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RESEARCH ARTICLE

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

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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 is 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.

Additional key words: sticky plant; squash real-time PCR; *Bactericera trigonica; Bactericera tremblayi; Bactericera nigricornis.*
Abbreviations used: HLB (Huanglongbing); PCR (polymerase chain reaction).
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Introduction

'*Candidatus* Liberibacter solanacearum' (Liefting *et al*., 2009), which is also known as '*Ca*. Liberibacter psyllaurous' (Hansen *et al*., 2008) (Bacteria: Proteobacteria: Alphaproteobacteria: Rhizobiales: *Rhizobiaceae*), is a Gram-negative bacterium restricted to plant phloem and insect hemolymph that currently cannot be cultured *in vitro* (Liefting *et al*., 2009). 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; 2012; Tahzima *et al*., 2014; Teresani *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*., 2014). The bacterium has been recorded in Fin-

land (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*., 2014), 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*., 2015) 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) 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*., 2014). 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*., 2012). 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.*, 2012). 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*., 2014). 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 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.

Material 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 cultivars 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 meddle 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).

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 se-

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	Middle	06/26	09/13
		Loretta	11010212	Villena	38º35'25'' N	0º52'18'' W	Late	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	Late	08/02	11/12
Carrot	2012	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	2012	Slaney	20120003	Tenerife	28º30'13'' N	16º21'55'' W	Middle	05/17	07/12
Carrot	2012	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.

lected 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 (Ribaut 1936, 1952; Ramírez, 1955, 1956, 1959; Shaposhnikov & Davletshina, 1967; Hodkinson, 1981; Hermoso de Mendoza, 1982; Ossiannilsson, 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 3MM 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*., 2014). The membrane containing squashed psyllids was carefully cut around the sample (~ 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*. (2014) 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).

Psyllid species composition

The overall numbers of captured psyllid species (15,900 individuals) in the seasonal surveys are shown

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.

in Table 3. The population dynamics of psyllid species captured in celery plots in Villena (2011) are presented in Figure 1A. 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. tremblayi* Wagner (225) and *B. nigricornis* Förster (2). 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 1A). The population dynamics of psyllid species captured on celery plots in Villena (2012) are presented in Figure 1B. 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.

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 1C). In the middle cycle of cultivation, 14,255 *B. trigonica* and seven 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.

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.

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 (Tables 3 & 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

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).

Crop-Location	Year	Cycle of cultivation	*B. trigonica*	*B. tremblayi*	*B. nigricornis*	Other[a]	Total
Celery-Villena	2011	Early	0+/36[b]	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)[c]	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[d]	53+/241 (14,401)

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

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 three

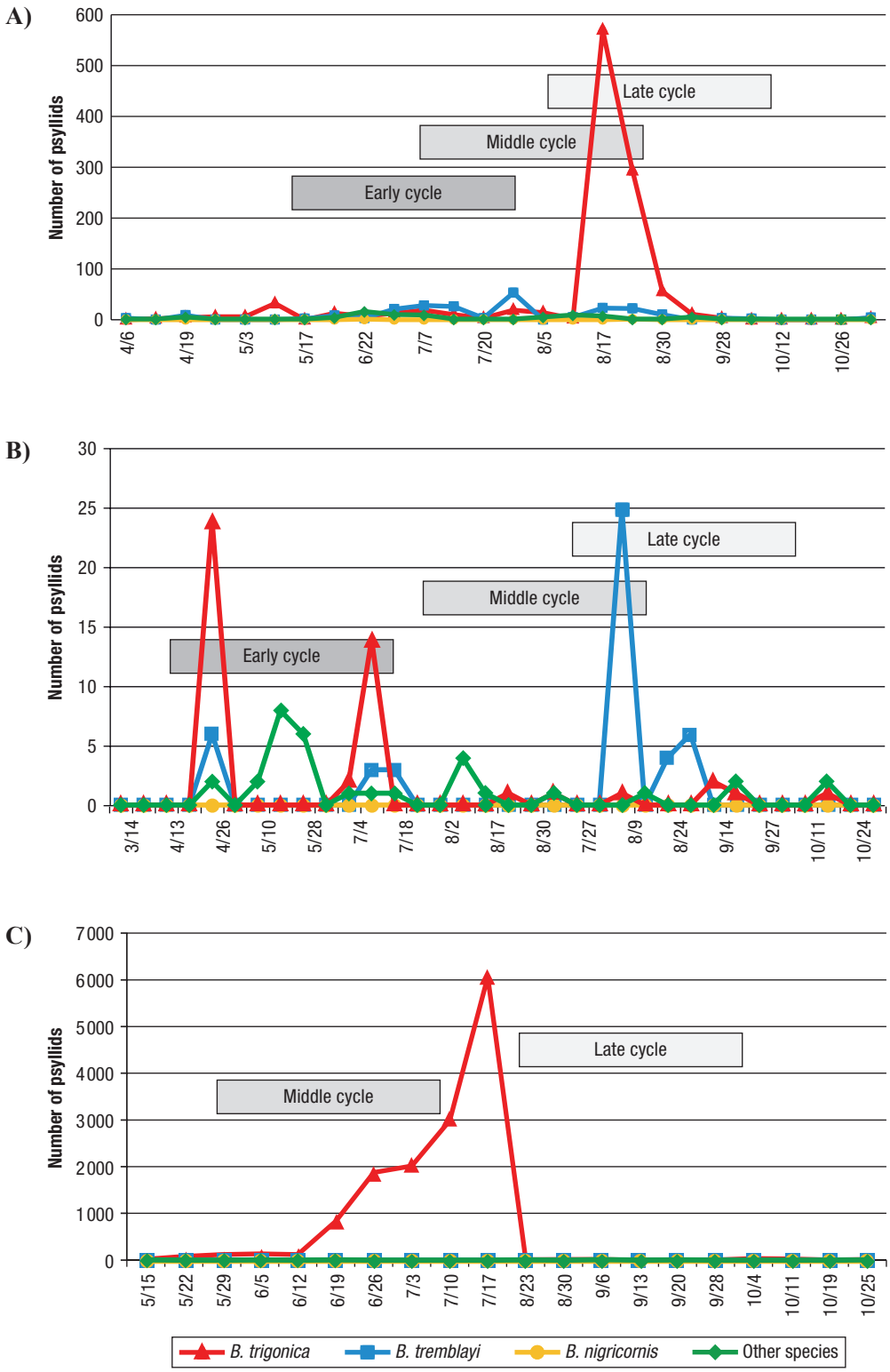

Figure 1. Population dynamics of psyllid species monitored using the sticky plant method. **A:** Population dynamics of psyllid species captured on celery plants in Villena in 2011 at the different cycles of cultivation. **B:** Population dynamics of psyllid species captured on celery plants in Villena in 2012 at the different cycles of cultivation. Combined data for the middle and late cycles. **C:** Population dynamics of psyllid species captured on carrot plants in Tenerife in 2012 at the middle and late cycles of cultivation.

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[b]	0+/0	0+/0	50+/70	0+/17	0+/2
Total	60+/63	0+/28	293+/590	35+/119	0+/11

[a] '*Candidatus* Liberibacter solanacearum' positive psyllid specimens/total of analysed 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.

specimens out of seven *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 addition, 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 although 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*., 2015). In both of the surveyed 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 (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 than 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 more 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*., 2012), *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*. (2015) 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 integrated disease management 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.* (2014) combined with real-time PCR described by Teresani *et al*. (2014) 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 (Font *et al*., 2010; Alfaro-Fernández *et al*., 2012a). 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 prevent 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.

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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