurs in autumn when the temperature is lower than in the previous cycles and the

rainfall is constant (data not shown). The mentioned factors could also justify why the population structure changed in the carrot crops in La Rioja between 2012 (*B. trigonica* was not captured and *B. nigricornis* was present) and 2013 (*B. trigonica* was the prevalent species and *B. nigricornis* was not captured). However, these factors did not affect *B. tremblayi* which maintained similar populations in both years.

The surveys were focused on arthropod families in which several species are described as vectors of plant pathogens. Psyllid species are cited as efficient vectors of the fastidious bacterium '*Ca*. Liberibacter' in economically important crops: *D. citri*, *T. erytreae* and *C. citrisuga* in citrus (Bové, 2006; Cen *et al*., 2012a), *B. cockerelli* in potato and tomato (Hansen *et al*., 2008) and *T. apicalis* in carrot (Nissinen *et al*., 2014). Moreover, recent studies suggest that Liberibacter species may be more widespread than previously thought, and vector species play an important role in bacterial spread. In addition, psyllids such as *B. cockerelli* and *T. apicalis* are able to transmit the same bacterium in distant and different geographical areas, highlighting the importance of the local identification of psyllid species and putative vectors. For this reason, we focused our interest in psyllid species. Bertolini *et al*. (2014b) reported that the presence of *B. trigonica* and other species was correlated with an increase in the prevalence of the bacterium from 2% to approximately 100% after six months of carrot cultivation. Other experiences with emerging diseases in the citrus and potato industries suggest that psyllid species, feeding briefly

outside their normal plant host range, could introduce a pathogen to another crop (Nelson *et al.*, 2013). This might present a serious threat for other economically important crops, such as potato, tomato and aubergine which could naturally be infected by '*Ca*. L. solanacearum' if psyllid species carrying the bacterium have the opportunity to reach the phloem of a potential host species.

Villaescusa *et al*. (2011) reported, using Moericke´s yellow traps, the arthropods occurring in the ambience of carrot and celery plots in Spain; psyllids represented 92.4% of the total number of captured arthropods. In our case, using sticky plants, psyllids landing on the plants represented 50.9% in 2011 and 8.2% in 2012 of the total number of the arthropods. Our data are essentially in agreement with the structure of the species found in the previous study, where *Bactericera* spp. (85% of the total psyllid caught), *Cacopsylla* sp. and *Trioza* sp. were caught. Althought the authors did not identify the *Bactericera* spp. Found, it is likely that *B. trigonica*, *B. tremblayi* and *B. nigricornis* were already present in the Villena area at that time.

To make decisions regarding disease integrated pest management control strategies, it is essential to identify the psyllid species that land on a particular crop and estimate the abundance of the different species and the percentage of specimens carrying the bacterium. In this context, the use of appropriate methodology is crucial. The use of the sticky host plants make it possible to more accurately determine the species that actually land on the plants. In addition, the squash protocol described by Bertolini *et al.* (2014a)

combined with real-time PCR described by Teresani *et al*. (2014a) has demonstrated their potential for the detection of '*Ca*. L. solanacearum' targets in psyllids. The squash procedure and subsequent detection by PCR-based methods yielded similar results using fresh or those preserved in alcohol for the detection of the viral targets (Marroquín *et al*., 2004). We assumed the non-effect of the treatment to remove the psyllids stuck on the plant and the preservation in alcohol. In fact, we were able to detect amplifiable '*Ca*. L. solanacearum' targets in 95.2% of the captured psyllids in La Rioja in 2012 using this methodology. The use of this technique allowed the estimation of the percentage of psyllids carrying the bacterium that could transmit it if given the opportunity to feed on the phloem of a host species. Although we have not yet performed transmission trials, the detection of targets is a strong indication that the squashed arthropods are harboring '*Ca*. L. solanacearum' acquired from infected plants; it is likely that it has multiplied in the insect to become detectable by real-time PCR. These facts represent the risk of bacterium transmission and disease spread.

B. tremblayi, *B. nigricornis* and *B. trigonica* are morphologically close psyllid species that belongs to the 'Bactericera nigricornis Förster group' (Hodkinson, 1981). They have polyphagous habits and show overlapping areas of distribution. These species were formally reported in Bosnia-Herzegovina, France, Greece, Iran, Italy, Serbia, Switzerland and Turkey (Ouvrard & Burckhardt, 2012). Here, we report the presence of *B. tremblayi* and *B. nigricornis* in Spain in addition to

B. trigonica, which was already reported by Alfaro-Fernández *et al*. (2012b) and is widely distributed in the Mediterranean region (Haapalainen, 2014). '*B. nigricornis* group' is composed of multivoltine species (Hodkinson, 2009), that feed on a variety of herbaceous plants, including beet, cabbage, carrot, onion, parsley or potato (Burckhardt & Lauterer, 1997), which are known hosts or potential hosts of '*Ca*. L. solanacearum'. Adults have also been recorded to overwinter on conifers (Reuter, 1908). This level of polyphagy is exceptional in Psylloidea, which are usually host specific (Hodkinson, 1974).

B. trigonica, B. tremblayi and *B. nigricornis* were found to be current visitors of the surveyed crops in continental Spain. In the Canary Islands, *B. trigonica* is the predominant species, which is in agreement with previous reports (Alfaro-Fernández *et al*., 2012a; Font *et al*., 1999). All these visitors, which carry '*Ca.* L. solanacearum', are potential vectors of the bacterium in different ecological areas. Only *B. tremblayi* is associated with Mediterranean climates; however, *B. trigonica* and *B. nigricornis*, which are also found in these regions, are associated with most temperate climates. This fact could suggest the climate adaptation of these species, which can be found in the north (La Rioja), Mediterranean coast (Valencia), continental country side (Villena) and the Canary Islands (Tenerife), representing a broad spectrum of climatic conditions.

Currently, there are no effective control strategies for plant protection against natural '*Ca*. Liberibacter' infection, except the

potential use of cultivation under insect-proof facilities. The "three-pronged system" (TPS) (Belasque *et al*., 2010) is used for HLB management in perennial plants could be adjusted for horticultural crops. This system involves the removal of inoculum sources, the replacement of infected trees with healthy trees and insecticide treatments aimed to reduce psyllid vector populations to mitigate the spread of the disease. The use of '*Ca*. L. solanacearum'-free carrot seed lots could be complemented with an accurate and timely detection of visitor psyllid species that may serve as a vector of the pathogen and with subsequent treatments to interfere with the transmission of the bacterium. The reduction of the psyllid population is critical independent of the efficiency of the transmission of the different vectors involved. Any vector species could play a role in the bacterium spread by compensating with their abundance poor transmission efficiencies (Marroquín *et al*., 2004).

This paper provides information about the psyllid species population that lands on celery, carrot and potato plants in Spain and reports, for the first time, *B. tremblayi*, *B. nigricornis* and *Bactericera* sp. as '*Ca*. L. solanacearum' carriers and potential vectors of the bacterium. However, experimental transmission assays are necessary to assess the vector ability of the psyllid species that have not been previously described as vectors of '*Ca*. L. solanacearum'.

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*The references of this paper (chapter) are included in the list of "Literature cited"

Transmission of '*Candidatus* Liberibacter solanacearum' by *Bactericera trigonica* Hodkinson. Threat to *Apiaceae* and *Solanaceae* crops

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To be submitted

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Abstract

The bacterium '*Candidatus* Liberibacter solanacearum' is a recent plant pathogen of several *Apiaceae and Solanaceae* crops and is associated with economically important losses. The bacterium is a carrot seed borne pathogen that can also be transmitted from potato mother tubers and by psyllid vectors. The psyllid *Bactericera trigonica* Hodkinson was recently described carring '*Ca.* L. solanacearum', bacteria associated with vegetative disorders in carrot and celery crops in Spain. Electrical Penetration Graphs showed that *B. trigonica* was able to feed in the phloem of carrot and celery and to probe (0.13% probability) in potato but not in the phloem of tomato plants. A series of transmission assays were conducted under controlled conditions to assess the ability of the psyllid to transmit the bacterium to several hosts. The bacterium was transmitted from infected carrots to carrot, celery, potato and tomato plants when single or groups of ten *B. trigonica* were forced to feed on these species. An inoculation access period of 24 hours was sufficient for a single *B. trigonica* to transmit the bacterium to carrot (67.8%), to celery (21.1%) and to potato and tomato (6.0%).

More efficient transmission occurred with ten individuals, and the transmission rates were 100% in celery, 80% in carrot and 10% in potato and tomato. Transmission efficiencies were higher with *Apiaceae* than with *Solanaceae* in assays that used carrot as the donor. The results confirm that *B. trigonica* is a vector of the bacterium to carrot and celery and represents a threat to potato and tomato crops.

Introduction

The bacterium '*Candidatus* Liberibacter solanacearum' (Liefting *et al*., 2009), also known as '*Ca*. Liberibacter psyllaurous' (Hansen *et al*., 2008), is a Gram-negative, phloem-limited, and psyllid-vectored bacterium in the family *Rhizobiaceae* of the α-Proteobacteria group. The bacterium is associated with zebra chip, which is one of the most economically important bacterial diseases of potato (*Solanum tuberosum* L.). The bacterium was also associated with vegetative disorders in other *Solanaceae* and *Apiaceae* crops in different areas worldwide (Haapalainen, 2014). The '*Ca*. L. solanacearum' is transmitted from potato mother tubers to growing plants and to progeny tubers (Pitman *et al*., 2011) and is a carrot seedborn pathogen (Bertolini *et al*., 2014b), which poses a high risk of introduction of the bacterium into new areas where seeds are commercialised. However, the bacterium is naturally spread by different psyllid species once it is established (Teresani *et al*., 2014b).

Psyllids (Hemiptera: Psylloidea) are small, 2-3 mm long

four-winged insects that feed on plants by sucking the phloem sap through a stylet. Currently, approximately three thousand species are known and classified in 11 families (Hodkinson, 2009; Ouvrard, 2014). The life cycles vary depending on the species and environmental conditions but it typically is approximately one month. The adults can fly over a 1 km distance and can be carried farther with the wind. The total number of eggs laid by a mated female ranged from one hundred to one thousand for most of the species studied (Hodkinson, 2009). Several species of psyllids are important agricultural pests that cause damage to the host plants with injections of toxic salivary secretions during feeding that produce necrosis, galling and plant malformations, in addition to transmitting pathogens (Munyaneza *et al*., 2010b).

Reports show that the fastidious bacterium '*Ca.* Liberibacter' is transmitted by a number of psyllid species that are associated with newly emerging and economically important diseases. The three '*Ca.* Liberibacter' species associated with huanglongbing (HLB) citrus disease are transmitted by *Diaphorina citri* Kumayama (Capoor, 1967), *Trioza erytreae* Del Guercio (McClean & Oberholzer, 1965), and *Cacopsylla citrisuga* Yang & Li (Cen *et al*., 2012). Additionally, *D. communis* Mathur was identified in Bhutan carrying '*Ca*. L. asiaticus' (Donovan *et al*., 2012), but the ability to transmit the bacterium was not reported. '*Ca*. L. solanacearum' is transmitted by *Bactericera cockerelli* Sulc in potato (Munyaneza *et al*., 2007b) and by *Trioza apicalis* Förster in carrot in Northern Europe (Nissinen *et al*., 2014). *Bactericera*

trigonica Hodkinson is associated with vegetative disorders of carrot in Spain (Alfaro-Fernández *et al*., 2012b). However, no studies on feeding behaviour or experimental transmission were conducted.

The detection methods for '*Ca*. L. solanacearum' include both conventional and real-time PCR of plant and insect tissues. The bacterium was detected in eggs, in different nymph instars and in adults of *B. cockerelli* (Hansen *et al*., 2008) and in field-collected and laboratory-reared *T. apicalis* by conventional PCR (Munyaneza *et al*., 2010b). The bacterium was also detected in unidentified psyllid species of the genera *Acizzia* and *Trioza* collected from *Acacia* and *Pittosporum* in New Zealand (Scott *et al*., 2009). The bacterium was also detected in *B. trigonica*, *B. tremblayi*, *B. nigricornis* and *Bactericera* sp. from Spain with squash real-time PCR (Teresani *et al*., 2014b).

The Electrical Penetration Graph (EPG) technique records changes in resistance, providing information on stylet tip position in specific plant tissues, time spent at each location, and stylet activity (Tjallingii, 1985). Waveforms were established by EPGs for several hemipteran species as aphids, whiteflies, trips or leafhoppers (i.e., Tjallingii, 1978; Prado & Tjallingii, 1994; Janssen *et al*., 1989; Kindt *et al*., 2003 & 2006; Miranda *et al*., 2009; Almeida *et al*., 2004). The EPG waveforms of psyllids were reported for *D. citri* (Bonani *et al*., 2010; Cen *et al*., 2012b), *Bactericera cockerelli* (Butler *et al*., 2012; Pearson *et al*., 2014; Sandanayaka *et al*., 2014), *Cacopsylla pyri* L. (Civolani *et al*. 2011) and *Psylla pyricola*

Förster (Ullman & McLean, 1988). To increase the understanding of pathogen transmission by *B. trigonica*, feeding behaviour parameters were studied using EPGs to assess the capacity of a *B. trigonica* individual to reach and feed from the phloem of different hosts to acquire and inoculate de bacterium effectively.

Mechanisms of transmission are best understood by considering the routes of pathogen movement in the insect and the sites of retention in insect tissues. The most fundamental distinction with regard to the mode of transmission is whether ingested particles are circulative or noncirculative in the vector, a distinction that focuses on the duration and/or site of retention and the route of movement within the insect (Blanc *et al*., 2014; Cooper *et al*., 2014). The '*Ca.* Liberibacter' spp. are transmitted through the psyllid vector to the plant host in a way similar to that described for persistently insect-transmitted plant viruses (Hogenhout *et al.*, 2008). For transmission success, the bacterial cells must pass through the alimentary canal wall and move through the haemolymph to reach the salivary glands, from where the bacteria are transmitted with salivary secretions into a new host plant during psyllid feeding (Haapalainen, 2014). '*Ca*. L. solanacearum' cells are increased from 0 to 2 weeks in *B. cockerelli* and a latent period of at least two-weeks is necessary for successfull transmission (Sengoda *et al*., 2014).

The actual risk of transmission of a pathogen to a crop under field conditions could be estimated by the vector intensity (Irwin & Ruesink, 1986), which is determined as the final product of two

factors: the vector activity (number of insects visiting the crop) and the vector propensity (probability of a vector transmitting a pathogen under field conditions). The number of insects carrying the bacterium can also influences the second factor.

Because of the recent association of '*Ca*. L. solanacearum' with several crops affected by psyllid species and the report of the association of *B. trigonica* with the bacterium in carrots in Spain, investigations were conducted to evaluate the vector propensity of this psyllid species on carrot, celery, potato and tomato and to evaluate the threat that this species represents to economically important solanaceous crops.

Materials and Methods

Source of insects

A *B. trigonica* laboratory colony was established from field-collected individuals obtained by sweep net sampling in commercial carrot fields (cv. Bangor) heavily infected with '*Ca*. L. solanacearum' (haplotype E) in the La Padilla region in Tenerife (Canary Islands) in 2013. The insects were reared for several generations on '*Ca.* L. solanacearum' infected carrot plants in insect cages and maintained in a controlled environment room at 25±2°C, 70±5% humidity with a photoperiod of 16:8 (L:D) at the ICIA facilities in Tenerife. Individuals from this colony were regularly tested with real-time PCR (see below) to confirm the presence of '*Ca*. L. solanacearum'.

Source of receptor plants

Potato (*Solanum tuberosum* L.) cv. Terrenta, tomato (*Solanum lycopersicum* L.) cv. Robin, carrot (*Daucus carota* L.) cv. Bangor and celery (*Apium graveolens* L.) cv. Monterrey were used in the following experiments. The plants were grown from '*Ca*. L. solanacearum'-free seeds or from potted seedlings that tested negative for the bacterium. The plants were grown in pots and maintained in an insect-proof greenhouse at the ICIA facilities to be used as healthy receptor plants at 2-3 full expanded leaves stage.

Source of '*Ca*. L. solanacearum' inoculum

To generate a source of inoculum for pathogen acquisition in the transmission studies, symptomatic and haplotype E of '*Ca*. L. solanacearum' real-time PCR positive carrot plants were collected in the field and maintained in pots in an insect-proof greenhouse at the ICIA facilities to be used as donor plants at 2-3 full expanded leaves stage.

Sample preparation, DNA purification and '*Ca.* L. solanacearum' detection

Samples (leaves or the entire plant) were collected from carrot, celery, potato and tomato plants and placed into separate plastic bags and stored at 4ºC for up to one week. The extracts were prepared using a Homex 6 (Bioreba, Switzerland) homogenizer to grind the plant material in PBS extraction buffer at 1:5-10 (w/v). One ml of extract from each plant was stored at -20°C until used.

The total DNA was purified from 200 µl of crude plant extract by using the cetyl trimethyl ammonium bromide (CTAB) protocol (Murray & Thompson, 1980). The purified DNA was stored at -20ºC.

Fresh or previously preserved in 70% ethanol psyllids were individually squashed on nylon membranes with the rounded end of an Eppendorf tube (Bertolini *et al*., 2008). The membranes were immediately processed or kept at room temperature in a dry, obscure place for a maximum of 1 month. The samples immobilised on membranes were carefully cut out and inserted into Eppendorf tubes containing 100 µl of distilled water (Teresani *et al*., 2014b). Three microliters of the extract was directly used as a template for real-time PCR analysis.

The detection of '*Ca*. L. solanacearum' was performed with the real-time PCR Kit CaLsol/100 (Plant Print Diagnòstics, Valencia, Spain) based on the protocol described by Teresani *et al*. (2014a). The assays were conducted with the StepOne Plus (Applied Biosystems, Foster City, CA, USA) machine, and the data acquisition and analysis were performed with the thermal cycler's software. The default threshold set by the machine was slightly adjusted above the noise in the linear part of the growth curve. Positive and negative controls included in the kit, PCR cocktail and pieces of membrane were simultaneously and similarly processed using healthy plants and noninfected psyllids.

Electrical Penetration Graphs (EPG) studies

To evaluate the phloem-feeding ability of *B. trigonica* on different plant species, the stylet penetration activities were monitored using the EPG technique on carrot, celery, potato and tomato plants. For the EPG-controlled acquisition experiments, adult psyllids were anesthetised with CO_2 for 3 s and immediately immobilised using a vacuum chamber. Under a dissecting microscope, a gold wire measuring 20 μm in diameter and 3 cm in length was attached (Heraeus, Hanau, Germany) to the pronotum with a drop of silver conductive paint (16034 Pelco Collodial Silver, Ted Pella Inc., Redding CA, USA). After a one-hour starvation period, the insects with the gold wire attached were connected to the EPG device after attaching the opposite end of the gold wire to a copper electrode. A second electrode was connected to a copper post that was inserted into the plant pot. The *B. trigonica* were placed on the abaxial surface of a young '*Ca*. L. solanacearum'-free carrot, celery, potato or tomato leaf. The EPG records were obtained with a DC-EPG device (EPG-Systems, Wageningen, The Netherlands; Tjallingii, 1978 & 1988), adjusted to 100 x gain. The monitoring system was assembled inside a Faraday cage (100 x 110 x 90 cm) to prevent electrical noise. The EPG data acquisition was conducted using Stylet+ software for Windows (EPG Systems, Wageningen, The Netherlands).

Each insect was monitored for 8 h in the laboratory starting immediately after the insects were placed on the leaf. The EPG waveforms previously described for psyllids (Bonani *et al*., 2010;

Civolani *et al*. 2011) were identified as follows: non-probing (np), intercellular apoplastic 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 variables were processed using the MS Excel Workbook for automatic EPG data calculations that was developed by Sarriá *et al*. (2009). Fifteen replicates per receptor plant species were performed. The insects that did not probe or became detached from the wire within the first 15 minutes of recording were replaced. Insects that did not show clear EPG waveforms due to poor electrical contact were excluded from analysis. The ability of *B. trigonica* to reach the phloem of the plants was determined by observation of the following parameters: number of E1 events, number of E2 events, total duration of E1, total duration of E2, percentage of probing spent in E1, percentage of probing spent in E2 and the number of probes.

Statistical analyses of the data were conducted using the R software package. Because the distributions were non-normal, the comparisons of means were performed using the Kruskal-Wallis test. When tests were significant, because the data were independent, multiple comparisons between the four treatments were made with the nonparametric Mann-Whitney U test. P-values less than 0.05 were statistically significant.

'*Candidatus* Liberibacter solanacearum' transmission studies in laboratory conditions

To determine if *B. trigonica* transmitted the bacterium from carrot to carrot and to other crops, transmission experiments were performed using healthy carrot, celery, potato and tomato as receptor plants. Before their use in the transmission studies, adult psyllids from the viruliferous colony were allowed to feed on '*Ca*. L. solanacearum' infected carrot donor plants for an acquisition access period (AAP) of 72 h. All experiments were conducted in a controlled environment room at 25±2°C, 70±5% humidity and a photoperiod of 16:8 h (L:D).

a) Restricted leaf exposure assays. Groups of 10 *B. trigonica* adults were released on leaves in clip-cages and allowed to feed for different inoculation access periods (IAP) of 24 h, 3 d, 7 d and until insect death (approximately 14d) on the same plant. Ten replicates of each receptor species (represented by 10 psyllid adults per clip-cage and four clip-cages per plant for a total of 40 psyllids per plant) were used. After each IAP, insects were removed from the leaf with an aspirator, and both the leaf and the insects were tested for '*Ca*. L. solanacearum' with real-time PCR. The plants were sprayed (1 g/1 of Confidor®, Bayer CropScience, Spain) outside the room after each transmission experiment to eliminate any nymphs emerging from the eggs and were grown in a greenhouse for one month and then were tested by real-time PCR to confirm '*Ca*. L. solanacearum' infection.

***b) Whole plant exposure, short or long, with groups or single* B. trigonica.** Groups of ten *B. trigonica* were confined in a small plant cage that permitted the insects free access to the entire plant until all the psyllids in the group died (approximately 14d, the long exposure). Ten plants for each receptor host were used. After this period, the psyllids were collected with an aspirator and analysed by squash real-time PCR. The plants were treated with the insecticide (1 g/1 of Confidor®, Bayer CropScience, Spain) and grown in an insect-proof greenhouse for one month, monitored for disease symptoms and tested to confirm bacterial infection.

Experiments with a single *B. trigonica* adult per plant were performed as described previously. The same insect was allowed to feed on a receptor plant for 24 h (short exposure) and then transferred to a second receptor plant in the same development stage until the psyllid death (approximately 14d, the long exposure). Thirty replications were performed. After this period, the single psyllids were analysed as described previously. The 60 plants were treated with the insecticide as described above, maintained in the controlled environment room for one month and tested to assess '*Ca*. L. solanacearum' infection. Three repetitions of the same experiment were performed.

Transmission studies in greenhouse conditions

Experiments were performed inside 1 x 1 x 1 m aphid-proof cages. Each cage contained a single '*Ca*. L. solanacearum'-infected carrot plant as the donor plant in the centre of 12 noninfected receptor plants (carrot, celery, potato or tomato). A group of 100 *B. trigonica* young adults collected from the '*Ca*. L. solanacearum' positive colony were confined for additional 72h on the carrot donor plant by a plant cage and then allowed to freely disperse into the cage and fly between the receptor plants and the carrot donor plant until the death of the insects (approximately 14d). Three repetitions of the same experiment were performed. Plant samples (2-3 expanded leaves) were collected 1.5 month after the beginning of the experiment and analysed with real-time PCR to assess the '*Ca*. L. solanacearum' infection.

***B. trigonica* performance in potato and tomato plants**

To assess the ability of *B. trigonica* to reproduce on potato and tomato plants, 18 new emerged psyllid couples were placed in individual plant cages with one seedling each of tomato or potato. The experiments were performed in climate chambers at 25°C, 70% humidity and a photoperiod of 14:10 h (L:D). The eggs were counted daily using a binocular stereomicroscope until all the insects died (approximately after 14d). The laid eggs were individually marked and monitored daily for development, moulting and mortality.

Results

Feeding behaviour on different hosts

No significant differences among the number of *B. trigonica* probes in the four plant species studied by EPG monitoring were found (Table 1). However, significant differences in the phloem-related activities of *B. trigonica* between *Apiaceae* (carrot and celery) and *Solanaceae* (potato and tomato) were found. Data did not show significant differences between experiments conducted on species belonging to the same botanical family. *B.trigonica* was able to reach the phloem sieve elements (E1) and remain in phloem sap ingestion events (E2) many times and longer when fed on carrot and celery than on potato and tomato plants ($P<0,05$). Data showed that *B. trigonica* was unable to ingest from the phloem of either potato or tomato plants as no E2 events were recorded When psyllids were allowed to probe on potato plants two out of 15 individuals was able to reach the phloem sieve elements and salivate once for a few seconds but phloem ingestion (E2) was never recorded. No phloem-related activity was observed when *B. trigonica* was exposed to tomato plants.

Table 1. Means (± SE) of nonsequential EPG variables for the probing behaviour of *Bactericera trigonica* on healthy carrot, celery, potato and tomato plants during an eight-hour recording (n = 15).

Nonsequential variables	Carrot	Celery	Potato	Tomato
	Mean ± SE			
Number of E1	2.80 ± 1.85 a	4.00 ± 3.76 a	0.13 ± 0.35 b	0.00 ± 0.00 b
Number of E2	1.87 ± 1.59 a	2.80 ± 3.32 a	0.00 ± 0.00 b	0.00 ± 0.00 b
Total duration of E	4389.00 ± 5010.17 a	3923.00 ± 6528.95 a	9.68 ± 26.58 b	0.00 ± 0.00 b
Total duration of E1	854.00 ± 1226.07 a	1127.00 ± 1477.59 a	9.68 ± 26.58 b	0.00 ± 0.00 b
Total duration of E2	3535.00 ± 4789.00 a	2797.00 ± 5901.00 a	0.00 ± 0.00 b	0.00 ±0.00 b
% of probing spent in E1	5.29 ± 7,70 a	6.35 ± 10.85 a	0.16 ± 0.47 b	0.00 ± 0.00 b
% of probing spent in E2	17.40 ± 21.20 a	11.12 ± 21.04 a	0.00 ± 0.00 b	0.00 ± 0.00 b
Number of probes	10.00 ± 4.70 a	9.13 ± 6.08 a	7.47 ± 4.29 a	5.86 ± 5.90 a

- E shows phloem-related activities; E1 correlates with salivation into phloem sieve elements and E2 correlates with ingestion from phloem.
- Values with different letters are significantly different ($P < 0.05$, Mann-Whitney U test).

Transmission studies in laboratory conditions

The detection of '*Ca*. L. solanacearum' in the *B. trigonica* adults used in the experiments ranged from 50% to 90%.

a) Restricted leaf exposure assays. The bacterium was transmitted from carrot to carrot, celery, potato and tomato in different percentages related to the IAP (Table 2). The bacterium was detected only in carrot and celery samples one month post-inoculation. No symptoms were observed in '*Ca*. L. solanacearum'-infected plants at the end of the experiment.

Table 2. Transmission of '*Ca*ndidatus Liberibacter solanacearum' from infected carrot to carrot, celery, potato and tomato plants by 40 adults of *Bactericera trigonica* with restricted plant exposure.

IAP[a]	Carrot (CaLsol[b]+/total)	Celery (CaLsol+/total)	Potato (CaLsol+/total)	Tomato (CaLsol+/total)
24 hours	6+/10[c]	2+/10	1+/10	6+/10
3 days	10+/10	3+/10	1+/10	5+/10
7 days	10+/10	6+/10	2+/10	4+/10
Approx. 14 days	7+/10	8+/10	3+/10	2+/10
Positive plants after 1 month	7+/10	10+/10	0+/10	0+/10

[a] IAP = Inoculation access period.
[b] CaLsol = '*Candidatus* Liberibacter solanacearum'.
[c] Number of positive detections of '*Candidatus* Liberibacter solanacearum' targets by real-time PCR in leaves/total number of assayed leaves.

b) Whole plant exposure assays, short or long, with groups or single B. trigonica. The bacterium was transmitted when ten *B. trigonica* fed on whole carrot, celery, potato or tomato plants for approximately 14d (the long exposure). The bacterium was detected in 8 out of 10 carrots, in 10 out of 10 celery plants, and in 1 out of 10 potato and tomato plants one month after exposure (Table 3).

Transmission of '*Ca*. L. solanacearum' by a single *B. trigonica* to the different plant species occurred independently of the IAP (short or long exposure). When the 24h IAP was assayed, 59 out of 87 carrots, 19 out of 90 celery plants and 5 out of 83 tomatoes tested positive for the bacterium. When the 14d IAP was assayed, 46 out of 70 carrots, 15 out of 90 celery plants and 4 out of 59 tomatoes tested positive (Table 3). The higest '*Ca*. L. solanacearum' transmission rates were obtained on carrot plants (67.8% and 65.7%

after 24h and 14d IAP, respectively), followed by celery (21.1% and 16.6% after 24h and 14d IAP, respectively). The '*Ca*. L. solanacearum' transmission rates to tomatoes were similar for both IAPs (approximately 6%, Table 3).

Table 3. Transmission of '*Ca*ndidatus Liberibacter solanacearum' from carrot to carrot, celery and tomato plants by a single or 10 adults of *B. trigonica* with 24h and ± 14d inoculation access periods (IAP) combined with whole plant exposure.

	CaLsol[b]+/total (% of transmission)		
	1 *B. trigonica*		10 *B. trigonica*
	IAP[a] 24h	IAP approx. 14d	IAP approx. 14d
Carrot	59+/87[c] (67.8%)	46+/70 (65.7%)	8+/10 (80%)
Celery	19+/90 (21.1%)	15+/90 (16.6%)	10+/10 (100%)
Potato	ND	ND	1+/10 (10%)
Tomato	5+/83 (6.0%)	4+/59 (6.8%)	1+/10 (10%)

[a] IAP = Inoculation access period.
[b] CaLsol = '*Candidatus* Liberibacter solanacearum'.
[c] Number of positive detections of '*Candidatus* Liberibacter solanacearum' targets by real-time PCR in leaves/total number of assayed leaves.

Transmission studies in greenhouse conditions

The '*Ca*. L. solanacearum' was transmitted to 10 out of 36 carrots, to 10 out of 36 celery plants and to 1 out of 36 tomatoes. The bacterium was not transmitted to the 36 potato plants assayed.

Reproduction rates in potato and tomato plants

Three of the 18 *B. trigonica* couples placed on potato plants laid eggs (the females of two couples laid two eggs and the female of one couple laid one egg). The nymphs lived only to the first nymph instar (N1). No eggs were found on the 18 tomato plants examined.

Discussion

A majority of plant pathogens are dependent on vectors for their spread and survival. In some specific pathosystems, aspects of the transmission mechanisms are well known; however, in many others, less information is available. Different diseases that cause important economic losses are associated with the emergent bacterium '*Ca*. Liberibacter solanacearum' that is naturally spread by different psyllid species. *B. trigonica* is the predominant species on *Apiaceae* in Spain (Teresani *et al*., 2014b), where the psyllid is associated with the transmission of the bacterium that causes vegetative disorders. For this reason, this species was selected to assess its vector ability.

Because phloem-feeding activities are associated with bacterial acquisition and inoculation (Sandanayaka *et al*., 2014), the stylet penetration activities of *B. trigonica* were monitored using the EPG technique on carrot, celery, potato and tomato plants. The acquisition of the bacteria has been associated with the phloem ingestión phase (E2) while its inoculation is likely associated with salivation into the phloem sieve elements (E1) (Sandanayaka *et al*.,

2014). Ours findings confirmed that *B. trigonica* is able to have a sustained phloem feeding in carrot and celery plants, and therefore the '*Ca*. L. solanacearum' acquisition and inoculation procces could occur in these host species. However, only occasional phloem salivation events (E1) were observed when *B. trigonica* was allowed to fed in potato plants, but this activity indicated that the psyllid was capable to inoculate the bacterium. Therefore, a *B. trigonica* that acquired the bacterium from other infected host species could potentially infect potato crops. In tomato plants, no phloem activities were recorded. Although no phloem activities of *B. trigonica* were detected in tomato plants, a transmission event occurred. The waveforms D (the initial contact of the stylet with phloem tissues) and the E1-like waveforms that occurred at the extracellular level within C (presumably extracellular salivation) were detected, as was reported previously for *B. cockerelli* and tomato (Sandanayaka *et al*., 2014).

Although no significant differences between the EPG variables in potato and tomato were observed, the psyllid feeding behaviour might have biological significance. Although only for approximately 4.9 seconds, the psyllid reached the phloem of the potato plants. This brief feeding was not likely related to no adaptation of the species to a new environment (Sandanayaka *et al*., 2013), such as suggests the few individuals of this species were captured on potato crops (Teresani *et al*., 2014b). However, the inoculation of the bacterium in potato during this short probe would be possible.

The EPG technique is a powerful tool to assess whether a species is able to reach the phloem, but it does not demonstrate actual transmission to a specific host. For this reason, conventional transmission assays were designed to assess whether *B. trigonica* acted as a '*Ca*. L. solanacearum' vector. The assays revealed that *B. trigonica* adults inoculated '*Ca*. L. solanacearum' into various host species independently of the IAPs or the quantity of psyllids involved.

The highest percentage of positive plants was found in carrot when the psyllids were fed a restricted leaf for 3d and 7d IAPs (100% transmission). In celery, the maximum transmission rates occurred for the 7d IAP and at insect death at approximately 14d (60% and 80%, respectively). The percentage of transmission was relatively low in the *Solanaceae*. The maximum detection in potato (30%) was observed after approximately 14d IAP, which suggested that that a longer period was necessary for more efficient inoculation. This behaviour could be because *B. trigonica* was not adapted to potato as discussed previously. Nevertheless, the maximum detection in tomato (60%) was observed after 24h IAP and decreased progressively with longer IAPs (3d to approximately 14d). In the "whole plant exposure" experiment with a group of 10 *B. trigonica* per plant, the bacterium was consistently detected after 1 month in carrot (80%) and celery (100%) but was detected in only 10% of potatoes and tomatoes. With a single *B. trigonica* per plant without removing the inoculum from plants, '*Ca*. L. solanacearum' was detected in tomato plants after 24h and after approximately 14d

IAP analysed one month after inoculation (approximately 6% detection rate). If the plant was not the preferred host for the bacterium, a longer post-inoculation period would be needed to multiply and move systemically into the plant. The lower rate of transmission of a single *B. trigonica* compared with that of ten psyllids was in agreement with the results reported by Rashed *et al*. (2012) who used *B. cockerelli*.

In addition to the abundance of *B. trigonica,* the feeding site on the plant also influenced the success of transmission of '*Ca*. L. solanacearum'. The effect of the feeding site on the acquisition and inoculation success was observed for other vector-borne pathogen systems where the pathogen was heterogeneously distributed within the host (Daugherty *et al*. 2010; Rashed *et al*., 2012), as could have been the case with *B. trigonica* on solanaceous hosts. The psyllid species is predominant on carrot and celery crops in Spain and is found in potato fields growing in the vicinity (Teresani *et al*., 2014b). Consequently, this pest could be a threat to other crops if the viruliferous psyllids flying from *Apiaceae*-infected hosts feed on the phloem of solanaceous hosts. These results suggest that carrots infected with haplotype E could represent a threat to other crops.

When *B. trigonica* reproduction was evaluated on potato and tomato, the psyllid laid eggs only on potato and was unable to complete the life cycle on this host because the progeny died at the nymph stage N1. In comparison to potato, *B. trigonica* laid an average of 235.1 eggs during the life span when placed on carrot

(data not shown) in the same conditions. This confirmed previous results that carrot was a good host for *B. trigonica* and for the bacterium. However, this does not exclude the possibility that psyllids will sporadically probe on potato, particularly if potatoes are the only available specie. Recently, the occasional detection of '*Ca*. L. solanacearum' haplotype E was reported in zebra chip symptomatic potato tubers in Spain (Palomo *et al*., 2014). This suggested that the transmission of non-solanaceous haplotypes from *Apiaceae* to, at least, potato is a real possibility in natural conditions. Moreover, the transmission of a non-solanaceous haplotype by *B. trigonica* to potato suggested that the same species could also act as a vector of solanaceous haplotypes. The rate of plant pathogen transmission and symptomatology are often correlated with vector density and pathogen infectivity (Jeger *et al*., 2004); therefore, *B. trigonica* could be a vector to crops other than *Apiaceae*.

Recommended strategies for the prevention of '*Ca.* L. solanacearum' introduction into areas where it is not present need to be determined. The importation of potato and other solanaceous and apiaceous plants (including seeds) from areas where '*Ca.* L. solanacearum' is present should be avoided. Carrot seeds as well as potato breeding material should be analysed according to post-entry quarantine requirements (EPPO, 2006) and should be free from '*Ca.* L. solanacearum' and the vectors *B. cockerelli, T. apicalis* and now, *B. trigonica*. Carrot seeds should be produced in a pest and bacterium free area or under special conditions (Bertolini *et al*.,

2014b), and seeds of other plants should be considered potential hosts because '*Ca.* L. solanacearum' was detected in chile pepper (*Capsicum annuum* L., Camacho-Tapia *et al*., 2011) and parsnip (*Pastinaca sativa*, unpublished data) seeds. Recently, special requirements that included testing by real-time PCR of carrot seed lots and of *in vitro* plants of carrot and celery were required to demonstrate the bacterium-free status before entry into Australia (M. Smyth, personal communication). When the bacterium is already present in a geographical region, several aspects such as climatic conditions, proximity of other bacterial hosts, and control of psyllid species should be assessed. The reduction of the psyllid population at the peak of maximum flight in the summer is critical for disease mitigation (Teresani *et al*., 2014b), particularly if known vectors are present. In conclusion, the psyllid *B. trigonica* must now be added to the list of vectors for '*Ca.* L. solanacearum'.

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*The references of this paper (chapter) are included in the list of "Literature cited"

Discussion

Discussion

The species of '*Ca*. Liberibacter' are associated with severe plant diseases in an increasing number of hosts. Although '*Ca.* L. solanacearum' has been reported being mainly pathogenic to *Solanaceae* (Liefting *et al.,* 2009b), it has also been associated with diseases in other botanical families, such as *Apiaceae*, in different geographical areas (Munyaneza, 2012).

Vegetative disorders causing economic losses in celery in Spain from 2008-2010 were the starting point of the studies of this thesis. Previous studies discarded viruses or fungus as causal agents, suggesting that phytoplasmas or bacteria could be involved in the disorders. To determine the etiology and to understand the epidemiology of the observed symptomatology, the development of reliable diagnostic methods is required. Therefore, in this work, a new real-time PCR protocol that was based on a TaqMan probe was developed for specific '*Ca*. L. solanacearum' detection, allowing for use with direct or traditional sample preparation methods. This new protocol uses for the first time a specific '*Ca*. L. solanacearum' TaqMan probe. A previously reported real-time PCR protocol (Li *et al*., 2009) for the same target used specific primers and a universal TaqMan probe that can react with other '*Ca*. Liberibacter' species. In fact, amplification was observed with '*Ca*. L. africanus' (unpublished data). The new real-time PCR protocol was specific and accurate for '*Ca.* L. solanacearum' detection, showing similar sensitivity to that of the protocol that was previously described by

Li *et al*. (2009) but higher than the most sensitive conventional PCR protocol that was tested (Ravindran *et al*., 2011a). The detection level decreased by two orders when the direct method (spot) was used. This result can be explained by the small amount of nucleic acid target that was available on the support. The use of this type of direct friendly method of sample preparation prior to real-time PCR is currently highly recommended for large-scale use (De Boer & López, 2012) and in the current EPPO/OEPP diagnosis protocol (standard) for '*Ca*. L. solanacearum' (EPPO, 2014). The simplicity in the sample preparation and the considerably lower cost of the direct method, together with the good agreement between the results that were obtained with spotted samples and DNA purification, confirm the appropriateness of the spot method for the detection of '*Ca*. L. solanacearum'. In addition, a commercially available complete kit based on the developed protocol showed an excellent sensitivity, specificity and 100% accuracy through intra-laboratory performance studies. In addition, in an international inter-laboratory ring-test with the participation of 27 laboratories from 15 countries, the kit showed an accuracy of 93% (data no shown).

Using this new real-time PCR protocol, we established a statistical association between the presence of '*Ca.* L. solanacearum' and the vegetative disorders that are found in celery but not with phytoplasmas. Although mixed infections can occur in the field, bacteria are more frequently found in carrot than in celery plants. Using electron microscopy, only typical '*Ca*. Liberibacter'-

like cells with an electron-dense layered cell wall with a triple-layered ultrastructure (Bové, 2006) were observed by TEM (bacterial-like organisms were observed by SEM in the phloem), confirming the association of '*Ca*. L. solanacearum' with the celery disorders. No phytoplasmas-like cells were observed in the analyzed samples. These data confirm the first report of the association of '*Ca*. L. solanacearum' with symptomatic celery. Once this association was confirmed, it became necessary to identify the pathway and epidemiology of the pathogen.

Four '*Ca*. L. solanacearum' haplotypes (designated A, B, C and D), which are distinguished by single nucleotide polymorphisms (SNPs) in three genome regions (16S, 50S and the 16S/23S intergenic region) (Nelson *et al*., 2011), have been described as affecting solanaceous and carrot crops worldwide. In celery samples that were infected with '*Ca*. L. solanacearum' haplotype (E), which was not previously found, was determined. This new haplotype is characterized by five nucleotide changes in the 16S rDNA, seven in the ISR-23S and five in the 50S rDNA regions compared with haplotype D, which was previously described in Spain (Nelson *et al*., 2012), assuming that E haplotype was recently introduced. The determination of this new haplotype in carrot plants occurring in the same areas in which celery was concomitantly cultivated suggests the natural transmission of the haplotype E between both of the botanical species, most likely by a same psyllid vector species. Recently, Lin *et al*. (2012), using simple sequence repeat (SSR) markers, reported the existence of 33

haplotypes. However, no clear genetic structure could be established among these '*Ca*. L. solanacearum' haplotypes based on geographical origin or host. Therefore, the method described by Nelson *et al*. (2012) based on three genomic regions was considered more appropriate for characterizing '*Ca*. L. solanacearum' haplotypes and for establishing a possible association among the hosts and geographical origins in this study.

In several attempts to detect '*Ca*. L. solanacearum' in celery seeds, no bacterial amplification occurred. The emergence of the disease in celery crops was associated with the proximity of carrot plots with a high prevalence of '*Ca.* L. solanacearum' and with the presence of *B. trigonica* populations in the same areas. At the same time, the reports of carrots that were infected with '*Ca.* L. solanacearum' in different geographical areas in Europe and in Spain suggest that carrot seeds should be the first source of inoculum in this crop. Therefore, we investigated the seed transmission of this pathogen in carrot.

The frequent detection of '*Ca.* L. solanacearum' in commercial carrot seed lots and even in individual carrot seeds was followed by a demonstration of the transmission from contaminated seeds to seedlings. Because '*Ca.* L. solanacearum' is a carrot seedborne pathogen, infected seeds act as a first source of inoculum for this crop and for the others that are cultivated in the vicinity. This, coupled with the abundance of psyllid species that could act as vectors, results in the natural spread of '*Ca*. L. solanacearum' and consequently of the disease to crops other than carrot.

The total number of '*Ca*. L. solanacearum' cells in individual carrot seeds was quantified by real-time PCR. The number of estimated bacterial cells in individual seeds was highly variable within the seed lot and between seed lots. As an example, the average number that was found in a bulk of lot 12/2012 was approximately 200 cells/seed but in individual seeds from the same lot, a high variability among the estimated number of cells/seed was observed (4-36,000 '*Ca*. L. solanacearum' cells/seed). In our work, we considered one copy of the 16S rRNA gene to be present in the bacteria genome, but Lin & Gudmestad (2013) reported that '*Ca*. L. solanacearum' has three copies of complete rRNA operons (16S, 23S and 5S). Consequently the number of cells/seed that was published in Bertolini *et al*. (2014b) (second chapter of this thesis) must be divided by three. A similar variability has been reported for other phytopathogenic bacteria (Dutta *et al*., 2013). No correlation was observed between the percentage of infected seeds and the average number of bacterial cells/seed. For example, the seed lot 10/2012 had the highest percentage of positive seeds and the lowest estimated average number of bacterial cells/seed.

To determine whether the total '*Ca*. L. solanacearum' cells that were detected in carrot seeds were alive and potentially able to cause disease, the cell viability was studied using propidium monoazide (PMA), a photoreactive DNA-binding dye that was used to detect viable microorganisms by PCR. Living organisms with an intact cell membrane are not affected by PMA. However, dead microorganisms have lost their capability to maintain their

membranes, and the naked DNA is covalently bonded with PMA, making the DNA non-amplifiable by PCR (Nocker *et al*., 2007). The use of this methodology permitted the quantification of the live '*Ca*. L. solanacearum' cells in carrot seeds, showing that the majority (approximately 95%) of the bacterial seed population was dead. Nevertheless, the remaining live cells (5%) were enough to cause infection in carrot seedlings that germinated from infected seeds. The results agree with Trivedi *et al*. (2009), who reported that only 17% of the '*Ca*. L. asiaticus' cells that were present in infected citrus material were live.

'*Ca.* Liberibacter'-like cells were visualized by TEM in the phloem of the carrot seed coat. It seems that the bacterium may penetrate undifferentiated cells of the seedling radicle in the very early stages of seed germination, eventually reaching the phloem of young seedlings to cause persistent infection, but further research is necessary to determine the mechanism of transmission by carrot seeds. This is the first report of a phloem-limited bacterium being transmitted in seeds, despite reports that assess the detection of '*Ca*. Liberibacter' spp. in citrus and pepper seeds.

Haplotypes D and E were observed in carrot seeds, suggesting the presence of a mixture of both of the haplotypes in the same carrot seed lot. This mixture could occur because some commercial carrot seed lots are a mixture of seeds from different origins and production years, impairing the identification of consistent relationships between haplotype, seed lot source and origin. The origin of '*Ca*. L. solanacearum' in Europe is unknown, but because

the bacterium has not yet been detected in any naturally occurring plant species in Europe, we speculate that it could have been introduced into Europe with carrot seeds, although the hypothesis of Nelson *et al*. (2013) assumes that carrot haplotype D is natural to Europe.

Because the same companies sell seeds worldwide, control of the seed lots is essential to prevent the introduction of the bacterium to new areas, to reduce the inoculum in carrot fields and to mitigate the spread to other potential hosts. Although the seeds bring the inoculum to the field, the presence of psyllid vectors is essential for the spread of '*Ca*. L. solanacearum' to other hosts of the same or different species. In an open field, our data show that the presence of psyllid vector species increased the prevalence of the bacterium from 2% in the first 60-90 days of cultivation to practically 100% at the end of carrot cultivation cycle. Consequently, there was a requirement to identify the species visiting *Apiaceae* and *Solanaceae* crops in ecologically diverse areas of cultivation in Spain because few or non-available information about the arthropod species that lands on these crops was available.

Seasonal and sporadic surveys were performed in celery, carrot and potato crops in Villena, Valencia and Tenerife. We included potato in the surveys because of the high risk of zebra chip disease for Spanish potato cultivation. In Villena (Alicante), the results of the captures of psyllids showed important differences between different years not only in the number of specimens but also in the most prevalent families that were captured. In 2011, a

high number of arthropods were captured, of which 50.9% belonged to the superfamily Psylloidea, while in 2012, although two celery plots were sampled in the middle cycle and three in the late cycle of cultivation, the prevalence of arthropods was very low compared with that of the previous year, and the major percentage (60.0%) of captured arthropods belonged to Cicadellidae family. This result was correlated in 2012 with a lower observed prevalence of symptoms in celery. In the carrot crop, the presence of symptomatic plants was similar to that in previous years, most likely because the primary infection was caused by carrot seed transmission. In this area, two psyllid species other than *B. trigonica* (previously associated with '*Ca.* L. solanacearum' transmission) were also captured: *B. tremblayi* and *B. nigricornis*. These three *Bactericera* species are morphologically close psyllid species that belong to the 'Bactericera nigricornis Förster group' (Hodkinson, 1981). In both of the surveyed years, the collected psyllid species showed the same psyllid population species structure: *B. trigonica* was always the prevalent species, followed by *B. tremblayi,* and *B. nigricornis* was the less frequent species. In addition, non-identified *Trioza* sp., *Psylla* sp. and *Bactericera sp.* (other than *B. cockerelli* and *T. apicalis*), were found on carrot, celery and potato.

In the surveys that were performed in Tenerife in 2012, 99.1% of the insects that were captured on carrot were psyllids, represented basically by *B. trigonica* (varying from 99.9% in the middle cycle to 85% in the late cycle of carrot cultivation). The population dynamics are basically concentrated in the medium

cycle of cultivation. Other psyllids were also found in the captures, such as *Bactericera* sp., *Ctenarytaina* sp., *Cacopsylla* sp. and *T. urticae.* The maximum flight of psyllid species was in summer both in mainland Spain and in the Canary Islands.

The same psyllid species visited potato crops in Valencia and in Tenerife, representing a risk for the crop if non-solanaceous haplotypes (D and E) were able to colonize potato plants.

Our data suggest that carrot is the preferential host for the psyllid species that are found in the monitored areas, as a higher number of individuals were caught in carrot plots. This hypothesis is confirmed by the higher number of psyllids that were captured in carrot than in celery plots that were grown in the vicinity. This preferential pattern is why the number of individuals who were captured in the surveys in Tenerife (carrot surveys) is much higher than that in Villena (celery surveys).

The detection of targets indicates that arthropods have acquired the bacterium, which has most likely multiplied in the insect to become detectable by real-time PCR. These results indicate that the bacterium can be transmitted by these psyllid species and spread disease in the field. The three psyllid species that were reported in this work (*B. trigonica*, *B. tremblayi* and *B. nigricornis*) were found as current visitors of '*Ca*. L. solanacearum' host crops in continental Spain as well as in the Canary Islands. This pattern, coupled with the fact that '*Ca.* L. solanacearum' targets were detected by squash real-time PCR in 19.5% of the

psyllids belonging to the different captured species, could mean that are potential vectors in different ecological areas.

B. trigonica was investigated regarding its capacity for bacterium transmission, although the other two species were also found to carry '*Ca.* L. solanacearum' targets. *B. tremblayi* and *B. nigricornis* are most likely also able to transmit the bacterium because they are able to acquire the bacterium, and the range of plant species that are naturally infected by a certain species of '*Ca.* Liberibacter' is restricted by the host range of the psyllid vector (Haapalainen, 2014). Other non-identified psyllid species (*Trioza* sp.*, Psylla* sp. and *Bactericera* sp.) that were found in the same fields carrying the bacterium could also be vectors. More studies should be conducted to accurately determine which species are and to determine their potential to transmit the bacterium. This thesis provides for the first time information regarding the psyllid species populations that land on celery, carrot and potato crops in Spain and reported new psyllid species that acquired '*Ca*. L. solanacearum' and that could consequently be considered as potential vectors of the bacterium.

Because Nelson *et al.* (2013) reported that other experiences with citrus and potato diseases suggest that psyllid species, proving and/or feeding briefly outside of their normal plant host range, could introduce a pathogen to other crops, we also considered the risks these species can represent for the Spanish potential host crops, which are frequently cultivated in the same area and share the same psyllid species. This fact was confirmed performing

transmission experiments from carrot to carrot, celery, potato and tomato using *B. trigonica*.

The vector ability of *B. trigonica*, the most frequent psyllid species in the Spanish areas where '*Ca*. L. solanacearum' causes vegetative disorders in *Apiaceae*, was studied in this thesis as well as its phloem-feeding activities. *B. trigonica* was able to feed continuously on the phloem of carrot and celery and to probe in the phloem of potato even for a few seconds. No feeding on tomato phloem was observed. The finding of *B. trigonica* performing E1 and E2 waveforms when feeding on carrot and celery plants indicates that the insect is able to inoculate the bacterium during salivation activity and to acquire the bacterium during the phloem sap uptake in these crops. However, in potato, where only E1 waveforms were observed, viruliferous *B. trigonica* could infect potato during salivation activity if the insect previously acquired the bacterium in another infected host species.

The brief salivation activity in the potato phloem is sufficient for bacterial transmission to potato plants under laboratory conditions. The short period of salivation could be due to the non-adaptation of the species to a new environment (Sandanayaka *et al*., 2013) because potato is not a host of *B. trigonica*. This fact is confirmed by the low number of individuals of this species that were captured on potato crops (Teresani *et al*., 2014b). The situation of *B. trigonica* being found in potato crops growing in the vicinity of infected carrot and celery could constitute a threat to this crop if viruliferous psyllids visit the potatoes. The fact that *B.*

trigonica can lay eggs in potato is further evidence of the threat that this species represents to potato crops. The recent detection of '*Ca*. L. solanacearum' haplotype E in symptomatic potato tubers in Spain (Palomo *et al*., 2014) confirms that an actual risk of transmission under natural conditions.

In spite of the feeding behavior of *B. trigonica* in tomato plants, the initial contact of the stylet with phloem tissues (waveform D) and the E1-like waveforms that occurred at the extracellular level within C could justify the bacterial transmission that was observed in the experiments. This behavior was also observed with *B. cockerelli* in tomato plants by Sandanayaka *et al*. (2014).

The non-adaptation of *B. trigonica* to potato could also influence in the IAP that is necessary to transmit the bacterium at higher levels than in carrot and celery, which are hosts of the vector. In contrast, in tomato, the majority of detection was observed with a short IAP, decreasing progressively with longer IAPs. This result could also suggest that haplotype E (used in the transmission experiments) is not adapted to *Solanaceae*.

The new information that is provided by this work performed in the context of this thesis could help in the design of new strategies for controlling the damages that are caused by '*Ca*. L. solanacearum' in Spanish agriculture. Actions to prevent the introduction of the bacterium in new areas are necessary, starting from the carrot seeds and potato breeding material as well as host plants free from '*Ca.* L. solanacearum' and its vectors. Imported

material should be analyzed before distribution. Currently, there are no effective control strategies for plant protection from natural '*Ca*. Liberibacter' infection except for the potential use of cultivation under insect-proof facilities. Nevertheless, something similar to the "three-pronged system" (TPS) (Belasque *et al*., 2010), a system that is used for HLB management, could be adjusted for horticultural crops. A possible strategy could involve 1) the exclusive use of '*Ca*. L. solanacearum' seed lots that are free of the viable bacterium in order to avoid inoculum sources and introduction to new areas or 2) insecticide or other treatments aiming to reduce psyllid vector populations or probing activity on the host plants, especially in crops that are grown during the population peak. The general aim of mitigation measures against '*Ca*. L. solanacearum' is not only to protect *Apiaceae* but also to protect *Solanaceae* crops that were demonstrated in this thesis as susceptible to non-solanaceous haplotypes of the bacterium. Integrated pest management is recommended to reduce the risk of spread of this emerging bacterium.

Conclusions

Conclusions

The results that are presented in this thesis permit the following general conclusions:

1) A new real-time PCR protocol for '*Ca*. L. solanacearum' detection for use with direct sample preparation methods or after DNA extraction was developed, validated and is commercially available.
2) This real-time PCR protocol enables the study of the association of '*Ca*. L. solanacearum' with vegetative disorders of celery in Spain, its detection in carrot seeds and the determination of putative vector psyllid species.

3) Using electron microscopy, '*Ca*. Liberibacter'-like cells were visualized in the phloem vessels of celery and on carrot seedlings and seeds.

4) A new '*Ca*. L. solanacearum' haplotype (E) was described. This haplotype is currently associated with vegetative disorders of celery and carrot and was also detected in carrot seeds and seedlings as well as in *B. trigonica*. In contrast, haplotypes A, B and C were not detected in Spain during the experimental period of the thesis.

5) '*Ca*. L. solanacearum' was demonstrated to be a seedborne pathogen, most likely infecting carrot seeds as the pathway of introduction of this bacterium in Spain and other countries. This fact justifies its currently high prevalence in carrot and the simultaneous detection in geographically distant areas in mainland Spain and in the Canary Islands.

6) Real-time PCR using propidium monoazide (PMA) was efficient in evaluating the number of viable '*Ca*. L. solanacearum' cells in carrot seeds. A minimal number of viable cells was able to cause symptoms in 12% of the symptomatic seedlings cv. Maestro after 150 days post-germination. The transmission efficiency varied among different carrot cultivars and seed lots.

7) The infected carrot plots very most likely acted as a source of inoculum for other carrot and celery plots. From this primary inoculum, the bacterium was naturally spread by psyllids. *B. trigonica*, *B. tremblayi*, *B. nigricornis* and *Bactericera* sp. were identified as carriers of the bacterium under natural conditions, suggesting that these species could act as potential vectors.

8) Electrical penetration graphs studies revealed that *B. trigonica* was able to feed on the phloem of carrot, celery, and potato but not on tomato plants. *B. trigonica* was demonstrated for the first time as an efficient vector of the bacterium between carrots and

from carrot to celery and could act as a vector from infected *Apiaceae* to *Solanaceae* crops.

9) The mitigation of the diseases that are associated with this bacterium should include 1) the exclusive trade and use of *'Ca*. L. solanacearum'-free seed lots, 2) treatments against psyllid species that have been identified as putative vectors during the period of maximum population, and 3) the production of associated hosts under insect-proof facilities or in areas that are free of the bacterium.

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List of publications

List of publications

Scientific (SCI) papers:

Bertolini, E.*, **Teresani, G.R.*,** Loiseau, M., Tanaka, F.A.O., Barbé, S., Martínez, C., Gentit, P., López, M.M., M. Cambra (2014). Transmission of '*Candidatus* Liberibacter solanacearum' in carrot sedes. Plant Pathology Article first published online: 22 Jun 2014, Doi: 10.1111/ppa.12245.

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Abstracts published in scientific meetings:

Alfaro-Fernández, A., Hernández Llopis, A. Serrano Fernández, A., Villaescusa, F.J., **Teresani, G.R.**, Bertolini, E., Martínez, M.C., Ferrándiz, J.A., Bartolomé, P., Sanjuán, S., Hermoso de Mendoza, A., López, M.M., Cambra, M. y Font, M.I. (2012). Patógenos detectados en cultivos de zanahoria españoles con amarilleos, enrojecimientos y deformaciones.

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