'Candidatus *Liberibacter solanacearum*' haplotypes D and E in carrot plants and seeds in Tunisia

Soukaina Ben Othmen, Félix E. Morán, Inmaculada Navarro, Silvia Barbé, Carmen Martínez, Ester Marco-Noales, Brahim Chermiti, et al.

ONLINE FIRST

Springer

Springer

CrossMark

'Candidatus Liberibacter solanacearum' haplotypes D and E in carrot plants and seeds in Tunisia

Soukaina Ben Othmen[1] · Félix E. Morán[2] · Inmaculada Navarro[2] · Silvia Barbé[2] · Carmen Martínez[2] · Ester Marco-Noales[2] · Brahim Chermiti[1] · María M. López[2]

Abstract

Vegetative disorders similar to those associated with the presence of *'Candidatus* Liberibacter solanacearum*'* (CaLsol) were observed in carrot plants in Kairouan, Tunisia from 2014 to 2016. Symptoms including leaf curling, yellowing, bronze and purplish discoloration, stunting of plants and roots, and proliferation of secondary roots, affected 20 to 40% of the carrots in some plots. In order to determine if these symptoms were associated with the presence of CaLsol, and/or *'Ca.* Phytoplasma spp.*'* and/or *Spiroplasma citri*, real-time PCR analyses were conducted using specific primers for these pathogens. CaLsol was detected for the first time in Tunisia and for the first time its haplotypes D and E were detected co-infecting a carrot plant. Furthermore, three samples of carrot seed produced in Kairouan tested positive for the haplotype D, showing a high percentage (35 to 63%) of viable bacterial cells after treatment with propidium monoazide. However, all the tests were negative for *'Ca.* Phytoplasma' spp. as well as for *S. citri*. The results highlight that several CaLsol haplotypes are emerging carrot pathogens in new areas.

Keywords *'Ca.* Phytoplasma' spp. · *Spiroplasma citri* · *Bactericera* sp. · real-time PCR

Introduction

Carrot is one of the most important crops in Tunisia. The average production is about 215,000 tons per year, from an area of 6319 ha (http://www.agriculture.tn, dates from 2015). The main production area of approximately 1730 ha is located in the centre of the country (Governorate of Sidi Bouzid). In 2014, 2015 and 2016, symptoms similar to those associated with the presence of *'Ca.* L. solanacearum' (CaLsol) were observed in the Governorates of Kairouan and Sidi Bouzid and, to a lesser extent, in several other carrot-growing areas such as Fahes (Governorate of Zaghouan) and Chott-Mariem (Governorate of Sousse). According to preliminary surveys, there were around 20% symptomatic plants during 2014, that

increased to 40% in 2015 and 2016. Symptoms were observed on plants of cultivars grown from local seeds as well as on commercial hybrids such as Maestro, Concerto and Olympus, imported from other continents.

CaLsol belongs to the order Rhizobiales of the α-2-subdivision of the Gram-negative Proteobacteria. Species in this bacterial genus alternate hosts in their life cycle between the plant phloem and the psyllid vector hemolymph (Liefting et al. 2009b; Lin et al. 2009) and, with the exception of the new species *Liberibacter crescens* (Fagen et al. 2014), have not yet been cultivated in axenic medium.

CaLsol is associated with various vegetative disorders in solanaceous crops including eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), potato (*S. tuberosum*), tamarillo (*S. betaceum*), tobacco (*Nicotiana tabacum*), tomatillo (*Physalis peruviana*), tomato (*S. lycopersicum*), wolfberry (*Lycium barbarum*), and weeds such as black nightshade (*S. ptychanthum*) and silver leaf nightshade (*S. elaeagnifolium*) (EPPO 2013; Haapalainen 2014). Symptoms in solanaceous plants are similar to those associated with a reduction in nutrient supply and also to those caused by *'Ca.* Phytoplasma spp.*'* and include: stunting, chlorosis and purpling of the foliage, upward rolling of the leaves, enlarged nodes, axillary branches or aerial tubers and production of poor quality fruits (Munyaneza et al.

S. Ben Othmen and F.E. Morán are first authors

✉ Félix E. Morán
  moran_fel@gva.es

1 High Agronomic Institute of Chott-Mariem, University of Sousse, 4042 Chott-Mariem, Tunisia

2 Instituto Valenciano de Investigaciones Agrarias (IVIA), CV-315 km 10.7, 46113 Moncada, Valencia, Spain

2007a, 2007b; Liefting et al. 2009a; Secor et al. 2009; Crosslin et al. 2010; Munyaneza et al. 2010a; Munyaneza 2012). CaLsol has also been associated with vegetative disorders in species of the family Apiaceae, including carrots (*Daucus carota*), celery (*Apium graveolens*) and parsnip (*Pastinaca sativa*) (EPPO 2013; Teresani et al. 2014; Alfaro-Fernández et al. 2011). In carrots, it has been reported that CaLsol symptoms also resemble those caused by a leafhopper-transmitted '*Ca.* Phytoplasma spp.' and *S. citri* (Font et al. 1999; Lee et al. 2003; Duduk et al. 2007, 2008; Cebrián et al. 2010; Abed et al. 2011; Munyaneza et al. 2011).

Five haplotypes of CaLsol have been described (A, B, C, D, and E) (Nelson et al. 2011, 2012; Teresani et al. 2014). Haplotypes A and B are associated with diseases of potatoes and other solanaceous crops (haplotypes A and B in America and only haplotype A in New Zealand), and are transmitted by the psyllid *Bactericera cockerelli*. In Europe, haplotypes C, D and E are associated with carrot, celery and parsnip disorders (Nelson et al. 2012; Teresani et al. 2014). Haplotype C has been found in Finland and is transmitted by the psyllid *Trioza apicalis* (Munyaneza et al. 2014; Haapalainen et al. 2016), whereas haplotypes D and E have been found in Spain, France and Morocco (Teresani et al. 2014; Loiseau et al. 2014; Tahzima et al. 2014), and the main vector seems to be *B. trigonica*, at least in Spain (Teresani et al. 2015). Given that the symptoms observed in Tunisian carrots resembled those associated with CaLsol, a set of analyses on plants as well as on local seeds for CaLsol detection, was carried out. In addition, since '*Ca.* Phytoplasma spp.' and *Spiroplasma citri* have also been reported affecting carrot with similar symptoms (Alfaro-Fernández et al. 2012), the detection of these two pathogens was also included.

Materials and methods

Plant extract preparation Inspections and samplings were carried out in carrot fields from 2014 to 2016 at Kairouan (Tunisia) (Fig. 1). General stunting, yellowing, purpling (Fig. 2a) and leaf curling (Fig. 2b), as well as shoot proliferation and hairy secondary roots (Fig. 2c) were frequently observed. A representative selection of symptomatic plants was collected. Mature leaf fragments were preserved in cryotubes with liquid nitrogen, and were sent in dry ice to the Bacteriology Laboratory of IVIA (Spain), where the samples were stored at −20 °C before analysis.

Plant material (1 g/plant) was ground in plastic bags (Bioreba, Switzerland) in 1:5 to 10 ml (w:v) of extraction buffer with phosphate-buffered saline (PBS) (NaCl, 8 g l^{-1}; NaH$_2$-PO$_4$.2H$_2$O, 0.4 g l^{-1}; Na$_2$HPO$_4$.12H$_2$O, 2.7 g l^{-1}; pH 7.2) using a Homex 6 homogenizer (Bioreba, Switzerland). Then, 1 ml of each leaf extract was stored at −80 °C until use.

Seed extract preparation Seed samples were prepared and analysed according to Bertolini et al. (2014b): 1 g (approximately 450 seeds) per lot was washed by shaking for 30 min in a tube containing 50 ml washing buffer (distilled water with 0.5% Triton X-100). Then, washed seeds were placed into plastic bags with 10 ml extraction buffer plus 0.2% sodium diethyl dithiocarbamate and 2% PVP-10. The seeds were slightly crushed with a hammer, and 1 ml of the extract from each seed lot was stored at −80 °C until use. Three seed lots of a local cultivar from the area of Rakada were analysed in 2015 and from Zaafrana in 2016.

Direct sample preparation without DNA purification (spot procedure) Freshly prepared extracts were immobilized on membranes (pieces of Whatman 3 MM filter paper–Healthcare, Europe) on which 5 μl of crude extract were loaded. Then, 100 μl of distilled water were added to each membrane in an Eppendorf tube and vortexed to extract the target DNA (Bertolini et al. 2014a; Teresani et al. 2014). The Eppendorf tubes were placed on ice and 3 μl of the extract was used as a template for real-time PCR assays. For further assays, the membranes were removed before storing the tubes at −20 °C.

DNA purification Total DNA was purified from 200 μl of crude plant or seed extract and 100 μl from the spot procedure, using the cetyltrimethyl ammonium bromide (CTAB) protocol (Murray and Thompson 1980). Purified DNA was stored at −20 °C until use.

Psyllid extract preparation Psyllids were captured from symptomatic plants using an entomological mouth aspirator. The putative individuals of *B. trigonica* and *B. nigricornis* were preserved in 70% ethanol. Psyllids were identified based on the male genitalia, according to Burckhardt and Freuler (2000). They were then squashed on Whatman 3 MM filter paper (HealthCare, Europe) with the rounded end of an Eppendorf tube according to Teresani et al. (2015). The membranes were carefully cut and inserted into Eppendorf tubes, distilled water (100 μl per tube) was added, the tubes vortexed, and the membranes removed before storing the tubes at −20 °C, until used for amplification.

Real-time PCR CaLsol real-time PCR assays were performed according to Li et al. (2006, 2009) using a Light Cycler 480 (Roche, Switzerland). The reaction mix consisted of 1X Quantimix (Biotools, Spain), 0.24 μM of each primer, 0.12 μM of TaqMan probe and 3 μl of the template (purified DNA or direct extraction from the spot) in a final volume of 12 μl. The real-time PCR amplification protocol included the following steps: 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 58 °C for 40 s. Primers and probe sequences were, respectively: LsoF, 5'-GTCGAGCGCTTATTTTTAAT
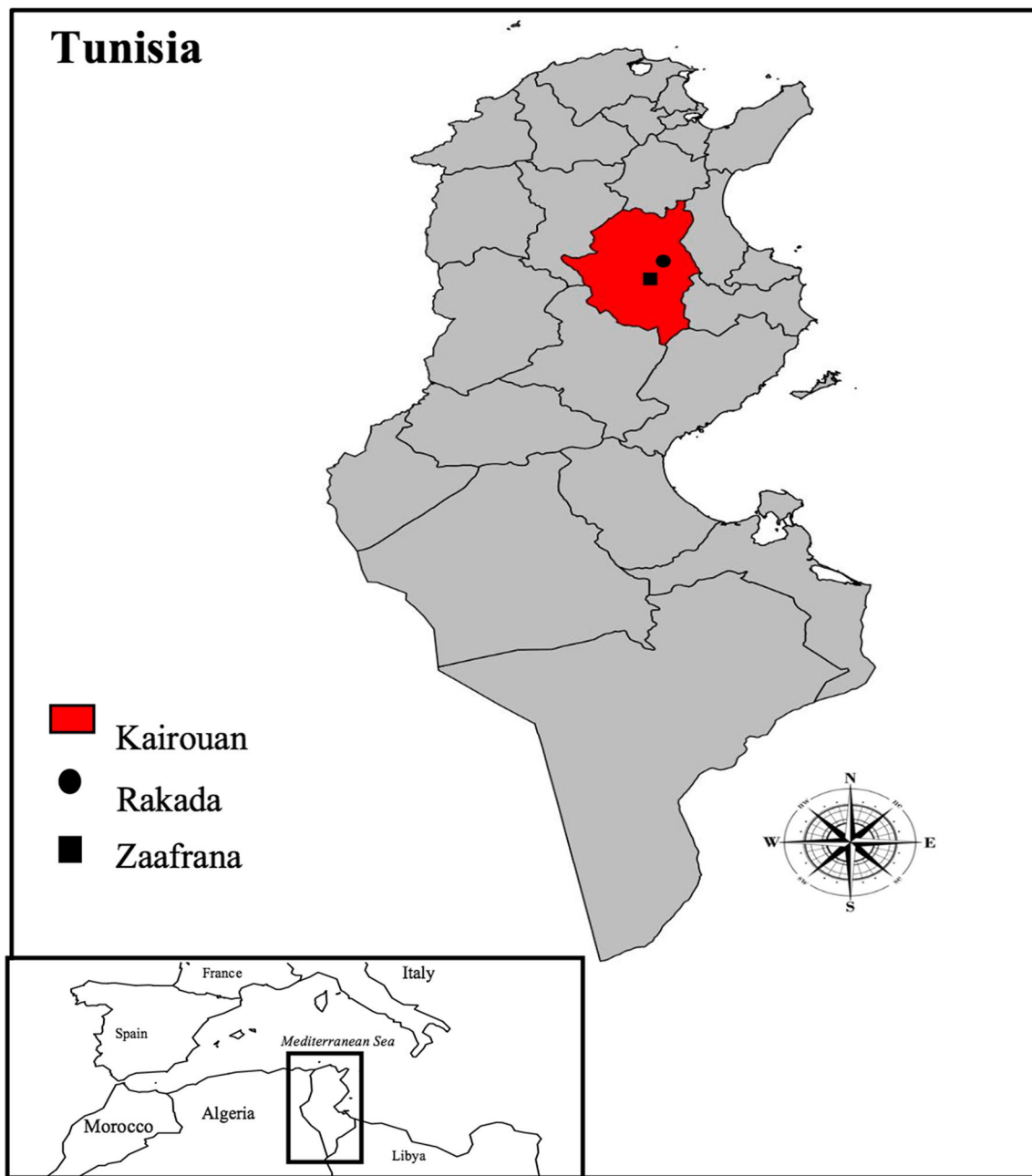
Fig. 1 Geographic location in Tunisia of the carrots fields sampled between years 2014 and 2016

AGGA-3'; HLBr, 5'-GCGTTATCCCGTAGAAAAAGG TAG-3'; and HLBp, 5'FAM-AGACGGGTGAGTAA CGCG-3'BHQ. The positive results (Ct values ≤ 40 and exponential curve) and the negatives were also checked by the real-time PCR protocol of Teresani et al. (2014). In this case, the reaction mix consisted of 1X Quantmix (Biotools, Spain), 0.5 μM of each primer, 0.16 μM TaqMan probe and 3 μl of the template. The amplification protocol consisted of 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers and probe sequences were the following: CaLsppF, 5'-GCAGGCCTAACACATGCAAGT-3'); CaLsppR, 5'-GCACACGTTTCCATGCGTTAT-3'; and the specific TaqMan probe CaLsolP, 5' FAM-AGCGCTTA TTTTTAATAGGAGCGGCAGACG-3' TAMRA (Teresani et al. 2014). Alternatively, a CaLsol/100 complete PCR kit (Plant Print Diagnostics, Spain) based on this last real-time PCR was used following the manufacturer's instructions.

Purified DNA and spots of crude extracts prepared from stored symptomatic carrot plants positive for CaLsol were used as positive controls in each PCR assay. DNase-free distilled water and spotted pieces of membrane with a crude healthy carrot extract were used as negative controls. All the samples were analysed at least three times.

Fig. 2 Yellowing symptoms on carrots collected from 2014 to 2016 at Kairouan (Tunisia). **a** yellowing and purpling on young and mature carrot leaves. **b** curling on new carrot leaves. **c** stunting of carrot shoots and abundance of stems and secondary roots

'*Ca. Phytoplasma spp.*' presence was screened by real-time PCR according to Hren et al. (2007) using the universal primers UniRNA. The reaction mix consisted of 1X Path-ID™ qPCR (Ambion, USA), 1 μM of each primer, 0.5 μM of TaqMan MGB probe and 3 μl of template. The amplification protocol consisted of 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, and 64 °C for 1 min. Primer and probe sequences were: UniRNA-F, 5'-AAATATAG TGGAGGTTATCAGGGATACAG-3'; UniRNA-R, 5'-AACCTAACATCTCACGACACGAACT-3' and the specific TaqMan MGB probe, 5'FAM-ACGACAACCATGCACCA-3' MGB.

S. citri presence was also screened by real-time PCR, following the protocol of Alfaro-Fernández et al. (2017). The reaction mix consisted of 1X Path-ID™ qPCR (Ambion, USA) 10 μM of each primer, 0.5 μM of TaqMan MGB probe and 3 μl of template. The amplification protocol consisted of 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 58 °C for 1 min. Primers and probe sequences were: SCPA-F, 5'-GAAATACTGATAATGGCAAACAGTTTG-3'; SCPA-R, 5'-ACTAGCACTATACCCACGGATATTGT-3'; and the specific TaqMan MGB probe SCPA-P, 5' FAM-ATATGAGCGAAAGCAAAT -3' MGB. The protocols of real-time PCR described for detection of *S. citri* and '*Ca. Phytoplasma spp.*' were used on a StepOne Plus thermal cycler (Applied Biosystems, USA) and the analyses were repeated at least twice.

Determination of bacterial viability To quantify the live CaLsol bacteria, 0.2 ml aliquots from seed samples were treated with propidium monoazide (PMA) at a final concentration of 100 μM. PMA selectively enters dead cells and, upon photo-activation, it intercalates and binds covalently to DNA strongly inhibiting its PCR amplification. Tubes were incubated at 30 °C in the dark for 10 min, with occasional inversion to allow the dye to intercalate with the DNA. After incubation, tubes were vortexed for 5 s and exposed to 100% light intensity for 15 min in a PhAST Blue apparatus (GenIUL, Spain). Subsequently, DNA was extracted from both PMA-treated and untreated samples using the CTAB method and samples were analysed by real-time PCR. Cells number were estimated using cycle threshold (Ct) values from these assays to estimate cells number from a modified standard curve according to Bertolini et al. (2014b), including corrections considering that each bacterial cell contains three copies of the 16S rRNA gene according to Nelson et al. (2015).

Haplotype characterisation and phylogenetic analyses To determine the genetic diversity of Tunisian CaLsol, haplotypes were characterized based on single nucleotide polymorphisms (SNPs) typing of partial regions of the genes 16S rDNA (16S rRNA), 16S–23S rDNA intergenic spacer region (IGS), and *rplJ* and *rplL* ribosomal protein genes (50S subunit) by phylogenetic analysis. The amplification of the 16S rRNA was performed using the forward primer designed by Li et al.

(2009), from a region of the 16S rRNA gene that is unique to CaLsol, in combination with the universal *Liberibacter* reverse primer of Jagoueix et al. (1996). The IGS was amplified using the primers of Ravindran et al. (2011). Finally, the 50S ribosomal protein genes *rplJ* and *rplL* were amplified with the new primers: CaLsol 50S-F (5'-CGAGACCATTGAAC ACAACG-3') and CaLsol 50S-R (5'- TGGAGGAG GAGTGAGAAT-3'). These primers were designed in this study from the complete genome of CaLsol strain R1 (GenBank accession No. CP002371) available in GenBank database with SnapGene software (GSL Biotech, USA). The *rplJ-rplL* locus was amplified by placing the reaction mixture conditions (final concentration of 1X Taq DNA polymerase buffer containing 2 mM $MgCl_2$, 400 nM of each primer, 400 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template) in a thermocycler GeneAmp PCR System 9600 (Applied Biosystem/ Perkin Elmer, USA) at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. Final elongation was 10 min at 72 °C, giving an amplicon of 946 bp. The results were compared with those obtained following Munyaneza et al. (2009). Purification of the amplified fragment from the PCR products was performed following the instructions provided by the Kit Invisorb Spin DNA extraction (Invisorb, Germany), and then quality tested with a NanoDrop spectrophotometer (Thermo Scientific, USA). The sequencing was performed with two readings (forward and reverse) by the Sequencing Service of the Institute of Molecular and Cell Biology of Plants (IBMCP) (Valencia-Spain) and the quality and detection of secondary peaks in chromatograms were determined with the package SangeranalyseR, tools for Sanger sequencing data (Hill et al. 2014) in R software (R Core Team 2016).

All sequences were subjected to molecular phylogenetic analysis performed with MEGA 6 software (Tamura et al. 2013) by Maximum Likelihood Method performed on 1000 replicates.

Results

Analyses of carrot plants The results of CaLsol detection in the carrot samples analysed in 2015 and 2016 are shown in Table 1. The data show the average Cts from three repetitions of each sample. Positive samples using the Li et al. (2009) protocol were detected both in 2015 and 2016 in the two surveyed and sampled areas. In 2015, four positive samples out of 43 were found, and in 2016 ten samples out of 50. Most of the samples were analysed directly from spots or after CTAB extraction. In some cases, a 1:10 and 1:100 dilution of the spots was evaluated in order to discount the presence of polymerase inhibitors as shown in the Table 1. Many negative and positive samples were also tested by the Teresani et al. (2014) assay, or with the Plant Print kit with the same results,

with the exception of eight clear positive samples by Li et al. (2009) that were only detected by Teresani et al. (2014) in late cycles.

All analysed carrot plants were negative for '*Ca*. Phytoplasma spp.*' using universal primers, and also for *S. citri* using specific primers (Table 1).

Analysis of seeds Three lots of seed samples analysed (SE in Table 1) in 2015 were negative for CaLsol, while the three lots of cv. Arbi Zaafrana analysed in 2016 were highly contaminated. Moreover, the viability of CaLsol cells in seeds was very high and ranged from 36% (SE.5) to 65% (SE.4), with an average value of 46% (Table 2, Fig. 3).

Analysis of psyllids A selection of 49 squashed and immobilized adults of *B. trigonica* (samples T in Table 1) and eight adults of *B. nigricornis* (samples N in Table 1) were analysed directly from spots for CaLsol, but all of them were negative.

Haplotype characterisation and phylogenetic analyses The haplotype was determined for 14 samples that were positive in real time PCR for CaLsol, using the three primer sets to ribosomal regions described previously (16S rRNA, IGS, and *rplJ* and *rplL* ribosomal protein genes), but in many cases amplification of all three regions was not achieved. Four samples (three carrot seeds SE4, 5 and 6 and the plant sample Z3), where amplification of all three regions was obtained, were selected for further investigation. Interestingly, the new primers designed for amplification and sequencing of *rplJ* and *rplL* ribosomal protein genes fragments gave stronger positive amplicons than the primers described by Munyaneza et al. (2009). As an example, the amplification obtained with primers to both *rplJ* and *rplL* ribosomal protein genes regions of infected carrot seeds are shown in Fig. 4.

Chromatograms from three carrot seed sequences, SE4, 5 and 6 showed that the three sequences were identical and were haplotype D. These were deposited in GenBank database: 16S rRNA partial gene (Acc. number: KY468271); IGS (Acc. number: KY464018) and *rplJ-rplL* ribosomal protein genes (Acc. number: KY753132).

For the carrot plant (Z3) the sequencing chromatograms obtained for the reverse and complementary strands, in the three ribosomal regions, showed two different signals in key SNPs used for haplotyping. These differences corresponded to haplotype D and haplotype E, indicating the possible presence of both haplotypes in the same sample (Fig. 5). Sequences deposited in GenBank database were: 16S rRNA partial gene (Acc. numbers: KY295907 and KY295908, respectively); IGS (Acc. numbers: KY296296 and KY296295, respectively) and *rplJ-rplL* ribosomal protein genes (Acc. numbers: KY777461 and KY777462, respectively). The phylogenetic relationships of these sequences with the five characterised

Table 1 Detection of 'Ca. Liberibacter solanacearum' by real -time PCR in carrot/insect samples during years 2015–2016 in Kairouan, Tunisia

Year	Origin	Type of sample	Sample name	Methodology	'Ca. Liberibacter solanacearum' Real time PCR Li et al. (2009)	'Ca. Liberibacter solanacearum' Real time PCR Teresani et al. (2014) or the Plant Print kit CaLsol 1/100	'Ca. Phytoplasma spp.' Real time PCR Hren et al. (2007)	Spiroplasma citri Real time PCR Alfaro-Fernández et al. (2017)
2015	Rakada	Plant material	C1; C2; C3; C4; C5; C6; C13; C14; C15; C16; C17; C18; C19; C20; C21; C24; C25; C26; C27; C28; C29; C30; C33; C34;S1;S2;S3;S4;S5;S6;S7;S8;S9;S10 SE1; SE2; SE3	Directly analysed and diluted 1/10 from spots, and after CTAB extraction	Negative	Negative	Negative	Negative
					Negative	ND		
			C22; C23; C31; C32	Directly analysed from spots and also after CTAB extraction	Positive (Cts: 36.4; 36.6; 35.2; 35.8)*	ND		
			S11; S12; S13; S14; S15	Analysis from spots	Negative	ND		
		Insects	T1; T2; T3 T5; T6; T7; T8; T9; T10; T11; T12; T13; T14; T15; T16; T17; T18; T19; T20; T21; T22; T23; T24; T25; T26; T27; T28; T29; Tb1; Tb2; Tk1; Tk2 Tk3; T271; T272; T2941; T2942; T2943 T2944 T2945 T2946 T2947; T2948; T2949; T29410; T29471; T29472; T29473 N1; N2; N3; N4; N5; N6; N7; N8	Analysis from spots	Negative	ND		
2016	Rakada	Plant material	R4; R15	Directly analysed and diluted 1/10 and 1/100 from spots, and after CTAB extraction	Positive (Cts: 38.6; 39.0)*	Positive (Cts: ND; 30.1)*		
			C1; C2; C3; C4; C5; C6; C7; C8; C9; C10; C11; C12 R1; R3; R5; R6; R7; R8; R9; R10; R11; R12; R13; R14; R15; R16; R17; R18	Analysis from spots and CTAB extraction	Negative	Negative		
	Zaafrana		Z2; Z3; Z7; Z8; Z14; Z15; Z18; Z19	Directly analysed and diluted 1/10 from spots, and after CTAB extraction	Positive (Cts: 36.0; 39.5; 38.4; 38.3; 40.0; 38.7; 38.7; 38.2)*	U		
			Z1; Z4; Z5; Z6; Z9; Z10 Z11; Z12; Z13; Z16; Z17; Z20	Analysis from spots and CTAB extraction	Negative	Negative		
		Seeds	SE4; SE5; SE6	Analysis from spots and CTAB extraction	Positive (Cts: 28.3; 34.6 29.2)*	Positive (Cts:27.0; 35.9; 29.6)*		

C, S, Z carrot samples, SE carrot seed samples, T Bactericera trigonica, N B.nigricornis, ND not determined

U unclear results because the Cts values were over 40 with exponential curve, ND Not determined, was not performed

* Average of CTs values from 3 replications

Table 2 Viability of '*Ca.* Liberibacter solanacearum' cells present in carrot seeds as determined by PMA methodology (Bertolini et al. 2014b)

Seed lot	Addition of PMA	Average Ct values*	Cell number	Viable cells (%)	Non viable cells (%)
SE.4	No	29.3 (SE = 0,20)	3.37×10^2	65.38	34.62
	Yes	29.9 (SE = 0,24)	2.21×10^2		
SE.5	No	29.3 (SE = 0,24)	3.30×10^2	36.37	63.63
	Yes	30.8 (SE = 0,11)	1.20×10^2		
SE.6	No	30.6 (SE = 0,36)	1.40×10^2	41.03	58.97
	Yes	31.8 (SE = 0,52)	5.81×10^1		
Average value				46.04	53.96

*Average of CTs values from 5 replications. SE: Standard error

haplotypes are shown in Fig. 6 and confirm their status as haplotypes D and E.

Discussion

The emerging bacterium CaLsol is associated with socio-economically important diseases in many crops (Munyaneza 2012; Haapalainen 2014). Its presence has been reported in several species of Solanaceae (Liefting et al. 2009b) and in plants of Apiaceae (Munyaneza et al. 2012a, b), being associated with vegetative disorders. In carrots, the bacterium has been detected in European countries such as Spain (Alfaro-Fernández et al. 2012), Finland (Haapalainen 2014), Sweden (Munyaneza et al. 2012a), and France (Loiseau et al. 2014), and was also reported from Morocco (Tahzima et al. 2014).

CaLsol-like symptoms were observed through 2014–2016 in carrots cultivated in different areas of Kairouan in Tunisia and the presence of CaLsol was confirmed in symptomatic carrot plants by real-time PCR. Moreover, the positive results obtained using the Li et al. (2009) protocol

Fig. 3 Figure 3. Standard curve including corrections according to Nelson et al. (2015): y = −1,412ln(x) + 37,623; $R^2 = 1$

were confirmed using the Teresani et al. (2014) assay or the Plant Print Diagnostics real-time kit, increasing the reliability of the diagnosis. These are protocols recommended by FAO-IPPC (2017).

However, CaLsol was not detected in all the symptomatic plants tested. This may be due to a number of factors: (i) high temperature, because temperatures close to 30 °C or higher may affect the prevalence of the high temperature sensitive CaLsol (Munyaneza et al. 2012c). At the time of sampling temperatures were above 30 °C, although temperatures during the growing season varied between 24 and 33 °C (www.meteo.tn/, dates from 2016); (ii) an uneven CaLsol distribution in the phloem, that could lead to samples testing negative; (iii) variable bacterial titer that may be below the level of detection according to the season and the plant organ infected (data not shown); (iv) time delay between samples collection and their analysis and/or the use of frozen samples, leading to CaLsol DNA degradation. As the disease in carrots has only recently been reported, the influence of these factors on CaLsol levels in plant tissues is not well understood. Furthermore, the presence of inhibitory compounds in plant tissues could also hamper detection, as they are a major impediment to successful DNA amplification. Consequently, 1:10 and 1:100 extract dilutions were tested to minimize the action of possible polymerase inhibitors but no improvement in amplification was found. In addition, in order to improve the efficiency of detection, different combinations of extraction procedures (spot PCR and modified CTAB extraction) were evaluated to optimize DNA amplification but with no improvement.

Some European laboratories have found difficulties in amplifying some regions used for haplotyping which may be due to the low concentration of the bacterium in the analysed samples. For this reason, a new PCR protocol for amplifying a fragment of the gene *rplJ-rpl* was designed because our PCR products were weak with protocol described in Munyaneza et al. (2009).
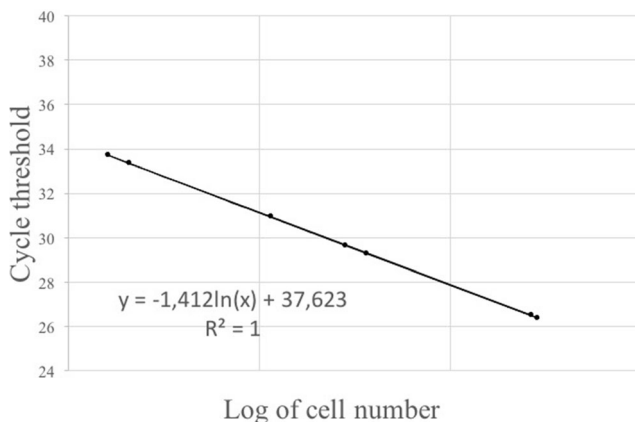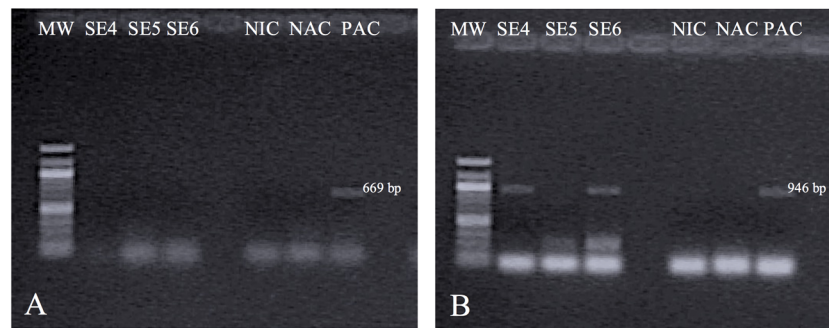
Fig. 4 Agarose gel electrophoresis of PCR amplification from DNA extracts of carrot seeds (SE4, SE5 and SE6) from Kairouan (Tunisia) with two primer pairs. **a** CL514F/R according to Munyaneza et al. (2009); (**b**) CaLsol 50SF/R designed in this study. Lane NIC depicts negative isolation control. Lane NAC depicts negative amplification control. Lane PAC depicts positive amplification control corresponding to a potato sample infected with CaLsol. MW depicts 100 bp DNA ladder
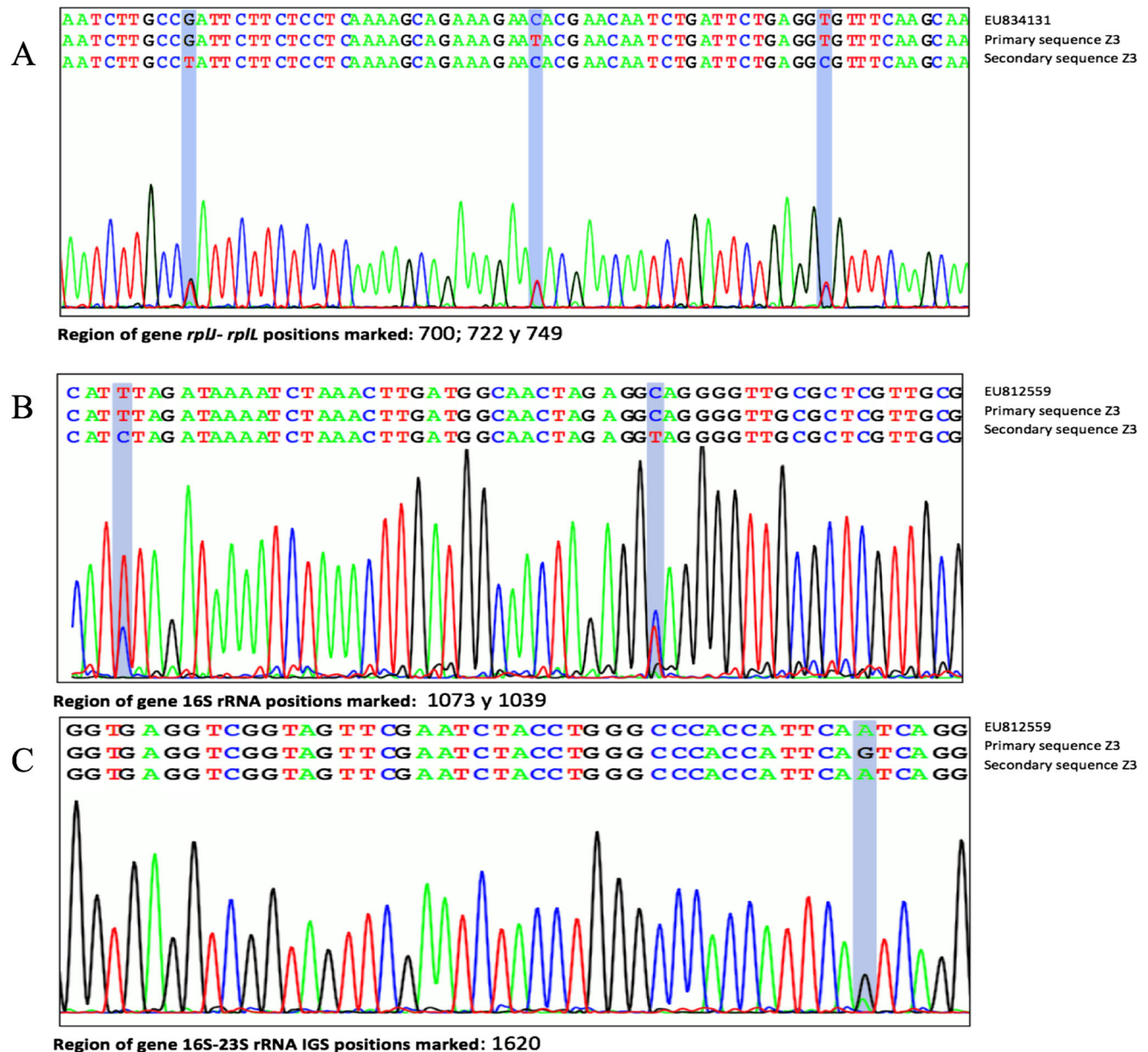
Fig. 5 Chromatogram of sequences of sample Z3 of partial regions of three genes: (**a**) ribosomal protein *rplJ* and *rplL* (50S rRNA); (**b**) 16S rDNA (16S rRNA gene) and (**c**) IGS where the key SNPs found in the primary and secondary sequences are marked with grey bars

Fig. 6 Phylogenetic tree using the Maximum Likelihood method based on the 3-parameter model using MEGA6. Bootstrap test of phylogeny was performed with 1000 replicates. The analysis involved nucleotide sequences from database GenBank of (**a**) the 50S ribosomal protein genes *rplJ* and *rplL* and (**b**) 16S rRNA partial genes of the haplotypes of CaLsol. Sequence of samples analysed in this study are marked with a large black dot
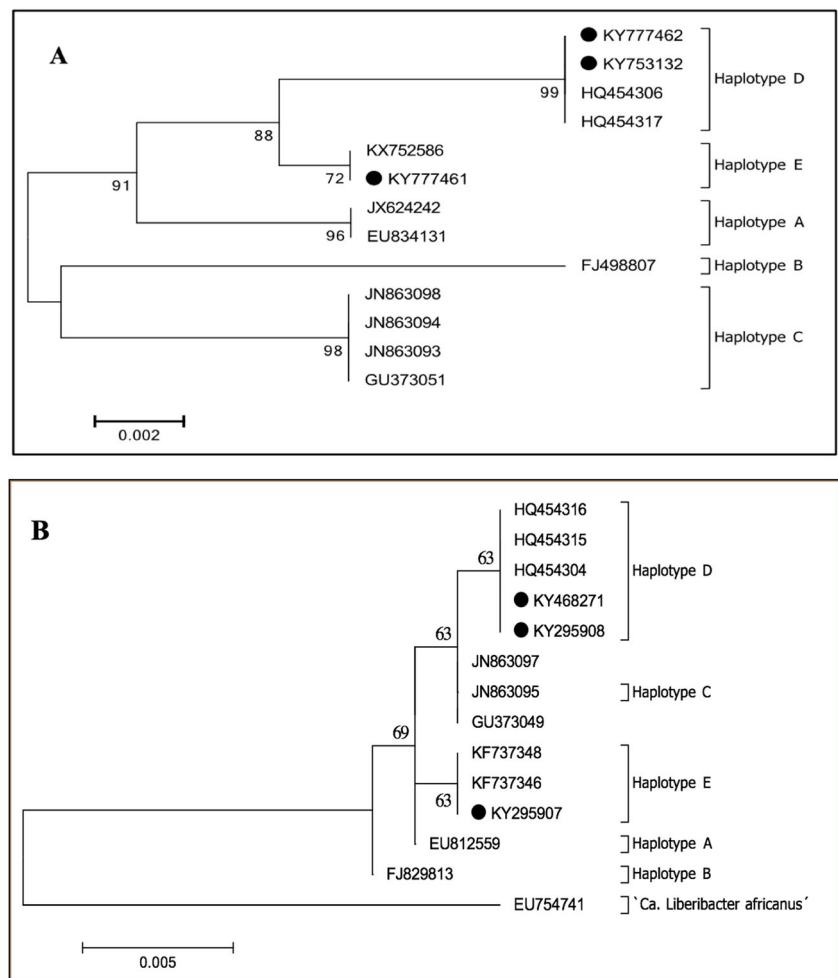
Analysis of CaLsol positives samples showed that only haplotype D was present in carrot seeds but haplotypes D and E were present in one carrot plant (Fig. 6). Due to the limited amount of DNA of this sample (Z3), we could not clone the amplicons to confirm the presence of both fragments of each haplotype. However, co-infection has already been described for haplotypes A and B in potato and potato-psyllid samples (Wen et al. 2013), but this is the first report of co-infection of haplotypes D and E in carrot. Since haplotype E is the most frequent haplotype found in carrot in Southern European countries (Alfaro-Fernández et al. 2012) and some of the cultivars found infected, such as cv. Maestro, are the same as those found infected in Europe, and were provided by the same companies, this haplotype may have been introduced through contaminated seeds (Bertolini et al. 2014b).

Using real-time PCR a high level of CaLsol was detected in 2016 in three carrot seed lots of the cv. Arbi Zaafrana, with a high number of quantified viable cells found after PMA treatment, higher than 35% in all cases and reaching 65% in one of the lots. These percentages are much higher than those reported by Bertolini et al. (2014b) and may be due to the lack of seed treatments performed by Tunisian growers on locally produced seed, in contrast to the treated commercially available seeds. The locally produced seeds had not been checked for CaLsol before sowing but, according to information from the growers, they were probably collected from infected mother plants.

Viable CaLsol cells in seeds were shown to be responsible for infection of carrot seedlings germinated from contaminated seeds (Bertolini et al. 2014b). However, transmission of this pathogen by carrot seeds seems to be not a major transmission pathway, in comparison to vector transmission, probably because other unknown parameters are influential in the development of the disease (Loiseau et al. 2017). If the primary inoculum of haplotype D in Tunisia is CaLsol contaminated seed, treatment of carrot seeds would be a key mitigation strategy for preventing the disease (Bertolini et al. 2014b; Ilardi et al. 2016), for example by hot water treatment (Australian Government Department of Agriculture and Water Resources 2017).

In Kairouan, it has only been possible to test a relatively low number of identified *B. trigonica* and *B. nigricornis* individuals for CaLsol by direct analysis of squashed

psyllids. As these insects were negative for CaLsol,, more individuals of these and other species should be collected and analysed to determine their possible role in transmission, because different psyllid species have been reported as vectors in several European countries (Munyaneza et al. 2010b; Teresani et al. 2015).

The negative results for the detection of '*Ca*. Phytoplasma spp.*' and *S. citri* suggests that CaLsol is the only bacterial agent associated with the symptoms observed in carrots in Kairouan, and with the commercial losses due to the poor quality of these plants. The information from the current study will help carrot growers to develop preventive and pest management strategies, such as analysis of seed lots, elimination of symptomatic plants, and analysis and control of potential vectors to reduce the incidence and spread of this emerging bacterium to other apiaceous or solanaceous hosts, and to other Tunisian regions. The recent discovery of this bacterium in Morocco (Tahzima et al. 2014), together with our results suggests that this emerging pathogen is spreading in North Africa and other Mediterranean countries. Thus, the National Plant Protection Organisations should be alerted to the threat that CaLsol poses to solanaceous and apiaceous cultivated plant species.

References

Abed G, Maslenin L, Weintraub PG, Mawassi M (2011) Phytoplasma and *Spiroplasma* diseases in open-field crops in Israel. Bull Insectol 64:53–54

Alfaro-Fernández A, Cebrián MC, Villaescusa FJ, Font MI (2011) Detection and identification of aster yellows and stolbur phytoplasmas in various crops in Spain. Bull Insectol **64**(Supplement):S63–S64

Alfaro-Fernández A, Cebrián MC, Villaescusa FJ, Hermoso de Mendoza FJ, Ferrándiz JC, Sanjuán S, Font MI (2012) First report of '*Candidatus* Liberibacter solanacearum' in carrot in mainland Spain. Plant Dis 96:582

Alfaro-Fernández A, Ibañez I, Bertolini E, Hernández-Llopis D, Cambra M, Font MI (2017) Transmission of *Spiroplasma citri* in carrot seeds and development of a real-time PCR for its detection. J Plant Pathol 99:371–379

Australian Government Department of Agriculture and Water Resources, 2017. Final pest risk analysis for '*Candidatus* Liberibacter solanacearum' associated with apiaceous crops. Department of Agriculture and Water Resources, Canberra, ACT, Australia,

Bertolini E, Felipe RTA, Sauer AV, Lopes SA, Arilla A, Vidal E, Mourão Filho FA, Nunes WMC, Bové JM, López MM, Cambra M (2014a) Tissue-print and squash real time polymerase chain reaction for direct detection of '*Candidatus* Liberibacter' species in citrus plants and psyllid vectors. Plant Pathol 63:1149–1158

Bertolini E, Teresani GR, Loiseau M, Tanaka FAO, Barbé S, Martínez MC, Gentit P, López MM, Cambra M (2014b) Transmission of '*Candidatus* Liberibacter solanacearum' in carrot seeds. Plant Pathol 64:276–285

Burckhardt D, Freuler J (2000) Jumping plant-lice (Hemiptera, Psylloidea) from sticky traps in carrot fields in Valais, Switzerland. Mitteilungen der Schweizerischen Entomologishchen Gesellschaft 73:191–209

Cebrián MC, Villaescusa FJ, Alfaro-Fernández A, Hermoso de Mendoza A, Córdoba-Sellés MC, Jorda C, Ferrándiz JC, Sanjuan S, Font MI (2010) First report of *Spiroplasma citri* in carrot in Europe. Plant Dis 94:1264

Crosslin JM, Munyaneza JE, Brown JK, Liefting LW (2010) Potato zebra chip disease: a phytopathological tale. Plant Health Progress. https://doi.org/10.1094/PHP-2010-0317-01-RV

Duduk B, Bulajić A, Duduk N, Calari A, Paltrinieri S, Krstić B, Bertaccini A (2007) Identification of phytoplasma belonging to aster yellows ribosmal group in vegetables in Serbia. Bull Insectol 60: 341–342

Duduk B, Perić P, Marčić D, Drobnjaković T, Picciau L, Alma A, Bertaccini A (2008) Phytoplasmas in carrots: disease and potential vectors in Serbia. Bull Insectol 61:327–331

EPPO (European and Mediterranean Plant Protection Organization) (2013) Data sheets on pests recommended for regulation. '*Candidatus* Liberibacter solanacearum'. Bull OEPP/EPPO 43: 197–201

Fagen J, Leonard MT, McCullough CM, Triplett EW, Davis MJ (2014) *Liberibacter crescens* gen. nov sp., nov., first cultured member of the *Liberibacter* genus. Int J Syst Evol Bacteriol 64:2461–2466

FAO-IPPC (International Plant Protection Convention), 2017. Diagnostic protocols DP 21 '*Candidatus* Liberibacter solanacearum'. ISPM27. Annex 21

Font MI, Abad P, Albiñana M, Espino AI, Dally DRE, Jordá C (1999) Amarilleos y enrojecimientos en zanahoria: una enfermedad a diagnóstico. Boletín de Sanidad Vegetal de Plagas 25:405–415

Haapalainen M (2014) Biology and epidemics of '*Candidatus* Liberibacter' species, psyllid-transmitted plant-pathogenic bacteria. Ann Appl Biol 165:172–198

Haapalainen M, Kivimäki P, Latvala S, Rastas M, Hannukkala A, Jauhiainen L, Lemmetty A, Pirhonen M, Virtanen A, Nissinen AI (2016) Carrot pathogen '*Candidatus* Liberibacter solanacearum' haplotype C is frequent and widely occurring in several areas in the southern part of Finland. Plant Pathol 66:559–570

Hill JT, Demarest BD, Bisgrove BW, Yi-chu S, Smith H, Yost J (2014) Poly peak parser: Method and software for identification of unknown indels using Sanger sequencing of polymerase chain reaction products. Dev Dyn 243:1632–1636

Hren M, Boben J, Rotter A, Kralj P, Gruden K, Ravnikar M (2007) Real-time PCR detection systems for flavescence dorée and bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. Plant Pathol 56:785–796

Ilardi V, Di Nicola E, Tavazza M (2016) First report of 'Candidatus Liberibacter solanacearum' in commercial carrot seeds in Italy. J Plant Pathol 98:374

Jagoueix S, Bové JM, Garnier M (1996) PCR detection of the two 'Candidatus Liberibacter' species associated with greening disease of citrus. Mol Cell Probes 10:43–50

Lee IM, Martini M, Bottner KD, Dane RA, Black MC, Troxclair N (2003) Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. Phytopathology 93:1368–1377

Li W, Hartung JS, Levy L (2006) Quantitative real-time PCR for detection and identification of 'Candidatus Liberibacter' species associated with citrus huanglongbing. J Microbiol Methods 66:104–115

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L (2009) Multiplex real-time PCR for detection, identification and quantification of 'Candidatus Liberibacter solanacearum' in potato plants with zebra chip. J Microbiol Methods 78:59–65

Liefting LW, Sutherland PW, Ward LI, Paice KL, Weir BS, Clover GRG (2009a) A new 'Candidatus Liberibacter' species associated with diseases of solanaceous crops. Plant Dis 93:208–214

Liefting LW, Weir BS, Pennycook SR, Clover GRG (2009b) 'Candidatus Liberibacter solanacearum', a Liberibacter associated with plants in the family Solanaceae. Int J Syst Evol Microbiol 59:2274–2276

Lin H, Doddapaneni H, Munyaneza JE, Civerolo E, Sengoda VG, Buchman JL, Stenger DC (2009) Molecular characterization and phylogenetic analyses of 16S rRNA from a new species of 'Candidatus Liberibacter' associated with zebra chip disease of potato (Solanum tuberosum L.) and the potato psyllid (Bactericera cockerelli Sulc). J Plant Pathol 91:215–219

Loiseau M, Garnier S, Boirin V, Merieau M, Leguay A, Renaudin I, Renvoisé JP, Gentit P (2014) First report of 'Candidatus Liberibacter solanacearum' in carrot in France. Plant Dis 98:839

Loiseau M, Isabelle R, Pascaline C, Pierre-Marie L, Aurélie F, Pascal G (2017) Lack of evidence of vertical transmission of 'Candidatus Liberibacter solanacearum' by carrot seeds suggests that seed is not a major transmission pathway. Plant Dis (in press) http://apsjournals.apsnet.org/doi/10.1094/PDIS-04-17-0531-RE

Munyaneza JE (2012) Zebra chip disease of potato: biology, epidemiology and management. Am J Potato Res 89:329–350

Munyaneza JE, Crosslin JM, Upton JE (2007a) Association of Bactericera cockerelli (Homoptera: Psyllidae) with "zebra chip", a new potato disease in southwestern United States and Mexico. J Econ Entomol 100:656–663

Munyaneza JE, Goolsby JA, Crosslin JM, Upton JE (2007b) Further evidence that zebra chip potato disease in the lower Rio Grande Valley of Texas is associated with Bactericera cockerelli. Subtrop Plant Sci 59:30–37

Munyaneza JE, Sengoda VG, Crosslin JM, Rosa-Lozano G, Sanchez A (2009) First report of 'Candidatus Liberibacter psyllarous' in potato tubers with Zebra chip disease in Mexico. Plant Dis 93:552

Munyaneza JE, Fisher TW, Sengoda VG, Garczynski SF (2010a) First report of 'Candidatus Liberibacter solanacearum' associated with psyllid-affected carrots in Europe. Plant Dis 94:639

Munyaneza JE, Fisher TW, Sengoda VG, Garczynski SF, Nissinen A, Lemmetty A (2010b) Association of "Candidatus Liberibacter solanacearum" with the psyllid Trioza apicalis (hemiptera: Triozidae) in Europe. J Econ Entomol 103:1060–1070

Munyaneza JE, Lemmetty A, Nissinen AI, Sengoda VG, Fisher TW (2011) Molecular detection of aster yellows phytoplasmas and 'Candidatus Liberibacter solanacearum' in carrots affected by the psyllid Trioza apicalis (Hemiptera: Triozidae) in Finland. J Plant Pathol 93:697–700

Munyaneza JE, Sengoda VG, Stegmark R, Arvidsson AK, Anderbrant O, Yuvaraj JK, Rämert B, Nissinen A (2012a) First report of 'Candidatus Liberibacter solanacearum' associated with psyllid-affected carrots in Sweden. Plant Dis 96:453

Munyaneza JE, Sengoda VG, Sundheim VGL, Meadow R (2012b) First report of 'Candidatus Liberibacter solanacearum' associated with psyllid-affected carrots in Norway. Plant Dis 96:454

Munyaneza JE, Sengoda VG, Buchman JL, Fisher TW (2012c) Effects of temperature on 'Candidatus Liberibacter solanacearum' and zebra chip potato disease symptom development. Plant Dis 96:18–23

Munyaneza JE, Sengoda VG, Sundheim L, Meadow R (2014) Survey of 'Candidatus Liberibacter solanacearum' in carrot crops affected by the psyllid Trioza apicalis (Hemiptera: Triozidae) in Norway. J Plant Pathol 96:397–402

Murray HG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325

Nelson WR, Fisher TW, Munyaneza JE (2011) Haplotypes of 'Candidatus Liberibacter solanacearum' suggest long-standing separation. Eur J Plant Pathol 130:5–12

Nelson WR, Sengoda VG, Alfaro-Fernández A, Font MI, Crossling JM, Munyaneza JE (2012) A new haplotype of "Candidatus Liberibacter solanacearum" identified in the Mediterranean region. Eur J Plant Pathol 135:633–639

Nelson WR, Wulff NA, Bové JM (2015) Intragenomic homogeneity on Liberibacter 16S rDNA confirms phylogeny and explains ecological strategy. bioRxiv. https://doi.org/10.1101/016188

R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ravindran A, Levy J, Pierson E, Gross DC (2011) Development of primers for improved PCR detection of the potato zebra chip pathogen, 'Candidatus Liberibacter solanacearum'. Plant Dis 95:1542–1546

Secor GA, Rivera VV, Abad JA, Lee IM, Clover GRG, Liefting LW, Li X, De Boer SH (2009) Association of 'Candidatus Liberibacter solanacearum' with the zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. Plant Dis 93:574–583

Tahzima R, Maes M, Achbani EH (2014) First report of 'Candidatus Liberibacter solanacearum' on carrot in Africa. Plant Dis 10:1426

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) Mega6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Teresani GR, Bertolini E, Alfaro-Fernández A, Martínez C, Tanaka FAO, Kitajima EW, Roselló M, Sanjuán S, Ferrándiz JC, López MM, Cambra M, Font MI (2014) Association of 'Candidatus Liberibacter solanacearum' with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. Phytopathology 104:804–811

Teresani GR, Hernández E, Bertolini E, Siverio F, Marroquín C, Molina J, Hermoso de Mendoza A, Cambra M (2015) Search for potential vectors of 'Candidatus Liberibacter solanacearum': population dynamics in host crops. Span J Agric Res 13:e10-002

Wen A, Johnson C, Gudmestad NC (2013) Development of a PCR assay for the rapid detection and differentiation of 'Candidatus Liberibacter solanacearum' haplotypes and their spatiotemporal distribution in the United States. Am J Potato Res 90:229–236