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'*Candidatus* Liberibacter solanacearum' Is Tightly Associated with Carrot Yellows Symptoms in Israel and Transmitted by the Prevalent Psyllid Vector *Bactericera trigonica*

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ABSTRACT

Carrot yellows disease has been associated for many years with the Gram-positive, insect-vectored bacteria, '*Candidatus* Phytoplasma' and *Spiroplasma citri*. However, reports in the last decade also link carrot yellows symptoms with a different, Gram-negative, insect-vectored bacterium, '*Ca*. Liberibacter solanacearum'. Our study shows that to date '*Ca*. L. solanacearum' is tightly associated with carrot yellows symptoms across Israel. The genetic variant found in Israel is most similar to haplotype D, found around the Mediterranean Basin. We further show that the psyllid vector of '*Ca*. L. solanacearum', *Bactericera trigonica*, is highly abundant in Israel and is an efficient vector for this pathogen. A survey conducted comparing conventional and organic carrot fields showed a marked reduction in psyllid numbers and disease incidence in the field practicing chemical control. Fluorescent in situ hybridization and scanning electron microscopy analyses further support the association of '*Ca*. L. solanacearum' with disease symptoms and show that the pathogen is located in phloem sieve elements. Seed transmission experiments revealed that while approximately 30% of the tested carrot seed lots are positive for '*Ca*. L. solanacearum', disease transmission was not observed. Possible scenarios that may have led to the change in association of the disease etiological agent with carrot yellows are discussed.

Yellows disease of carrots was recorded in the literature over 70 years ago and was subsequently reported from around the globe including North America (Ivanoff and Ewart 1944), Spain (Font et al. 1999), Lithuania (Valiunas et al. 2001), and Israel (Orenstein et al. 1999). During the 20th century, the suspected causal agent for carrot yellows disease were the Gram-positive wall-less bacteria '*Candidatus* Phytoplasma', nonculturable Mollicutes vectored mainly by leafhopper (Cicadellidae) and planthopper (Fulgoromorpha) insects (Tsai 1979; Weintraub and Beanland 2006). At the beginning of the 21st century, several incidences of carrot yellows infections were also associated with *Spiroplasma citri*, a wall-less Gram-positive bacterium with helical structure, transmitted by leafhoppers (Gera et al. 2011; Lee et al. 2006). Since carrot yellows control strategies were mostly targeted to reduce the populations of the insect vector, the association of yellows with a second Mollicute did not call for major changes in disease management practices.

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In 2010, a study from northern Europe reported for the first time the association of a Gram-negative bacterium, '*Ca.* Liberibacter solanacearum', with carrot yellows symptoms (Munyaneza et al. 2010). While '*Ca.* Liberibacter spp.' are phylogenetically very distant from the Gram-positive phytoplasma and spiroplasma, they share several similar biological traits, i.e., they are phloem-restricted, intracellular pathogens that depend on phloem feeding insect vectors for dissemination. Later studies have also reported the association of '*Ca.* L. solanacearum' with carrot yellows in Finland, Norway, Sweden, Austria Spain, Canary Islands, France, Africa, Germany, and Greece (Alfaro-Fernandez et al. 2012a, b; EPPO 2015; Holeva et al. 2017; Loiseau et al. 2014; Munyaneza et al. 2010, 2012, 2013, 2015; Tahzima et al. 2014).

'*Ca.* L. solanacearum' populations can be categorized into groups of genetic variants called haplotypes, which are determined based on single-nucleotide polymorphism (SNP) of three genomic regions: 16S rDNA, 50S rDNA rplJ-rplL, and 16S/23S ISR (Nelson et al. 2011; 2013). Thus far, five different '*Ca.* L. solanacearum' haplotypes, associated with plant disorders, have been described (Teresani et al. 2014). Haplotypes A and B are associated with zebra-chip disease in solanaceous crops in North America and New Zealand (Liefting et al. 2008), while haplotypes C to E are associated with yellows disease in apiaceous crops (mainly carrot) in Europe, Africa, and the Mediterranean (Haapalainen et al. 2016; Teresani et al. 2014).

Differently from phytoplasma and spiroplasma, all '*Ca.* L. spp.' are vectored by psyllids (Psyllidae). However, while the solanaceous-associated haplotypes A and B are vectored by the same psyllid, *Bactericera cockerelli*, haplotypes C and D/E are vectored by different psyllids, *Trioza apicalis* and *B. trigonica*, respectively (Alfaro-Fernandez et al. 2012b; Hansen et al. 2008; Munyaneza et al.

2010; Nelson et al. 2013). *B. trigonica* was reported in Africa and southern Europe as the vector that transmits '*Ca.* L. solanacearum' to carrot, however, in northern and western Europe *T. apicalis* is the '*Ca.* L. solanacearum' vector (Alfaro-Fernandez et al. 2012a, b; Munyaneza et al. 2010; Tahzima et al. 2014; Teresani et al. 2014).

In Israel, carrot yellows was first reported in 1999 and has since appeared sporadically causing significant economic losses. Typical disease symptoms include shoot proliferation, abnormal root shape and some leaf discoloration, resulting in reduced quality and yield. While the disease has been associated with phytoplasma (Orenstein et al. 1999) and/or spiroplasma (Gera et al. 2011), high percentages of phytoplasma/spiroplasma-free symptomatic carrot plants were observed (Gera et al. 2011; Weintraub and Orenstein 2004). The presence of *B. trigonica* in Israel (Halperin et al. 1982) and recent reports on the association of '*Ca.* L. solanacearum' with carrot yellows symptoms and seeds (Bertolini et al. 2015) prompted us to examine the association of '*Ca.* L. solanacearum' and psyllids with carrot yellows incidence in Israeli fields. Clarifying the etiological agent(s) of carrot yellows is crucial for understanding disease epidemiology and damage, and is essential for providing efficient management practices to control the disease.

MATERIALS AND METHODS

Carrot sample collection from commercial fields. Symptomatic and asymptomatic carrot plants were collected during April and June 2015, from 18 commercial carrot fields in north and south Israel, respectively (Fig. 1A). In total, 59 symptomatic and 10 asymptomatic plants were analyzed by PCR for the association with '*Ca.* L. solanacearum' as described below.

Carrot DNA extraction and PCR analysis. DNA from carrots was extracted using a modified CTAB (cetyltrimethylammonium bromide) procedure (Zhang et al. 1998). Two hundred milligram of leaf and petiole tissue was ground in liquid nitrogen using a mortar and pestle, and transferred into a 2-ml Eppendorf tube containing 1 ml of prewarmed (65°C) CTAB with 10 mM DTT (Dithiothreitol). The tube was vortexed thoroughly and incubated at 65°C for 45 min with occasional mixing by tube inversion. Content was then centrifuged at 4°C for 10 min at 21,000 × *g* and the resulting supernatant (~600 µl) was transferred into a new 2-ml tube containing a similar volume of chloroform/isoamyl alcohol (IAA) (24:1). The content was mixed and centrifuged at 4°C for 10 min at 21,000 × *g*. The supernatant (~400 µl) was mixed with 0.7 volumes of isopropanol and incubated at −80°C for 2 h or at −20°C overnight. The sample was centrifuged at 4°C for 20 min at 21,000 × *g* to pellet DNA. The liquid phase was discarded and the pellet was washed with 500 µl of 70% ethanol and centrifuged for 3 min at 4°C for 10 min at 21,000 × *g*. The DNA pellet was dried at room temperature for 10 min and resuspended with 40 µl of water and incubated at 55°C for 10 min. PCR was performed on 20 ng of template DNA using the DreamTaq Green PCR Master Mix (Fermentas) in a final reaction volume of 10 µl. The presence of '*Ca.* L. solanacearum' was determined using the OA2/OI2c primers. A '*Ca.* Liberibacter solanacearum'-positive carrot sample was used as a positive control for PCR analysis. The presence of phytoplasma and spiroplasma was determined using conventional nested-PCR with primers P1/P7 followed by U3/U5 or ScR16F1/ScR16R1 followed by ScR16F1A/ScR16R2, respectively. All primer sequences and references are listed in Supplementary Table S1.

Haplotyping analysis of '*Ca.* Liberibacter solanacearum'-positive samples. A subset (n = 20) of the '*Ca.* Liberibacter solanacearum'-positive carrot samples listed in Table 1 and a subset (n = 14) of the '*Ca.* L. solanacearum'-positive psyllid samples (described in the section below) were haplotyped according to Teresani et al. (2014). The 16S rRNA (EU812559.1) and the 50S rDNA rplJ-rplL (EU834131.1) regions were PCR-amplified. PCR products were purified using the PureLink PCR Purification kit (Thermo) and directly sequenced. The haplotype was determined by analyzing SNPs according to Teresani et al. (2014) using SnapGene software (GSL Biotech).

Psyllid monitoring, sampling, DNA extraction, and PCR analysis. Two carrot fields in southern Israel (Fig. 1A) were selected for monitoring psyllid population dynamics. Field A, located next to Moshav Shibolim (31°23′8.20′′N, 34°36′25.71′′E) was sown on 26 November 2015 with the carrot cultivar Nairobi and harvested on 23 May 2016. The average max/min temperature and

Fig. 1. Map of Israel indicating sampled fields and representative symptomatic and asymptomatic carrot plants. **A,** Partial map of Israel, black circle represents the 18 commercial fields sampled and described in Table 1. Open circles designated A and B show the location of fields A and B used for psyllid monitoring survey and disease incidence determination. **B,** Early stage axillary shot proliferation (circled); **C,** Carrot plants showing extensive axillary shot proliferation, a week before harvest. **D,** Uprooted symptomatic and asymptomatic carrot plants. The first two plants from the left show abnormal root structure and typical axillary shot proliferation, the three plants on the right show no disease symptoms.

TABLE 1. Carrot samples collected from commercial fields

Number	Region	Location[a]	Symptoms	PCR/qPCR with '*Ca.* L. solanacearum' primers	Haplotype by 16S[b]	Haplotype by 50S rplJ and rplL[b]
1	North	Tel Yossef	+	+	D	N/D
2	North	Tel Yossef	+	+		
3	North	Tel Yossef	+	+	D	D
4	North	Bet HaShitta	+	+	D	N/D
5	North	Bet HaShitta	+	+	D	D
6	North	Bet HaShitta	+	+		
7	North	Newe Oor (N)	+	+	D	N/D
8	North	Newe Oor (N)	+	+		
9	North	Newe Oor (N)	+	+	D	N/D
10	North	Newe Oor (S)	+	+	D	N/D
11	North	Newe Oor (S)	+	+		
12	North	Newe Oor (S)	+	+	D	D
13	North	Newe Oor (S)	+	+		
14	North	Gazith organic	+	+	D	D
15	North	Gazith organic	–	–		
16	North	Gazith organic	–	–		
17	North	Gazith	–	–		
18	North	Gazith	–	–		
19	North	Gazith	–	–		
20	North	Gazith	–	–		
21	North	Nahal Harod	+	–		
22	North	Nahal Harod	–	–		
23	North	Nahal Harod	–	–		
24	North	Nahal Harod	–	–		
25	North	Geshser	–	–		
26	North	Geshser	+	+		
27	North	Geshser	+	+		
28	North	Geshser	+	+	N/D	D
29	North	Ein Harod	+	+	D	N/D
30	North	Ein Harod	+	+	D	N/D
31	North	Ein Harod	+	+	D	N/D
32	North	Ein Harod	+	+	D	D
33	North	Meirav	+	+	D	N/D
34	North	Meirav	+	–		
35	North	Meirav	+	+		
36	North	Meirav	+	+		
37	North	Barkai	+	+	D	D
38	North	Barkai	+	+	D	D
39	North	Barkai	+	+	D	N/D
40	North	Barkai	+	+	D	N/D
1	South	Negba	+	+	D	N/D
2	South	Negba	+	+		
3	South	Negba	+	+		
4	South	Negba	+	+		
5	South	Shikmim	+	+		
6	South	Shikmim	+	+		
7	South	Shikmim	+	+		
8	South	Shikmim	+	+		
9	South	Dorot	+	+		
10	South	Dorot	+	+		
11	South	Dorot	+	+		
12	South	Dorot	+	+		
13	South	Beeri	+	+		
14	South	Beeri	+	+		
15	South	Beeri	+	+		
16	South	Beeri	+	+		
17	South	Sde Zvi	+	+		
18	South	Sde Zvi	+	+		
19	South	Sde Zvi	+	+		
20	South	Sde Zvi	+	+		
21	South	Broor Hayl	+	+		
22	South	Broor Hayl	+	+		
23	South	Broor Hayl	+	+		
24	South	Broor Hayl	+	+		
25	South	Saad	+	+		
26	South	Saad	+	+		
27	South	Saad	+	+		
28	South	Saad	+	+		
29	South	Saad	+	+		

[a] (N), north; (S), south.
[b] N/D, not determined.

humidity throughout the duration of cultivation in the closest meteorological station (Dorot, 12 km north of field A) was 23.8/10.5°C and 66.9%, respectively. Field B, located next to Kibbutz Negba (31°40′8.7′′N, 34°42′2.98′′E), was sown on 8 December 2015 also with cultivar Nairobi and harvested on June 1st, 2016. The average max/min temperature and humidity throughout the duration of cultivation in the meteorological station located at Negba was 22.8/9.9°C and 69.7%, respectively. Field A was grown under organic farming practices (i.e., without insecticide spraying); field B was grown under conventional farming practices including 10 insecticide applications. In each field four yellow sticky traps (20 × 14 cm), designated A1 to A4 and B1 to B4, were placed. Traps 1 and 2 in each field were placed in the same row ~30 m from each other. Traps 3 and 4 were placed a further six rows away (~15 m) and were also ~30 m apart. Traps were collected and replaced every 2 weeks. Psyllids captured on the traps were identified morphologically and counted manually. To determine '*Ca.* L. solanacearum' presence in psyllids, a batch of 10 psyllids from each trap was placed in 500 µl of ethanol at room-temperature overnight to remove residual glue from specimens. DNA was extracted from psyllids according to Lidor et al. (2017). In brief, each group of 10 psyllids was crushed using a blue pellet pestle and 200 µl of CTAB buffer containing 10 mM DTT. Homogenate was incubated for 2 h at 37°C and centrifuged at 4°C for 5 min at 21,000 × *g*. The supernatant was transferred into a new tube containing a phenol/chloroform (1:1) solution with the same volume and lightly mixed. Content was centrifuged at 4°C for 5 min at 21,000 × *g* and the resulting supernatant (~185 µl) was collected and transferred into a new tube containing the same volume of chloroform. Contents were lightly mixed and centrifuged again as above. The upper yellow phase was collected into a new tube containing the same volume of isopropanol. NaCl (5 M) was added to the tube at 0.2 volumes of the isopropanol solution and lightly mixed. Content was incubated at –20°C for 2 h and centrifuged at 4°C for 10 min at 21,000 × *g*. The DNA pellet was washed twice with 300 µl of 70% ethanol, air dried and resuspended with 40 µl of water. Resuspended DNA was left at 4°C overnight. PCR analysis to detect '*Ca.* L. solanacearum' in the sampled psyllids was performed using the Hy-Taq Ready Mix (HyLabs) and as indicated above.

Estimation of disease incidence in carrot fields. During the week preceding harvest, disease incidence was estimated in fields A and B mentioned above. In each field, four, randomly selected, 12-m-long stretches, containing approximately 300 plants each, were visually inspected and the number of carrot plants showing typical shoot proliferation symptoms was scored. The percentage of symptomatic plants in each field (i.e., disease incidence) was calculated by averaging the percent symptomatic plants in the four stretches. The Welch test was carried out to determine whether the mean disease incidence in fields A and B were statistically different (α level = 0.05).

Psyllid rearing and '*Ca.* L. solanacearum' transmission assays. Insects were collected from carrot fields using an insect net and placed in an empty insect rearing cage (BugDorm). Psyllids were transferred from the cage using an empty 50-ml Falcon tube into new BugDorm cages containing '*Ca.* L. solanacearum'-free or '*Ca.* L. solanacearum'-positive carrot plants in a greenhouse (24 to 28°C). When the caged carrot plants started showing severe decline, they were replaced with a batch of new plants. For '*Ca.* L. solanacearum' transmission assays, we used '*Ca.* L. solanacearum'-free carrot plants, germinated from a '*Ca.* L. solanacearum'-free commercial Nairobi seed lot. Plants were inoculated at the third to fourth true leaf stage using 10 psyllids collected from a '*Ca.* L. solanacearum'-positive colony, attached to one of the leaves using a small rearing bag. Psyllids were removed after 72 h or when dead (approximately 7 days) and plants were then treated with insecticides to prevent a second generation of psyllids developing. Untreated carrot plants were used as control. The appearance of carrot yellows symptoms was monitored for 8 weeks and plant tissue was sampled upon symptom appearance and tested by PCR for '*Ca.* L. solanacearum'. This experiment was performed at least three times with six or more plants each time.

Species identification of psyllids collected in Israeli carrot fields. Psyllid specimens collected from fields and reared as above were selected for species analysis (n = 13). Voucher specimens were retained after DNA extraction with DNeasy Blood & Tissue Kit (QIAGEN) using the nondestructive DNA extraction method as previously described (Sjölund et al. 2016). Vouchers of the specimens were deposited at the Natural History Museum in London. The *internal transcribed region 2* (ITS2) including partial regions of the rRNA 28S and 5.8S genes were sequenced using CAS5p8sFcm and CAS28sB1d primers. The consensus sequence from the 13 specimens were aligned with those from six *Bactericera* spp. (*Bactericera albiventris*, *B. cockerelli*, *B. silvarnis*, *B. nigricornis*, *B. tremblayi*, and *B. trigonica*) and a pairwise identity of the ITS2 region (662 to 667 bp) was conducted using the Genius software (Biomatters Ltd) to confirm species identification (Supplementary Table S2).

Fluorescence in situ hybridization of '*Ca.* L. solanacearum' in carrot plants. One-millimeter width and 15 mm longitudinal sectioning of petioles and adventitious roots from symptomatic and asymptomatic carrot plants were performed using a sterile razor blade. Cut specimens were soaked in 1 ml of fixation solution (95% ethanol and 5% acetic acid) inside a 2-ml Eppendorf tube connected to an Edwards freeze dryer (Edwards), and vacuum was applied for 1 h with 15 min interruption intervals. Fixation solution was replaced with fresh solution and specimens were kept at 4°C for 1 h. Specimens were washed briefly with 1 ml of hybridization solution (20 mM Tris, 0.01% SDS, 0.9 M NaCl, 15% formamide) before adding fresh hybridization buffer containing a final concentration of 1 ng/µl of each fluorescently-labeled probe (Lso-50S-R1Cy3 and Lso-50S-R2Cy3). Specimens were incubated in hybridization buffer with probes overnight at 4°C. The following day, specimens were washed with fresh, prewarmed (45°C) hybridization buffer using vacuum for 15 min. Then specimens were placed on glass slides, and observed under an Olympus confocal laser scanning microscope (Tokyo, Japan) using wavelengths of 543 and 570 nm for excitation and emission, respectively.

Scanning electron microscope analysis of '*Ca.* L. solanacearum' in carrot plants. Petioles of carrot leaves from symptomatic and asymptomatic plants were fixed in 4% glutaraldehyde and 4% formaldehyde prepared from paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C. Vacuum was applied for the rapid infiltration of fixative into the tissue. The fixed tissue was washed three times with 0.1 M phosphate buffer (pH 7.2). The tissue was then postfixed in 0.1% OsO4 in 0.1 M phosphate buffer (pH 7.2) for 1 h, followed by washing three times with phosphate buffer. Tissue was dehydrated in an ethanol series to 100% ethanol, then critical-point dried. Transverse sections were prepared by cutting with a razor blade, sections were mounted on aluminum stubs and sputter-coated with iridium. Observations and digital photos were made with a JEOL 7800 HRSEM (JEOL).

Diagnostic analysis of imported carrot seed lots. To determine the association of '*Ca.* L. solanacearum' with imported carrot seeds, total DNA was purified from different seed lots as detailed here. Carrot seeds (120 mg) were weighed and placed inside a Bioreba extraction bag (Bioreba AG, Switzerland). Seeds were crushed by a hammer to small pieces and then transferred into a 2-ml tube. The Nucleospin plant II kit (Machery-Nagel, Germany) was used according to manufacturer's instructions, with the exception of the first step in which the amount of PL1 buffer added to the crushed seeds was doubled (800 µl instead of 400 µl). For conventional PCR analysis, we diluted the DNA prep 50-fold and used 4 µl of the diluted DNA in a final PCR volume of 20 µl using the OA2/OI2c primers. For quantitative real-time PCR (qPCR) we used either the LsoF/HLBr primers or the RecA primer set CKC_05085 F/R. For the reference gene, we used either the carrot tubulin gene

with primers Dau c TUB f/r, or the ITS region using primers DcITS1-F/R. qPCR was performed using a StepOnePlus Real-Time PCR System (Thermo) with the following program: 95°C for 15 s, (95°C for 3 s, 60°C for 30 s) ×40, 95°C for 15 s, 60°C for 60 s and melting curve analysis with 0.3°C step increases from 60°C to 95°C with 15 s in each step. '*Ca.* L. solanacearum' infection in seeds was calculated using the ΔΔCt (cycle threshold) formula, where the carrot tubulin/ITS gene was used as a host reference gene and a noninfected carrot plant as a control. For example: '*Ca.* L. solanacearum' Ct – carrot Ct = ΔCt → ΔΔCt = ΔCt uninfected carrot – ΔCt tested sample. In the case where the ΔΔCt was higher than two, the sample was considered positive for '*Ca.* L. solanacearum'.

Seed transmission evaluation. To determine whether '*Ca.* L. solanacearum' is transmitted from seeds to seedlings, we sowed a subset of ~200 seeds from nine '*Ca.* L. solanacearum'-positive and two '*Ca.* L. solanacearum'-negative seed lots. Six seed lots (numbers 16, 23, 34, 44, 65, and 75; Table 2) were sown on October 2015 in an insect proof facility with temperatures ranging from 18 to 38°C. For this batch, three plants of each lot were tested for '*Ca.* L. solanacearum' by conventional PCR 60 days after sowing. Plants were further grown until maturity and observed for disease symptom appearance. Three additional positive seed lots (78, 79, and 90; Table 2) were sown in a temperature-controlled greenhouse (24 to 28°C). Six seedlings of each lot were sampled at 35, 60, and 95 days after sowing and tested by qPCR for '*Ca.* L. solanacearum'. Plants were kept in the greenhouse until 120 days-old and monitored for disease symptom appearance.

Statistical analysis. The statistical significance of differences in psyllid capture numbers in field A versus field B was calculated using the JMP software (SAS Institute, Cary, NC) and the Welch's unequal variances *t* test, as standard deviations were not equal. The alpha level for statistical significance was 0.05.

RESULTS

Association of '*Ca.* L. solanacearum' with carrot yellows symptoms in Israel. To determine '*Ca.* L. solanacearum' association with carrot yellows in Israel, symptomatic and asymptomatic carrot plants were collected for PCR analysis from 18 different

TABLE 2. Carrot seed lots tested for '*Candidatus* Liberibacter solanacearum' using qPCR

Number[a]	Date tested	Cultivar	Seed lot production year	'*Ca.* L. solanacearum' presence[b]	Number[a]	Date tested	Cultivar	Seed lot production year	'*Ca.* L. solanacearum' presence[b]
1	6.7.2014	Nairobi	2014	–	47	1.8.2014	New castle	N/A	–
2	6.7.2014	Nairobi	2013	–	48	1.8.2014	Vac62	N/A	+
3	6.7.2014	Nairobi	2013	–	49	1.8.2014	Maymee	N/A	–
4	6.7.2014	Nairobi	2013	–	50	8.7.2014	Zetor RZ F1	2006	N/D
5	6.7.2014	Nairobi	2013	–	51	8.7.2014	Interceptor F1	2006	N/D
6	6.7.2014	Nairobi	2013	–	52	8.7.2014	Nairobi F1	2008	+
7	6.7.2014	Nairobi	2013	–	53	8.7.2014	Newark F1	2010	+
8	6.7.2014	Nairobi	2013	–	54	8.7.2014	Nairobi F1	2011	+
9	6.7.2014	Nairobi	2013	–	55	8.7.2014	Musico (Vac59 F1)	2013	+
10	6.7.2014	Nairobi	2013	–	56	8.7.2014	Chantenay	2014	–
11	6.7.2014	Nairobi	2013	–	57	13.7.2014	Favor hybrid F1	1989	+
12	6.7.2014	Nairobi	2013	–	58	13.7.2014	CLX 3110-F1	1995	–
13	6.7.2014	Nairobi	2013	–	59	13.7.2014	Predor F1	1995	–
14	6.7.2014	Nairobi	2013	–	60	13.7.2014	Nickerson Berton F1	1995	–
15	2.7.2014	Maestro	2013	+	61	13.7.2014	Starca F1	1993/94	+
16*	2.7.2014	Maestro	2013	+	62	13.7.2014	Primo F1	1995	
17	2.7.2014	Maestro F1	2014	+	63	13.7.2014	Nairobi F1	1994	+
18	2.7.2014	Maestro F1	2014	–	64	13.7.2014	Concerto F1	2005	+
19	2.7.2014	Maestro F1	2014	+	65*	13.9.2014	Exelso F1	2007	+
20	2.7.2014	Maestro F1	2013	N/D	66	13.9.2014	Asnastasia	2007	–
21	2.7.2014	Newark	2013	–	67	13.9.2014	Alessia	2007	–
22	17.7.2014	CLX31633	2014	–	68	13.9.2014	Niagana F1	2008	–
23*	17.7.2014	CLX31695	2014	+	69	13.9.2014	Kagan F1	2008	–
24	17.7.2014	CLX31810	2014	+	70	13.9.2014	Kamasan F1	2008	–
25	17.7.2014	Chantenay	2013	–	71	13.9.2014	Newhall F1	2010	–
26	17.7.2014	Concerto	3013	–	72	13.9.2014	Siroco F1 (Vac30)	N/A	+
27	17.7.2014	Concerto	2014	+	73	13.9.2014	Zetor RZ F1	N/A	–
28	17.7.2014	Concerto	2014	+	74	13.9.2014	Dordogne	N/A	
29	17.7.2014	Concerto F1	2014	-	75*	13.9.2014	Vac 44	N/A	+
30	17.7.2014	Concerto F1	2014	+	76	13.9.2014	Elegance	N/A	–
31	17.7.2014	Dordogne	2014	–	77	13.9.2014	Nerja F1	2013	–
32	17.7.2014	Dordogne	2014	–	78*	19.4.2016	Nantes	2015	+
33	17.7.2014	Evora	2013	–	79*	19.4.2016	Amsterdam	2015	+
34*	17.7.2014	Excelso	2013	+	80	19.4.2016	Nostel	2015	–
35	3.8.2014	Excelo F1	2014	–	81	19.4.2016	Nairobi	2015	–
36	3.8.2014	Excelo F1	2014	N/D	82	19.4.2016	Newcastle	2015	–
37	3.8.2014	HCM	2014	+	83	19.4.2016	Honeysnax	2015	–
38	3.8.2014	Heneysnax	2013	N/D	84	19.4.2016	Yellow bunch	2015	–
39	3.8.2014	Jerada	2013	–	85	19.4.2016	Nairobi	2015	–
40	3.8.2014	Maverick	2013	–	86	19.4.2016	Maverick	2015	–
41	3.8.2014	Maxi	2013	+	87	19.4.2016	CR10348	2015	–
42	3.8.2014	Mokum	2013	–	88	19.4.2016	Salto	2015	–
43	3.8.2014	Morelia	2014	N/D	89	19.4.2016	Romance	2015	–
44*	3.8.2014	Musico	2013	+	90*	19.4.2016	Soprano	2015	+
45	3.8.2014	NUN8806	2013	N/D	91	30.11.2015	Maestro	2015	+
46	1.8.2014	Nairobi	N/A	–					

[a] Seed lots tested for seed transmission are indicated by an asterisks (*).
[b] N/D, not determined (qPCR results were inconclusive).

commercial carrot fields (Fig. 1; Table 1). Diseased plants showing symptoms of axillary shoot proliferation and some yellow discoloration of leaves (Fig. 1) were observed in 16 out of 18 fields. '*Ca.* L. solanacearum' was detected in the majority of symptomatic plants (57/59) and not among the asymptomatic plants (0/10). None of these samples tested positive for either phytoplasma or spiroplasma.

Haplotyping analysis of '*Ca.* L. solanacearum' from Israeli fields. All the haplotyped carrots samples (n = 20) were most similar to haplotype D, previously reported in Spain, Canary Islands, and Africa in carrot and celery (Table 1). Interestingly, all sequenced samples had one mismatch compared with the reported sequences of haplotype D (Hajri et al. 2017; IPPC-FAO 2017; Nelson et al. 2013). This is a previously unreported SNP at position 1,119 (G→A) of the 16S sequence. All the tested psyllid samples (n = 14) had identical sequences to those found in carrots. Sequences representing the haplotype found in Israel were deposited as GenBank accession numbers MG657031 (16S) and MG657027 (50S).

Temporal distribution of psyllids in carrot fields in southern Israel, association with '*Ca.* L. solanacearum', and disease incidence estimation. Psyllids were captured on yellow sticky traps from the first sampling point (31 January 2016) in both fields A and B, when seedlings were still small (two to three true leaves) (Fig. 2A and B). Psyllid capture rate gradually rose over time in both fields, peaking at around 5 weeks before harvest and then declining (Fig. 2C). Significantly higher numbers of psyllids were captured on sticky traps in the organic field A versus the conventional field B at three time points (28 February 2016, P = 0.0256; 14 March 2016, P = 0.0124; and 18 April 2016, P = 0.0099) (Fig. 2C). The maximal average number of captured psyllids in the two fields was 330 psyllids per trap per day in field A (18 April 2016) and 120 psyllids per trap per day in field B (16 May 2016). These values were significantly different (P = 0.016). Only few leafhopper vectors of phytoplasma were captured on traps throughout the survey. When examined for the presence of '*Ca.* L. solanacearum', psyllids from both fields were found to be '*Ca.* L. solanacearum'-positive from the first sampling time-point and throughout the course of the survey, except for one time-point in each field (18 April 2016 in field A and 2 May 2016 in field B). Carrots showing typical axillary shoot proliferation symptoms were first observed a month before harvest in both fields. Disease incidence was evaluated a week before harvest and was found to be significantly higher in field A (18%) than in field B (4%).

Determining psyllid species. The sequences of the ITS2 region from the psyllid specimens collected in Israel displayed a pairwise identity of 99.8%. When compared with five other *B. trigonica* specimens collected elsewhere, the pairwise identity was 99.7%. This high identity was also supported by morphological identifications using classification keys (Hodkinson 1981). The *B. trigonica* consensus sequence had the highest pairwise identity with *B. tremblayi* (94.2%) and the lowest with *B. cockerelli* (81.5%). It should be noted that, to date, there are no entries for *B. trigonica* in the NCBI database. Photos of greenhouse-reared *B. trigonica* adults, nymphs, and eggs deposited on carrot leaves are shown in Supplementary Fig. S1.

Transmission of '*Ca.* L. solanacearum' by psyllids. *B. trigonica* collected from the field and reared on '*Ca.* L. solanacearum'-positive carrot plants were used to test '*Ca.* L. solanacearum' transmission. All inoculated carrot plants showed axillary shoot proliferation 30 to 45 days postinoculation when grown at 24 to 28°C (Fig. 3A and B). Later, plants developed more substantial symptoms, which included leaf curling and discoloration (Fig. 3C) and extensive proliferation of axillary shoots (Fig. 3D). Symptomatic plants were positive for '*Ca.* L. solanacearum', indicating transmission of '*Ca.* L. solanacearum' by *B. trigonica* specimens from Israel. Uninoculated control plants did not develop shoot proliferation symptoms (Fig. 3D) and were PCR-negative for '*Ca.* L. solanacearum'.

Fluorescent in situ hybridization and scanning electron microscopy of '*Ca.* L. solanacearum' in infected carrot tissue. Fluorescent in situ hybridization (FISH) analysis, showed that '*Ca.* L. solanacearum' was present along phloem sieve elements of leaf petioles of symptomatic carrot plants (Fig. 4A and B). Red fluorescence, indicative of '*Ca.* L. solanacearum' cells, was restricted to sieve elements and was not seen in leaf petiole preparations from asymptomatic control carrot plants (Fig. 4C). '*Ca.* L. solanacearum' detection rate in leaf petioles was on average 1 out of 30 preparations. In lateral root preparations, the detection rate of '*Ca.* L. solanacearum' was even lower (1:55).

Scanning electron microscopy (SEM) observations of infected leaf petiole samples revealed bacterium-like organisms (BLOs) ranging in length from 1.5 to 3 µm long and 0.1 to 0.25 µm in diameter (Fig. 5). These BLOs were detected in the vascular tissue of petioles in close proximity to xylem vessels. BLOs were not seen in uninfected control carrot plants. In some instances, the plant cell plasma membrane appeared to have detached from the cell wall and collapse inward to form cylindrical structures. BLOs were observed from within the collapsed cylindrical membrane structures, suggesting that they are intracellularly localized (Fig. 5B and D). Additionally, some plant cells were filled with a fibrous structure that appeared to have clogged the cell or vascular element (Supplementary Fig. S2). BLOs were not seen in uninfected control carrot plants.

Association of '*Ca.* L. solanacearum' with carrot seeds and evaluation of seed transmission. To determine whether '*Ca.* L. solanacearum' was imported to Israel via contaminated seed, several seed lots of different sources, varieties and production years were tested. Initially, samples were tested by conventional PCR and all, except one, were found to be negative for '*Ca.* L. solanacearum'. However, with the use of qPCR, 28 of 91 seed lots tested positive for the presence of '*Ca.* L. solanacearum' (31%), as described in Table 2. The 16S sequence of the one sample that could be amplified by conventional PCR (number 91 in Table 2), most resembled that of haplotype E, found in celery and carrot in Spain (Teresani et al. 2014). This sample had an additional SNP (A→G) at location 115 of the 16S sequence compared with the reported haplotype E sequence. Seeds from eight '*Ca.* L. solanacearum'-positive lots were sown to test for seed transmission. None of the germinated seedlings developed typical yellows symptoms such as induced shoot branching or leaf discoloration. Additionally, all the seedlings (n = 72) that were sampled for PCR/qPCR analyses throughout the cultivation of these plants, tested negative for '*Ca.* L. solanacearum'.

DISCUSSION

Characterization of a disease caused by, or associated with, obligate, nonculturable pathogens poses certain difficulties. The main obstacle with such pathogens is the difficulty of completing Koch's postulates and to determine with certainty the causal agent of a disease. Such is the case with yellows disease of carrot. This study describes the presence and association of '*Ca.* L. solanacearum' with carrot yellows in Israel and illustrates the complications of studying phytopathogenic fastidious prokaryotes.

Carrot yellows disease was first noticed in Israel in 1995 and reported by Orenstein et al. (1999). In their work, a sample of 500 plants, displaying proliferation of axillary shoots and secondary hairy roots symptoms, were all positive for phytoplasma by PCR. Due to this overwhelming association and due to previous reports linking phytoplasma with carrot yellows symptoms (Ivanoff and Ewart 1944; Gabelman et al. 1994), phytoplasma was considered as the etiological agent of carrot yellows in Israel.

Five to six years later, a survey focusing on identifying potential leafhopper vectors of phytoplasma in Israeli carrot fields was conducted. Vacuum sampling did not retrieve any leafhopper vectors and sticky traps had a very low capture rate of leafhopper

vectors during the whole season (maximum ~5 per day per trap) (Weintraub and Orenstein 2004). Despite the low abundance of phytoplasma leafhopper vectors in that survey, disease incidence in the two fields was 12 and 21%. Furthermore, the majority of the symptomatic plants collected during that study tested negative for phytoplasma by PCR. The following observations may suggest the involvement of another biotic factor with carrot yellows: (i) low incidence of phytoplasma vectors relative to disease incidence

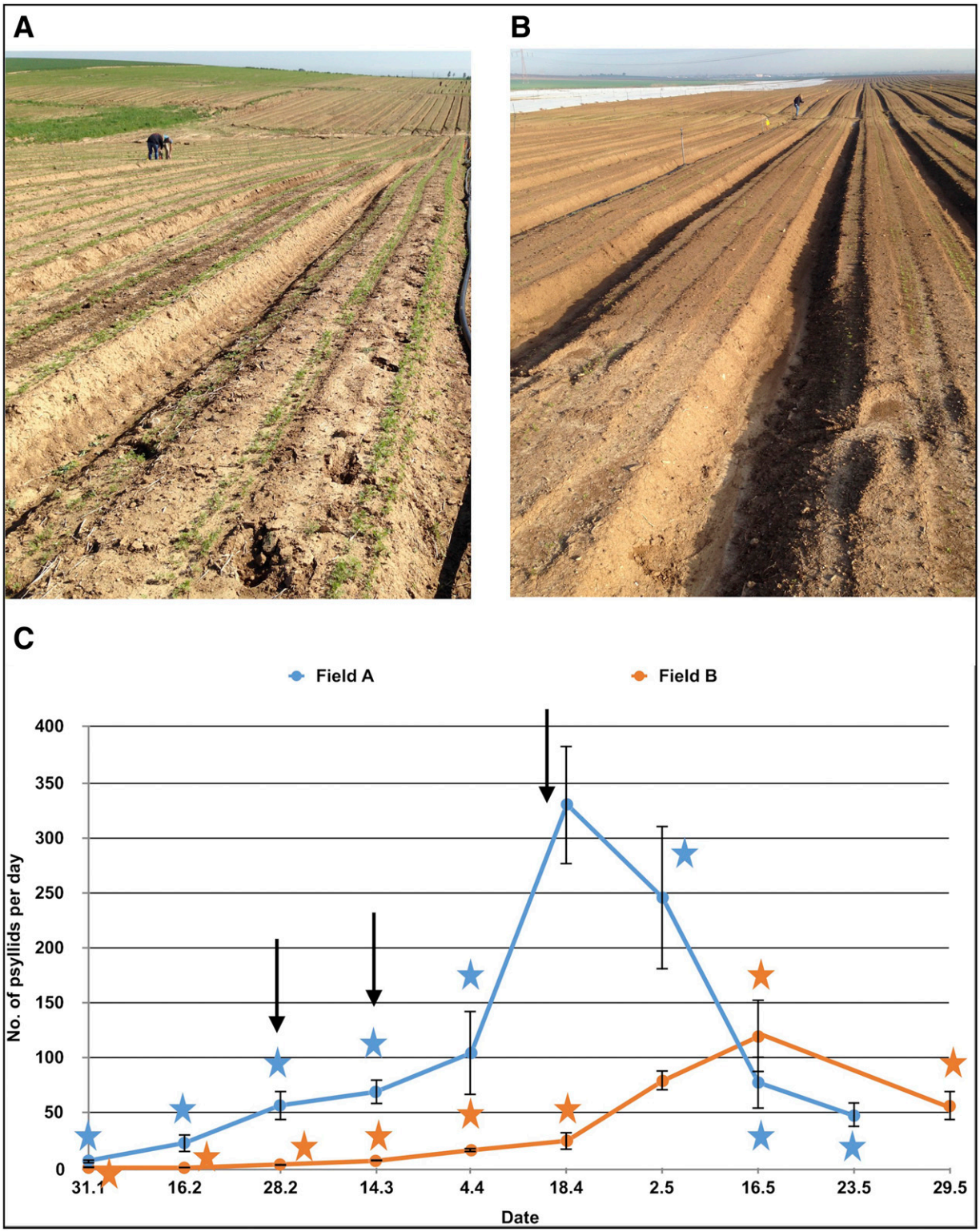

Fig. 2. Field survey for psyllid abundance, dynamics and association with '*Candidatus* Liberibacter solanacearum'. **A,** A photo of field A, an organic carrot field, taken on 17.1.2016 upon survey initiation, 7 weeks after sowing. **B,** A photo of field B, a conventional carrot field, taken on 17.1.2016 upon survey initiation, 5 weeks after sowing. **C,** Mean and standard error of psyllids caught per day per trap, from four yellow sticky traps spread in each field and collected at approximately 2-week intervals. Final sampling point was made a week before harvest. Star indicates sampling points where '*Ca.* L. solanacearum' was detected in at least one trap in the corresponding field. Dates in which a statistical ($P < 0.05$) difference in psyllid number between the two fields was present are marked with a black arrow.

and (ii) a significant proportion of symptomatic plants that tested negative for phytoplasma. In a more recent study, Gera et al. (2011), collected 50 carrot plants showing typical yellows symptoms from different fields and examined them for association with phytoplasma and spiroplasma by nested-PCR (Gera et al. 2011). Here too, a very large proportion (80%) of the tested plants were negative for phytoplasma, while 56% were infected with another pathogen from the Mollicutes group *Spiroplasma citri*. Still, 24% of the total symptomatic plants did not test positive for either phytoplasma or spiroplasma. Overall, these three past studies (1995 to 2011), which were all conducted in the same carrot growing region as this study was, revealed a trend of decreased association of phytoplasma with carrot yellows symptoms in Israel (Table 3).

Our study shows this trend to have continued. In the years 2015 to 2016 none of the symptomatic plants tested were positive for phytoplasma, while nearly 100% were positive for '*Ca.* L. solanacearum'. We propose two possible scenarios that may have led to the shift in pathogen association with carrot yellows. The first is that '*Ca.* L. solanacearum' was only recently introduced into Israeli carrot fields by either infected plant material, psyllid migration or other means. The local prevalence of an efficient psyllid vector for '*Ca.* L. solanacearum', *B. trigonica*, in the same area, facilitated the wide dissemination of '*Ca.* L. solanacearum' to all carrot growing regions in Israel. The dominance of *B. trigonica*, which feed and propagate on carrots, over the occasionally hosted leafhopper vectors of Mollicutes, may subsequently have led to the dominance of '*Ca.* L. solanacearum' over phytoplasma in Israel carrot fields. A second possible scenario, is that '*Ca.* L. solanacearum' was already present in Israel a long time ago, and that the cause of its current dominance was a change in the relative abundance of vector populations occurring in the past decade or two. For many years, carrot growers in Israel and around the world linked carrot yellows symptoms with leafhoppers and phytoplasma (Wally et al. 2004). Consequently, insecticide spraying regimes were aimed mainly to limit the leafhopper populations. It is possible that these management practices allowed other, nontarget insects to thrive in carrot fields and increase their relative abundance. A survey conducted in Israel by a private company (A. Ovadia and O. Bahar, Agronomia-Agricultural Services (2001) Ltd., *personal communication*), revealed that in 2009, the psyllid populations in tested carrot fields were one to two orders of magnitude greater than that of each of the possible phytoplasma leafhopper vectors. Hence, increasing psyllid populations in recent years may have resulted in a more efficient dissemination of their associated microbial pathogen, '*Ca.* L. solanacearum', which subsequently became the dominant bacterial pathogen in carrot fields.

Disease symptoms were first noticed about a month before harvest. However, '*Ca.* L. solanacearum'-positive psyllids were recorded in the fields three months earlier. Our '*Ca.* L. solanacearum' psyllid transmission experiments show that under controlled conditions, symptoms develop as early as 30 days postinoculation. It is possible that the low temperatures of January to April suppress '*Ca.* L. solanacearum' transmission, infection, and symptom development. It is also possible, that the physiological age of the young carrot plants does not support symptom development and these can only appear at a later physiological stage.

The peak in psyllid population was also recorded about a month before harvest in each of the tested fields. Considering the time from inoculation to symptoms development, and the early presence of infected psyllids in the field, we speculate that the majority of the symptomatic plants that were recorded a week before harvest, were not a result of inoculation that occurred during the psyllid peak but prior to it. Further, we speculate that if plants were allowed to grow further in the fields, for an additional month, disease incidence would have grown substantially, as a result of the development of symptoms in plants that were inoculated during the psyllid peak.

The predominant '*Ca.* L. solanacearum' haplotype found in symptomatic carrots and in psyllids collected in Israel during this study was most similar to haplotype D. A previously unreported SNP at position 1119 of the 16S sequence was found. When we examined previously deposited sequences of haplotype D, we noticed that while some accession sequences cover the 1119 position (and do not contain an SNP) others do not (Hajri et al. 2017; Monger and Jeffries 2017). This could be a result of the close proximity of this SNP to the end of the PCR product (~49 bp before

Fig. 3. '*Candidatus* Liberibacter solanacearum' transmission by *Bactericera trigonica.* **A,** Carrot plants were inoculated by attaching a rearing bag containing 10 *B. trigonica* psyllids collected from a '*Ca.* L. solanacearum'-positive colony to one carrot leaf for 72 h. **B,** Typical axillary shoot proliferation was observed 30 to 45 days postinoculation (circled). **C,** Leaf curling and discoloration observed under greenhouse inoculation conditions. **D,** A representative example of symptomatic versus asymptomatic carrot plants following psyllid inoculation. On the right, inoculated plants showing extensive axillary shoot proliferation and on the left asymptomatic control plants. Photo was taken 60 days postinoculation.

the end), especially if the PCR product was directly sequenced and not cloned beforehand. Interestingly, we found one accession (KY624596), which was deposited and designated as haplotype D (Monger and Jeffries 2017), but contained the same SNP at position 1119. This indicates that this SNP is also present in the D haplotype in other regions of the world. This finding warrants a more careful examination of the 16S sequence in both past and future examinations of '*Ca.* L. solanacearum' samples. Reanalysis of previous samples along with careful examination of new samples will enable to determine whether this SNP is common to all haplotype D sequences, or whether haplotype D may have two possible nucleotides (A or G) at this position.

The origin of '*Ca.* L. solanacearum' in Israel is unknown. Bertolini et al. (2015) reported that '*Ca.* L. solanacearum' can be detected in carrot seeds, transmitted to the emerging seedling, and subsequently cause disease symptoms. In the present work, '*Ca.* L. solanacearum' was detected in a significant percentage of imported carrot seed lots, including seed lots from over 20 years ago. This indicates that '*Ca.* L. solanacearum' was associated with carrot cultivation at least 20 years ago, as suggested by another recent study (Monger and Jeffries 2017). Unfortunately, due to the inability to PCR-amplify sufficient DNA from seed samples, we could only determine the haplotype of one seed lot. The haplotype identified was most similar to haplotype E with an addition of a previously unreported SNP at position 115. Teresani et al. (2014), who first reported haplotype E, deposited two accessions of this haplotype; one from celery (KF737346) and one from carrot (KF737348). While the accession from celery does not cover position 115, the accession from carrot does, and it also contains the same SNP we report here. Hence, it appears that the 115 A→G SNP was previously overlooked and should be added to haplotype E.

Israel imports carrot seeds from multiple countries known to have '*Ca.* L. solanacearum'. Therefore, it is very likely that other haplotypes such as haplotype C, D, and E are also associated with imported seed lots, as recently demonstrated (Monger and Jeffries 2017). Our seed transmission experiments suggest that '*Ca.* L. solanacearum' is not effectively transmitted from seed to seedlings. This result is in agreement with the fact that only one haplotype was identified in Israel and in accordance with a recent study reporting that '*Ca.* L. solanacearum' is not seed-transmitted under laboratory conditions (Loiseau et al. 2017). Since carrot is grown in Israel throughout the year, '*Ca.* L. solanacearum' and psyllids can perpetuate from one year to another without the need for reintroduction of the pathogen or vector every year. This suggests that in Israel, seed transmission, if occurs at all, is not a major contributor to carrot yellows epidemics. Taken together, these results provide further support for the hypothesis that the change in disease etiology may be a result of vector population shift, rather than a recent introduction of a new pathogen.

The three known haplotypes infecting carrot (C, D, and E) can be divided into the northerly European (Sweden, Norway, Finland, and Germany) haplotype C transmitted by *T. apicalis* and the southerly European/Mediterranean (Spain, Canary Islands, Africa, and Israel) haplotypes D and E transmitted by *B. trigonica* (Alfaro-Fernandez et al. 2012a, b; Hajri et al. 2017; Munyaneza et al. 2010, 2012, 2015; Tahzima et al. 2014). The finding of a variant of haplotype D in Israel is therefore not surprising, and falls in line with this geographical separation. It is currently unknown what determines the geographical separation of haplotypes; however, vector distribution and pathogen-vector compatibility may be drivers of this phenomenon.

Further distinctions between the pathology of '*Ca.* L. solanacearum'-infected carrots in Israel and in north European countries are reflected in a slightly different symptomology. While leaf curling and purple discoloration are common symptoms in carrot fields in northern Europe (Munyaneza et al. 2010; Nissinen et al. 2014; Wang et al. 2017), these were rarely observed in our field surveys. Nevertheless, under greenhouse conditions, when both bacterial and insect load can be very high, both leaf curling and purple discoloration can be observed. On the other hand, the most characteristic symptom indicative of '*Ca.* L. solanacearum' infection in Israel is the appearance of axillary shoot proliferation (i.e., witch's-broom). Shoot proliferation appears to be more common around the Mediterranean basin (Bertolini et al. 2015; Tahzima et al. 2014; Wang et al. 2017) while in north European countries, where the C haplotype is dominant, this symptom is absent (Munyaneza et al. 2010; Nissinen et al. 2014). Additionally, carrot yellows infection in northern Europe leads to reduced tap root size and overall stunting of the plant (Munyaneza et al. 2010); however, in Israel, the infected plants have bigger tap roots and have far more foliage than healthy plants (Weintraub and Orenstein 2004).

FISH analysis with newly designed probes, specifically detected '*Ca.* L. solanacearum' in phloem sieve elements of leaf petioles from symptomatic carrots. The relatively low detection rate of '*Ca.* L. solanacearum' in symptomatic plants using this method supports

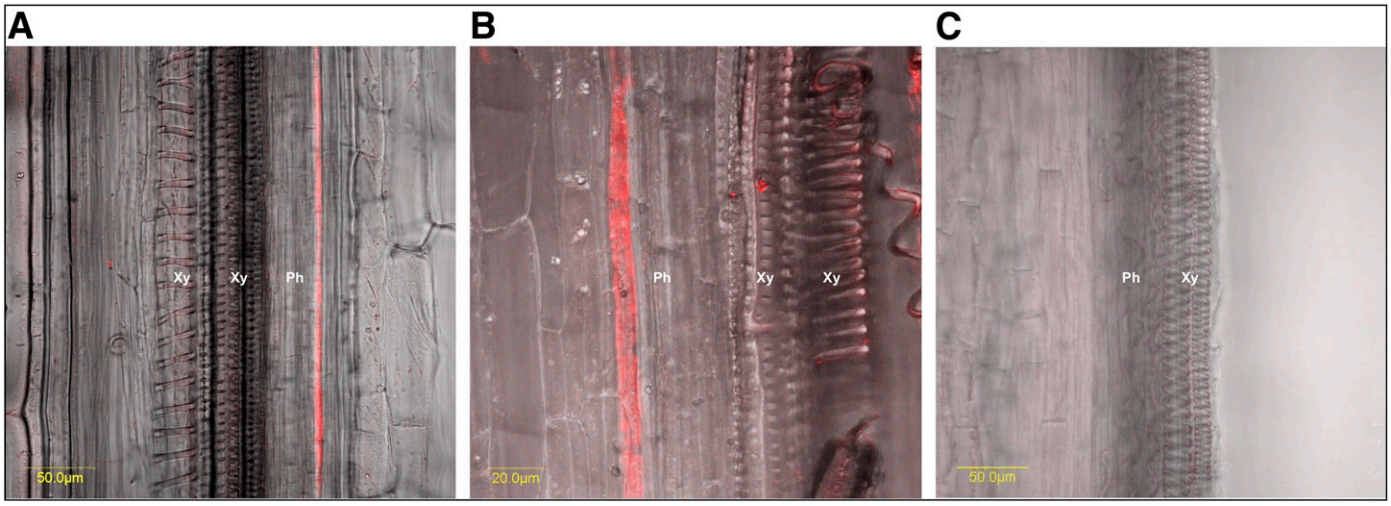

Fig. 4. Fluorescent in situ hybridization analysis of carrot leaf petioles. Longitudinal sectioning of **A and B,** symptomatic and **C,** asymptomatic carrot leaf petioles were hybridized with fluorescently labeled '*Candidatus* Liberibacter solanacearum'-specific probes and visualized using a confocal microscope. Red fluorescence, indicative of '*Ca.* L. solanacearum' was detected in infected petioles along phloem sieve elements and was not detected in asymptomatic plants. Size bars are located at the bottom left corner of each image. Xy, xylem; Ph, phloem.

the notion that '*Ca.* L. spp.' distribution throughout the plant is intermittent (Garnier and Bové 1983). SEM observations revealed the presence of elongated BLOs with similar dimensions to those observed in previous reports (Cicero et al. 2016; Garnier and Bové 1983; Liefting et al. 2009a, b). BLOs were located in the vascular tissue of infected plants and in many instances appeared enfolded in the plant cell plasma membrane. This further supports the claim that '*Ca.* L. solanacearum' colonizes the intracellular milieu. We also observed fibrous structures clogging few of the plant cells from symptomatic plants. Similar structures were observed by Etxeberria et al. (2009) in citrus leaves; however, in that report these cells originated from control plants.

Our results add further emphasis to the vast spread of '*Ca.* L. solanacearum' around the world. Together with previous studies, our results display a fairly rapid change in disease etiology which occurred in carrot yellows in Israel in the last two decades, and may have occurred (or will occur) in other locations around the world. Further studies on this complex system would help determine how the various factors contribute to the change in carrot yellows disease etiology and what governs haplotype distribution and host range.

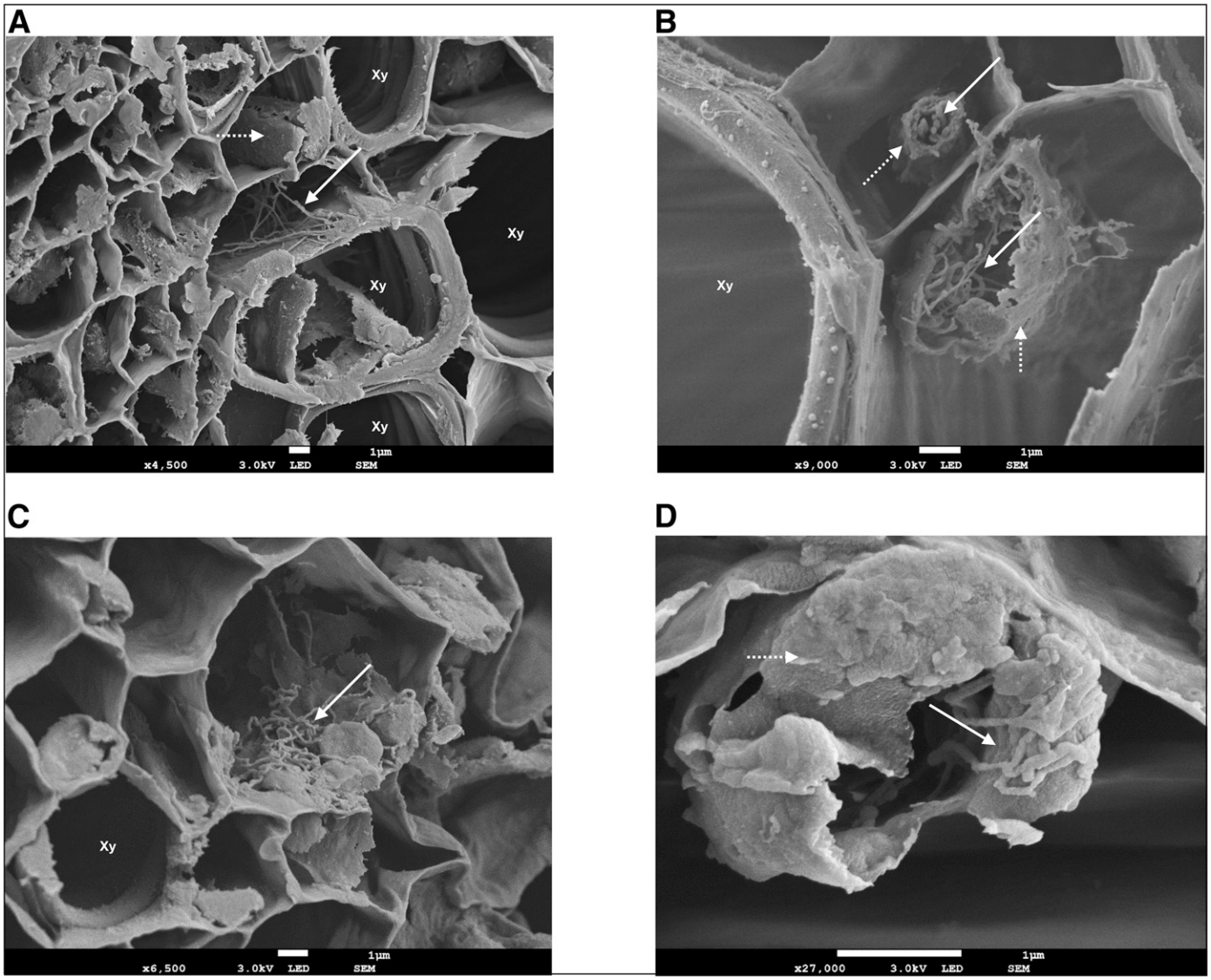

Fig. 5. Scanning electron micrographs of '*Candidatus* Liberibacter solanacearum'-positive symptomatic carrot leaf petioles. **A to D,** Scanning electron micrographs of infected carrot petiole cross sections show bacterium-like organisms (BLOs) indicated by white arrows. **A, B, and D**, Cylindrical membrane structure that appear to have detached from the cell wall and collapsed inwards are indicated by dashed white arrows. **B and D,** BLOs can be seen from within the cylindrical membrane structure. Size bar (1 µm) and magnification value are indicated at the bottom of each image. Xy, xylem.

TABLE 3. Association of phytoplasma, spiroplasma and '*Candidatus* Liberibacter solanacearum' with carrot yellows symptoms in Israel carrot fields from 1995 to 2016[a]

Pathogen/years	Phytoplasma (%)	Spiroplasma (%)	'*Ca.* L. solanacearum' (%)	Undetermined (%)	Sample size	Reference
1995–1997	100	N/D	N/D	0	>500	Orenstein et al. 1999
1999–2000	47	N/D	N/D	53	15	Weintraub and Orenstein 2004
2009–2010	20	54	N/D	26	50	Gera et al. 2011
2015–2016	0	0	97	3	59	Current study

[a] N/D, not determined.

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