



Transmission of ‘*Candidatus Liberibacter solanacearum*’ by *Bactericera trigonica* Hodkinson to vegetable hosts

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Abstract

The bacterium ‘*Candidatus Liberibacter solanacearum*’ is a recent plant pathogen of several crops in Solanaceae and Apiaceae and is associated with economically important diseases. 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 described carrying CaLso associated with vegetative disorders in carrot and celery crops in Spain and its competence to transmit this phloem-limited bacterium among vegetables is currently being investigated. Here electrical penetration graphs showed that *B. trigonica* fed in the phloem of carrot and celery and probed the phloem in potato, but not in tomato plants. The bacterium was efficiently transmitted to carrot and celery plants when either single *B. trigonica* or groups of ten fed 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%), celery (21.1%) and eventually to potato and tomato (6.0%). Higher transmission rates were obtained with 10 individuals on celery (100%), carrot (80%), potato (10%) and tomato (10%). *Bactericera trigonica* laid eggs, and the hatched nymphs develop into adult on carrot and celery, but not on potato and tomato. CaLso was detected in 20% of the eggs laid by females carrying the bacterium. The results confirmed that *B. trigonica* is a vector of the bacterium to carrot and celery, and it is discussed the potential role of this psyllid in the transmission of the pathogen to potato and tomato plants.

Additional keywords: electrical penetration graph; haplotype E; psyllids; real-time PCR; transovarial passage.

Abbreviations used: CaLso (‘*Candidatus Liberibacter solanacearum*’); EPG (electrical penetration graph); HLB (Huanglongbing); IAP (inoculation access period); PCR (polymerase chain reaction).

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Introduction

The bacterium ‘*Candidatus Liberibacter solanacearum*’ (CaLso) (Liefting *et al.*, 2009), also known as ‘*Ca. Liberibacter psyllaurosus*’ (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 is also associated with vegetative disorders in other Solanaceae and Apiaceae crops in different areas worldwide (Haapalainen, 2014). CaLso is transmitted from potato mother tubers to growing plants and to progeny tubers (Pitman *et al.*, 2011) and is a carrot seed borne pathogen (Bertolini *et al.*, 2014), which poses a high risk of introduction of the bacterium into new areas in which contaminated carrot seeds are commercialized. The bacterium is also horizontally and naturally spread by different psyllid species (Hemiptera:

Psylloidea) following establishment (Hansen *et al.*, 2008; Alfaro-Fernández *et al.*, 2012; Nissinen *et al.*, 2014).

Psyllids are small, 2-3 mm long four-winged insects that feed on plants by ingesting the phloem sap with piercing-sucking mouthparts. The life cycles vary depending on the species and environmental conditions but last 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 deposited by a mated female ranges from one hundred to one thousand for most of the species studied (Hodkinson, 2009).

Previous reports show that '*Ca. Liberibacter* spp.' associated with newly emerging and economically important diseases are transmitted by several psyllid species. The three '*Ca. Liberibacter*' species associated with huanglongbing (HLB) citrus disease are transmitted by *Diaphorina citri* Kuwayama, *Trioza erythrae* Del Guercio, and *Cacopsylla citrisuga* Yang and Li (Cen *et al.*, 2012a). Additionally, *Diaphorina communis* Mathur was identified in Bhutan carrying '*Ca. L. asiaticus*' (Donovan *et al.*, 2012), but the ability to transmit the bacterium was not reported. CaLso is transmitted by *Bactericera cockerelli* Sulc in potato and tomato (Secor *et al.*, 2009) and by *Trioza apicalis* Förster in carrot in northern Europe (Nissinen *et al.*, 2014). *Bactericera trigonica* Hodkinson is associated with vegetative disorders of carrot and celery in Spain (Alfaro-Fernández *et al.*, 2012; Teresani *et al.*, 2014), and Antolínez *et al.* (2017a, 2017b) described the feeding behaviour of *B. trigonica* on carrot plants and reported that the transmission of CaLso was not affected by the vector gender. Moreover, Munyaneza *et al.* (2016) assessed the ability of the potato psyllid *B. cockerelli* to transmit CaLso from potato to carrot, and Antolínez *et al.* (2017b) the risk of transmission of CaLso from Apiaceae to potato by *B. trigonica*.

The detection methods for CaLso include both conventional and real-time Polymerase Chain Reaction (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.*, 2010). The bacterium was also detected in *B. trigonica*, *B. tremblayi*, *B. nigricornis* and *Bactericera* sp. by squash real-time PCR (Teresani *et al.*, 2015).

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, which is 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 similar way to that described for persistently and propagative 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 which the bacteria are transmitted with salivary secretions into a new host plant during psyllid feeding (Hall *et al.*, 2013). Cell titre of CaLso increases from 0 to 2 weeks in *B. cockerelli* and a latent period of at least two weeks is necessary for successful transmission (Sengoda *et al.*, 2014).

Considering that *Ca. L. solanacearum* is phloem-limited, transmission to certain host plants depends on the vector ability to feed in their sieve tube elements. Vector feeding behaviour on particular host can be determined by analyzing the electrical penetration graph (EPG) waveforms, which are formed as a result of two signal component variables. One is the fluctuation in the electric circuit resistance (Ohm's law), and the other one is the emf component, derived from fluctuation in the electromotive forces. These components provide information on stylet tip position in specific plant tissues, time spent at each location, and stylet activity (Tjallingii, 1985). The EPG waveforms of psyllids are reported for *D. citri* (Bonani *et al.*, 2010; Cen *et al.*, 2012b), *B. cockerelli* (Butler *et al.*, 2012; Pearson *et al.*, 2014; Sandanayaka *et al.*, 2014; Mustafa *et al.*, 2015), *C. pyri* L. (Civolani *et al.*, 2011) and *Psylla pyricola* Förster (Ullman & McLean, 1988). Comparison by EPGs waveforms of the probing behaviours of males and females of *B. trigonica* in carrot has been recently reported (Antolínez *et al.*, 2017a) and the characterization of the electrical penetration graphs (Antolínez *et al.*, 2017b).

Because of the recent association of CaLso with several crops affected by psyllid species and the report of the association of *B. trigonica* with the bacterium in carrots and celery, investigations were conducted to increase the understanding of vector feeding behaviour and transmission of this pathogen in carrot, celery, potato and tomato and to evaluate the threat that this psyllid species could represent to economically important solanaceous crops.

Material 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 heavily infected with CaLso (haplotype E) in the La Padilla region in Tenerife (Canary Islands, Spain) in 2013. The laboratory colony was maintained on CaLso positive carrot plants in a controlled environment room at 25±2°C, 70±5% humidity and with a photoperiod of 16:8 h (L:D) at the ICIA facilities in Tenerife. Used in the following experiments, individuals from this colony were regularly tested by real-time PCR (see below) to confirm the presence of CaLso. The CaLso haplotype found in the colony was the haplotype E, which was determined according to Teresani *et al.* (2014).

Source of receptor plants

Potato 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 CaLso-free seeds or 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 the stage of 4-5 fully expanded leaves stage.

Sample preparation, DNA purification and CaLso 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 4°C for up to one week. The extracts were prepared using a Homex 6 (Bioreba, Reinach, Switzerland, CH) 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 use. The total DNA was purified from 200 µL of crude plant extract using the cetyl trimethyl ammonium bromide (CTAB) protocol (Murray & Thompson, 1980). The purified DNA was stored at -20°C until use.

Psyllids, either fresh or preserved in 70% ethanol, were individually squashed on nylon membranes with the rounded end of an Eppendorf tube (Teresani *et al.*, 2015). The membranes were immediately processed or kept at room temperature in a dry and dark place for a maximum of one month. The samples immobilized on membranes were carefully cut out and inserted into Eppendorf tubes containing 100 µL of distilled water to release the DNA targets (Teresani *et al.*, 2015). Three microliters of the extract were directly used as a template for real-time PCR analysis.

The detection of CaLso was performed with a complete real-time PCR Kit CaLso/100 (Plant Print Diagnostics) according to the manufacturer's

instruction and based on the protocol described by Teresani *et al.* (2014). The assays were conducted with the StepOne Plus (Applied Biosystems) machine, and the data acquisition and analyses 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 noncontaminated 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 experiments, adult psyllids were starved for a 1 hour at 25±2°C before the experiment, anesthetized with CO₂ for 3 s and immediately immobilized using a vacuum chamber. Under a dissecting microscope, a 20 µm diameter and 3 cm long gold wire (Heraeus Holding, Hanau, Germany) was attached to the pronotum of *B. trigonica* adults with a drop of silver conductive paint (16034 Pelco Colloidal Silver, Ted Pella Inc., Ladd Research Industries, Williston, VT, USA). The insects with the gold wire attached were connected to the EPG probe 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 CaLso-free carrot, celery, potato or tomato leaf. The EPG recordings were obtained with a DC-EPG device (Giga-4; EPG Systems, Wageningen, The Netherlands), adjusted to 100x gain. The monitoring system was assembled inside a Faraday cage (100 × 110 × 90 cm) to prevent electrical noise. The EPG data acquisition was conducted with a PC computer using Stylet+software for Windows (EPG Systems, Wageningen, The Netherlands).

Insect probing and feeding behaviour 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; Antolínez *et al.*, 2017c) were identified as follow: non-probing (np), intercellular apoplastic stylet pathway (C waveform), initial contact with phloem tissue (D waveform), salivation into phloem sieve elements (E1 waveform), passive phloem sap uptake from the phloem sieve elements (E2 waveform) and active intake of xylem sap from xylem elements (G waveform). The behavioural variables were processed using the MS

Excel Workbook for automatic EPG data calculations according to Sarriá *et al.* (2009). Fifteen replicates from both sexes per plant species were performed. 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 probes, number of E1 and E2 events, total duration of E1 and E2 per insect, and percentage of probing spent in E1 and in E2.

The number and total duration per insect (mean \pm SE) of selected EPG variables were calculated using the SPSS statistical software package according to Backus *et al.* (2007): NWEI number of waveform events per insect, which is the sum of the number of events of a particular waveform divided by the total number of insects under each treatment; and WDI, waveform duration (sec) per insect, which is the sum of durations of each event of a particular waveform made by each individual insect that produced that waveform divided by the number of insects that performed that particular waveform under each treatment. 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 performed with the nonparametric Mann-Whitney U test. P-values less than 0.05 were statistically significant.

‘*Ca. Liberibacter solanacearum*’ transmission studies

To determine whether *B. trigonica* transmitted the bacterium to carrot and to other hosts, transmission experiments were performed using healthy carrot, celery, potato and tomato as receptor plants. All experiments were conducted in a controlled environment room at $25\pm 2^\circ\text{C}$, $70\pm 5\%$ humidity and with a photoperiod of 16:8 h (L:D). Plants without insects were used in all experiments as negative controls.

a) Restricted leaf exposure to *B. trigonica*. Groups of 10 *B. trigonica* adults were confined on leaves inside clip-cages (3.5 cm diameter \times 4 cm high) and allowed to feed for different inoculation access periods (IAP) of 24 h, 3 d, 7 d and until 14 d or insect death on the same plant. Ten replicates of each receptor plant species (represented by 10 psyllid adults per clip-cage/leaf 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 the leaf was detached, the surface cleaned and immediately tested for CaLso by real-time PCR. The plants were sprayed

(1 g/L of Confidor®, Bayer CropScience) 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 new leaves were tested by real-time PCR to confirm CaLso infection.

b) Whole plant exposure to *B. trigonica* (no-choice assay). A single *B. trigonica* adult was confined in a small plant cage (5.5 cm diameter \times 15 cm high) that permitted the insect free access to the entire plant during a 24-h IAP. The same insect was then transferred to a second receptor plant in the same development stage and allowed to feed during 14 d or until the psyllid death. Thirty psyllids/receptor plant were used for each exposure period, and three repetitions of the same experiment were performed. The plants were sprayed as described above, maintained in the controlled environment room for one month, and then new leaves from each plant were sampled and pooled for real-time PCR analysis to confirm CaLso infection.

Additionally, groups of ten *B. trigonica* were confined in a small plant cage (as described before) and allowed to feed during 14 d or until the psyllid death. Ten plants for each receptor host were used. The plants were treated with insecticide as previously described and grown in an insect-proof greenhouse for one month, monitored for disease symptoms and then new leaves from each plant were sampled and pooled for real-time PCR analysis to confirm CaLso infection.

c) Whole plant exposure to *B. trigonica* (choice assay). Experiments were performed inside aphid-proof cages (140 cm high \times 100 cm long \times 100 cm deep). Each cage contained a single CaLso-infected carrot plant surrounded by 12 non-infected receptor plants of each plant species tested (carrot, celery, potato or tomato). The receptor plants were placed at similar distance to the carrot plant. A group of 100 *B. trigonica* young adults collected from the CaLso positive colony was released inside the cage and allowed to move freely among the receptor plants and the carrot plant until the death of the insects. One cage was psyllid-free to serve as the control treatment. Cages were replicated three times and arranged in a randomized block design. Plant samples were collected 1.5 months after the beginning of the experiment and analysed by real-time PCR to assess the newly emerged leaves for CaLso.

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

To assess *B. trigonica* reproduction on potato and tomato plants, 18 newly emerged psyllid couples were individually confined on a seedling of tomato or potato inside plant cages (5.5 cm diameter \times 15 cm high) for 14 days. The same was performed on carrot seedlings as

the control host using 11 newly emerged psyllids couples. Using a binocular stereomicroscope, the eggs laid by each couple were counted daily and individually marked and monitored for hatching and nymphal development and mortality. The experiments were performed in climate chambers with controlled conditions at $25\pm 2^\circ\text{C}$, 60-70% humidity and with a photoperiod of 16:8 h (L:D).

Transovarial passage

Twenty adult females from the bacteriferous colony were used to assess the transovarial passage of CaLso in *B. trigonica*. Females were individualized on a healthy carrot seedling inside a plant cage and allowed to lay eggs during a week. The females were then removed from the cages and individually analysed for CaLso. Eggs from each female were dissected from the leaves using a heat-sterilized insect pin and then individually squashed on nylon membranes with the aid of a sterile plastic tip and allowed to dry. Ten microliters of distilled water were added to the membrane in an Eppendorf tube, mixed with a vortex, and three microliters of the extract was analysed by real-time PCR as described before. The experiment was performed in climate chambers with controlled environment at $25\pm 2^\circ\text{C}$, 60-70% humidity and with a photoperiod of 16:8 h (L:D).

Results

Feeding behaviour on different hosts

EPG studies to evaluate the phloem-feeding ability of *B. trigonica* on different vegetable hosts were

performed. Fifteen psyllids per receptor plant species were analysed for 8 h. The percentage of psyllids probing before the first 15 min and showing clear EPG waveforms was similar between host and non-host plants (61.8% in carrots, 64.5% in celery, 68.8% in potato and 64.0% in tomato). When EPG variables were analysed, the number of probes of *B. trigonica* on the four different plant species used in the study decreased in the order carrot > celery > potato > tomato, but no significant differences were found among them (Table 1). Similarly, no differences were found for the activity in the xylem (number and total duration of G). However, significant differences in the phloem-related activities of *B. trigonica* were found in Apiaceae (carrot and celery) compared with Solanaceae (potato and tomato) species, but not between species belonging to the same botanical family (Table 1). *Bactericera trigonica* reached the phloem sieve elements (E1 and E2 waveforms) when fed on carrot and celery, but when psyllids probed potato plants, only two of 15 individuals reached the phloem sieve elements and each salivated (E1) once for a few seconds (53.3 and 92.0 s); however, phloem ingestion (E2 waveform) was never detected in the 8 h of recordings. No phloem-related activity (E1 or E2 waveforms) was observed when *B. trigonica* was placed on tomato plants.

'Ca. Liberibacter solanacearum' transmission studies

The detection of CaLso in the *B. trigonica* adults used in the experiments ranged from 50% to 90%.

a) Restricted leaf exposure to *B. trigonica*. The bacterium was detected in leaves in different

Table 1. Means (\pm 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 |
|----------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Number of probes (NWEI) | 10.00 \pm 4.70 a | 9.13 \pm 6.08 a | 7.47 \pm 4.29 a | 5.86 \pm 5.90 a |
| Number of G (NWG) | 2.60 \pm 1.18 a | 2.20 \pm 1.56 a | 1.60 \pm 1.88 a | 2.33 \pm 1.34 a |
| Total duration of G (WDG) | 3408.90 \pm 1638.83 a | 2878.29 \pm 1429.42 a | 2998.64 \pm 1810.82 a | 3218.43 \pm 2218.07 a |
| Number of E1 (NWEI) | 2.80 \pm 1.85 a | 4.00 \pm 3.76 a | 0.13 \pm 0.35 b | 0.00 \pm 0.00 b |
| Number of E2 (NWEI) | 1.87 \pm 1.59 a | 2.80 \pm 3.32 a | 0.00 \pm 0.00 b | 0.00 \pm 0.00 b |
| Total duration of E (WDI) | 4389.00 \pm 5010.17 a | 3923.00 \pm 6528.95 a | 9.68 \pm 26.58 b | 0.00 \pm 0.00 b |
| Total duration of E1 (WDI) | 854.00 \pm 1226.07 a | 1127.00 \pm 1477.59 a | 9.68 \pm 26.58 b | 0.00 \pm 0.00 b |
| Total duration of E2 (WDI) | 3535.00 \pm 4789.00 a | 2797.00 \pm 5901.00 a | 0.00 \pm 0.00 b | 0.00 \pm 0.00 b |
| % of probing spent in E1 | 5.29 \pm 7.70 a | 6.35 \pm 10.85 a | 0.16 \pm 0.47 b | 0.00 \pm 0.00 b |
| % of probing spent in E2 | 17.40 \pm 21.20 a | 11.12 \pm 21.04 a | 0.00 \pm 0.00 b | 0.00 \pm 0.00 b |

NWEI, number of waveform events per insect; WDI, waveform duration (seconds) per insect; waveform E1, salivation into phloem sieve elements; waveform E2, passive phloem sap uptake from the sieve elements. Values with different letters in the same row are significantly different ($p < 0.05$, Mann-Whitney U test).

percentages that varied depending on the plant species and IAP (Table 2). The bacterium was detected only in carrot and celery newly emerged leaves one month post-inoculation. Carrot and celery receptor plants showed symptoms, but symptoms were not observed at the end of the experiment in either potato or in tomato plants in which DNA targets were detected.

b) Whole plant exposure to *B. trigonica* (no-choice assay). The bacterium was detected in newly formed leaves of the experimental plants one month after the IAP when ten *B. trigonica* fed on whole plants of carrot, celery, potato and tomato plants for approximately 14 d (the long exposure). The bacterium was detected in eight out of 10 carrots, in 10 out of 10 celery plants, and in 1 of 10 potato and tomato plants (Table 3).

Detection of CaLso after one month occurred in different plant species independently of the IAP when a single *B. trigonica* was used (short or long exposure). When the 24 h IAP was assayed, 59 of 87 carrots, 19 of 90 celery plants and five of 83 tomatoes tested positive for the bacterium. When the 14 d IAP was assayed, 46 of 70 carrots, 15 of 90 celery plants and four of 59 tomatoes tested positive (Table 3). The highest CaLso detection rates were obtained on carrot

plants (67.8% and 65.7% after 24 h and 14 d IAPs, respectively), followed by celery (21.1% and 16.6% after 24 h and 14 d IAPs, respectively). The CaLso detection rates in tomatoes were similar for both IAPs (approximately 6%, Table 3). No bacterium was detected when a single insect fed on potato plants. Plants without insects used as negative controls always tested negative.

c) Whole plant exposure to *B. trigonica* (choice assay). CaLso was detected in 10 of 36 carrots plants, 10 of 36 celery plants and 1 of 36 tomato plants. The bacterium was not detected in any of the 36 potato plants assayed.

Reproduction rates on 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.

When the control host (carrot) was analysed, the 11 *B. trigonica* couples laid 235.1 ± 40.81 eggs per female on carrot seedlings and 85% of them completed their development from eggs to adult.

Table 2. Detections of ‘*Candidatus Liberibacter solanacearum*’ (CaLso) in carrot, celery, potato and tomato plants in restricted leaf exposure to *B. trigonica*.

| IAP ^a | Carrot | Celery | Potato | Tomato |
|--------------------------------------------|--------------------|--------|--------|--------|
| | (CaLso +/- Total) | | | |
| 24 hours ^b | 6+/10 ^d | 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 ^c | 7+/10 | 10+/10 | 0+/10 | 0+/10 |

^aIAP = Inoculation access period. ^bEach receptor plant was inoculated in four leaves, by confining 10 adults per leaf inside a clip cage. Right after the IAP, the inoculated leaves were assayed by real-time PCR. ^cone month after the IAP, newly-formed leaves of each receptor plant were also tested by PCR. ^dNumber of positive detections of CaLso targets by real-time PCR in leaves/total number of assayed leaves.

Table 3. Detections of ‘*Candidatus Liberibacter solanacearum*’ (CaLso) in carrot, celery, potato and tomato plants in whole plant exposure assays to a single or 10 adults of *B. trigonica* at 24 h or 14 d of inoculation access periods (IAP)

| | CaLso +/- Total (% of transmission) | | |
|--------|-------------------------------------|----------------|------------------------|
| | 1 <i>B. trigonica</i> | | 10 <i>B. trigonica</i> |
| | IAP 24h | IAP ~ 14d | IAP ~ 14d |
| Carrot | 59+/87 (67.8%) ^a | 46+/70 (65.7%) | 8+/10 (80%) |
| Celery | 19+/90 (21.1%) | 15+/90 (16.6%) | 10+/10 (100%) |
| Potato | 0+/90 (0%) | 0+/90 (0%) | 1+/10 (10%) |
| Tomato | 5+/83 (6.0%) | 4+/59 (6.8%) | 1+/10 (10%) |

^aNumber of infected plants/total number of assayed plants. Results of three repetitions are presented together.

Table 4. Transovarial passage of 'Candidatus Liberibacter solanacearum' (CaLso) by *Bactericera trigonica*. Eggs laid by 20 adult females.

| Adult <i>B. trigonica</i> | N° of females | N° of females that laid eggs | N° of analyzed eggs | N° of eggs CaLso + |
|---------------------------|---------------|------------------------------|----------------------|--------------------|
| CaLso + | 15 | 8 (4) ^a | 74 (50) ^b | 15 |
| CaLso – | 5 | 4 (0) | 22 | 0 |

^a In brackets, the number of females that laid positive eggs. ^b In brackets, the number of eggs laid by females that laid positive eggs.

Transovarial passage

Eggs laid by adult females were analysed to detect CaLso (Table 4). Fifteen of 20 females tested positive, and five tested negative. From the 15 positive females, only eight laid eggs. Eggs laid by CaLso-negative females were all negative, and the bacterium was detected in 20.3% of the eggs laid by CaLso-positive females.

Discussion

Many plant pathogens depend on an insect vector for their spread, transmission and survival. The transmission of CaLso, which causes vegetative disorders in Apiaceae in Spain, has been associated with *B. trigonica*, the predominant psyllid species on carrot and celery (Alfaro-Fernández *et al.*, 2012; Teresani *et al.*, 2015). In addition, several studies have recently been published about sex-specific probing behaviour and the characterization of the EPG of *B. trigonica* on carrots (Antolínez *et al.*, 2017a,c), and a risk of bacterial transmission from Apiaceae to potato by this psyllid and *B. tremblayi* (Antolínez *et al.*, 2017b). However, it is advisable to have more outcomes for the transmission of this bacterium and particularly after the first outbreak in Spain of zebra chip (http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/candidatus_liberibacter_solanacearum/), that reveal the existence of this disease caused by CaLso (haplotype E) in field potatoes.

The stylet penetration activities of *B. trigonica* were investigated in this work using the EPG technique on carrot, celery, potato and tomato plants, because these activities are associated with bacterial acquisition and inoculation of CaLso (Sandanayaka *et al.*, 2014; Antolínez *et al.*, 2017a,c). In our work, EPG waveforms were analogous to those described for other psyllids (Bonani *et al.*, 2010; Civolani *et al.*, 2011; Pearson *et al.*, 2014; Mustafa *et al.*, 2015) and those described for *B. trigonica* by Antolínez *et al.* (2017c). Acquisition

of the bacteria is associated with the phloem ingestion (E2 waveform), whereas inoculation is associated with salivation into the phloem sieve elements (E1 waveform) (Sandanayaka *et al.*, 2014; Antolínez *et al.*, 2017c). Therefore, because our findings confirmed that *B. trigonica* is able to sustained salivation and ingestion in the phloem of carrot and celery plants, the psyllid could acquire and inoculate CaLso in these host species. No phloem salivation (E1) or acquisition (E2) were detected on tomato and only sporadic phloem salivation events (E1 waveform) were observed when *B. trigonica* was forced to feed on potato plants (one event each for two psyllids for 53.3 and 92 s). These results would discard the possibility that the psyllid acquire the bacteria from both Solanaceous crops and neither inoculate it in the case of tomato. Short periods of E1 activity in the phloem of potato plants indicate the possibility of transmission, although at a low efficacy, since the success of the inoculation is directly correlated with the duration of this period of salivation. However, we must consider that the insect can inoculate the bacterium in a period as short as 30 seconds in a preferred host such as carrot (Antolínez *et al.*, 2017c), and that these studies only covers a limited period of 8 h.

Similar phloem-feeding behaviour occurred when bacteriferous individuals of the potato psyllid *B. cockerelli* were exposed to carrot, a non-preferred host plant, to assess the probability of CaLso cross transmission. Three of 23 tested psyllids salivated (E1 waveform) from 4-5 min to 2.3 h in carrot phloem, and one of 23 *B. cockerelli* ingested from carrot phloem (E2 waveform) for approximately 1 h (Munyanza *et al.*, 2016), compared with our work in which *B. trigonica* did not ingest and only salivated into potato phloem tubes for a few seconds.

When EPG experiments are conducted, insects are forced to feed on the plants attached to a gold wire and sporadic probing and feeding may occur as insects have no other choice (no-choice conditions assay). This technique is a powerful tool to assess whether a species can feed in the phloem, but does not demonstrate actual transmission to a specific host. Therefore, conventional transmission assays were designed to assess whether *B. trigonica* acted as a CaLso vector.

The highest percentage of positive plants was found in carrot when the psyllids fed on a restricted leaf for 3 and 7 d IAPs (100% detection). In celery, the maximum detection rates occurred for the 7 d IAP and at insect death at approximately 14 d (60% and 80%, respectively). The percentage of transmission was relatively low in the Solanaceae. The maximum detection in potato (30%) was observed after an approximately 14 d IAP and the

maximum detection in tomato (60%) was observed after a 24 h IAP and then decreased progressively with longer IAPs (3 to approximately 14 d). In the “whole plant exposure” experiment in which a single *B. trigonica* was used per receptor plant, CaLso was detected in tomato one month after inoculation when the IAP was 24 h or 14 d (approx. 6% detection rate). In the same experiment with a group of 10 *B. trigonica* per plant, the bacterium was consistently detected in carrot (80%), in celery (100%) and was detected in 10% of potatoes and tomatoes in newly emerged leaves. Furthermore, all the experiments had negative controls (cages with plant, but no insects), resulting all negative, independently of the IAP.

The high detection rate of CaLso by real-time PCR in leaves just after the insects were removed might be due to its inoculation into the phloem, to releasing of bacteria in other plant tissues due to the activity of bacteriliferous insects, or even detection of non-viable bacteria. However, pathogen detection in newly formed leaves can confirm systemic transmission of the pathogen to the plant. Thus our experimental transmission assays revealed that *B. trigonica* adults inoculated CaLso (haplotype E) into various host species based on the appearance of typical symptoms in health plants, where the infected insects had been feeding, and also by the detection of CaLso by real-time PCR in newly formed leaves.

When the plant is not the preferred host for the bacterium, a longer post-inoculation period may be required to multiply and move systemically into the plant, or its percentage may be reduced because of likely poor fitness of haplotype E to colonize and survive in some solanaceous species.

Remarkably, Sandanayaka *et al.* (2014) working with *B. cockerelli* and Antolínez *et al.* (2017b) with *B. trigonica* observed also a low percentage of tomato and potato plants positive for CaLso in transmission assays, respectively, despite no phloem activities were recorded for those psyllids in eight hours' EPG studies, suggesting that few individual of the psyllids might reach the phloem of the host plants on longer observation periods.

In the field, phloem-restricted pathogens are transmitted efficiently only by colonizing species, and the transmission of a persistent phloem-restricted plant pathogen by non-colonizing species is very unlikely to occur from an epidemiological point of view (Irwin *et al.*, 2007). Only psyllid species that voluntarily land and feed on potato plants would be capable to drive CaLso epidemics. Although the primary transmission from Apiaceae to Solanaceae mediated by *B. trigonica* would be unlikely, as also considered by Antolínez *et al.* (2017a), we have to keep in mind that *B. trigonica*

is predominant on carrot and celery crops in Spain and is consistently found in potato fields growing in the vicinity (Teresani *et al.*, 2015). The possibility that psyllids will sporadically probe on potato should not be excluded, particularly when potatoes are the only available crop or when the population of psyllids is large, and a whole field of infected carrot or celery is harvested, forcing the population to move to another plant species grown nearby. 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 an occasional vector to crops other than Apiaceae although unable to cause epidemics in a non-colonizable plant.

The results of our studies could justify cases of zebra chip disease in potato tubers detected recently in Spain and that secondary dispersion within potato crop might be unlikely done by *B. trigonica*. However, other *Bactericera* species has been recorded, that might act as more effective vectors within potato (Teresani *et al.*, 2015). Although this option should be discarded for *B. tremblayi* in view of recent findings by Antolínez *et al.* (2017b), there is still a need to study the role of *B. nigricornis* among other potential vectors naturally present in the crop.

When *B. trigonica* reproduction was evaluated on different hosts over 14 days, the psyllids laid no eggs on tomato plants and much fewer eggs on potato (five eggs in three couples) than on carrots (average of 235.1 ± 40.81) and the few eclosed nymphs on potato died during the first instar. These results confirm that potato and tomato are not good hosts for *B. trigonica* since the females hardly ever or never laid eggs on potato and tomato, respectively, most likely because they found them no suitable for feeding, and the nymphs failed to thrive, even in conditions of non-choice.

CaLso is transmitted by psyllids to the plant host and among psyllids (Mann *et al.*, 2011), but can also pass transovarially to the progeny of a bacteriliferous female. Hansen *et al.* (2008) found evidence of transovarial CaLso transmission by *B. cockerelli*, detecting the bacterium in 20 of 25 eggs. By contrast, no evidence of ‘*Ca. L. asiaticus*’ transovarial passage or a low rate of transmission is observed in *D. citri* (Hung *et al.*, 2004; Pelz-Stelinski *et al.*, 2010). In our study, CaLso was detected in 20.3% of eggs laid on carrot by females contaminated with the pathogen, with females are known to probe more times, to ingest longer from phloem sieve elements and to reach phloem tissues more frequently than males (Antolínez *et al.*, 2017a). Thus, the PCR assays showed that the bacterium was vertically transmitted in *B. trigonica*. The increase of detection in nymphs and adults could be explained by bacterial multiplication in these life stages and by the

horizontal transmission through psyllids feeding on an infected plant, such as described for 'Ca. L. psyllauros' on tomato (Hansen *et al.*, 2008). To illustrate the potential of *B. trigonica* to spread the bacteria in carrot, initial prevalence of 3-4%, under a high population of bacteriferous *B. trigonica* resulted in a final prevalence of approximately 90% after six months of cultivation (Bertolini *et al.*, 2014; Teresani *et al.*, 2014).

Recommended strategies to prevent the introduction of CaLso into areas in which it is currently absent must be determined. Carrot seeds and potato breeding material should be analysed according to post-entry quarantine requirements (EPPO, 2006) and should be free from CaLso and the vectors *B. cockerelli*, *T. apicalis* and now, *B. trigonica*.

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