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

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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 Zaghuan) 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 (Liefing 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 (Munyanza et al.

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2007a, 2007b; Liefing 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} ; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 g l^{-1} ; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 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 $5\text{ }\mu\text{l}$ of crude extract were loaded. Then, $100\text{ }\mu\text{l}$ 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 $3\text{ }\mu\text{l}$ 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 $200\text{ }\mu\text{l}$ of crude plant or seed extract and $100\text{ }\mu\text{l}$ 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 ($100\text{ }\mu\text{l}$ 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), $0.24\text{ }\mu\text{M}$ of each primer, $0.12\text{ }\mu\text{M}$ of TaqMan probe and $3\text{ }\mu\text{l}$ of the template (purified DNA or direct extraction from the spot) in a final volume of $12\text{ }\mu\text{l}$. 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'-GTCTGAGCGCTTATTTTAAAT

Fig. 1 Geographic location in Tunisia of the carrots fields sampled between years 2014 and 2016

AGGA-3'; HLB_r, 5'-GCGTTATCCCGTAGAAAAAGG TAG-3'; and HLB_p, 5'-FAM-AGACGGGTGAGTAA CGCG-3'BHQ. The positive results (C_t 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 μ M of each primer, 0.16 μ M TaqMan probe and 3 μ l 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: CaLsp_pF, 5'-GCAGGCCTAACACATGCAAGT-3'); CaLsp_pR, 5'-GCACACGTTTCCATGCGTTAT-3'; and the

specific TaqMan probe CaLsol_p, 5' FAM-AGCGCTTA TTTTAATAGGAGCGGCAGACG-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'-CGAGACCATTGAACACAACG-3') and CaLsol 50S-R (5'-TGGAGGAGGAGTGAGAAT-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 MgCl₂, 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'	'Ca. Phytoplasma spp.'	<i>Spiroplasma citri</i>
					Real time PCR Li et al. (2009)	Real time PCR Hren et al. (2007)	Real time PCR Alfaro-Fernández et al. (2017)
					or the Plant Print kit CaLsol 1/100		
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
			C22; C23; C31; C32	Directly analysed from spots and also after CTAB extraction	Negative	Negative	Negative
			S11; S12; S13; S14; S15		Negative	Negative	Negative
			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; T61; T62; Tk1; Tk2 Tk3; T271; T272; T2941; T2942;		Positive (Cts: 36.4; 36.6; 35.2; 35.8)*		
			T2943 T2944 T2945 T2946 T2947; T2948; T2949; T29410; T29471; T29472; T29473	Analysis from spots	Negative	Negative	
		Insects	N1; N2; N3; N4; N5; N6; N7; N8				
			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	Analysis from spots and CTAB extraction	Negative	Negative	
			R1; R3; R5; R6; R7; R8; R9; R10; R11; R12; R13; R14; R15; R16; R17; R18				
			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; 38.2)*	U	
2016	Rakada	Plant material	Z1; Z4; Z5; Z6; Z9; Z10 Z11; Z12; Z13; Z16; Z17; Z20	Analysis from spots and CTAB extraction	Negative	Negative	
			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)*	
		Seeds					

C, S, R, Z carrot seed 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 (Munyanzeza 2012; Haapalainen 2014). Its presence has been reported in several species of Solanaceae (Liefting et al. 2009b) and in plants of Apiaceae (Munyanzeza 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 (Munyanzeza 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

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 (Munyanzeza 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 Munyanzeza et al. (2009).

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

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 (Munyanza 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.

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