Short Communication

OCCURRENCE AND MOLECULAR DETECTION OF *SPIROPLASMA CITRI* IN CARROTS AND ITS INSECT VECTOR, *CIRCULIFER TENELLUS,* IN MEXICO

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SUMMARY

In 2014, carrot plants in Zacatecas, Mexico, were found with yellow, brown, or purple-colored leaves, which were occasionally smaller and rolled. Roots of these affected plants were hairy, deformed, and small. Molecular diagnostics failed to detect phytoplasmas in these samples, but identified *Spiroplasma citri* in 58 and 94% of the samples using PCR targeting the spiralin and adhesion-related protein 1 (*arp1*) genes, respectively. Sequence analysis confirmed the presence of *S. citri*, and identified a novel, putative *arp* gene in one carrot sample. *S. citri* is a phytopathogenic mollicute transmitted by leafhopper species. Beet leafhoppers (*Circulifer tenellus* Baker) collected in the same state of Zacatecas, Mexico, were subsequently tested for *S. citri* infection, and 36.5% were positive using PCR targeting the *arp1* gene. Sequencing analysis confirmed the presence of *S. citri* in the leafhoppers. This is the first report of *S. citri* in carrot and *C. tenellus* in Mexico. Previously in the Americas, *S. citri* in carrot was only reported in Washington and California in the United States. The presence of *S. citri* in carrots and the beet leafhopper in Mexico, suggests that this pathogen could become a threat to vegetable industries in this region of Mexico, including the carrot industry.

Keywords: carrot purple leaf disease, beet leafhopper, Mollicutes, spiralin

In the United States, carrot plants (*Daucus carota* L.) with brown, purple, or chlorotic leaves, excessive proliferation of side roots, or virescence and phyllody of the flowers have been associated with phytopathogenic mollicute infection by phytoplasmas or spiroplasmas (Lee *et al*., 2006; Crosslin *et al*., 2006; Mello *et al*., 2008). Organisms of the class Mollicutes are wall-less prokaryotes that have small genomes and are found in various animal, arthropod, and plant hosts.

Phytoplasmas can be separated into phylogenetic groups and cause diseases in a variety of plant hosts, of which aster yellows phytoplasma, from the aster yellows group 16SrI, and beet leafhopper-transmitted virescence agent (BLTVA), from the clover proliferation group 16SrVI, have been found in carrot in the United States (Shaw *et al*., 1990; Lee *et al*., 2003). Phloem feeding insects transmit phytoplasmas to their plant hosts, including leafhoppers, such as the aster leafhopper (*Macrosteles fascifrons* Stål) and the beet leafhopper (*Circulifer tenellus* Baker; Crosslin *et al*., 2005; Lee *et al*., 1998; Munyaneza and Upton, 2005). While aster yellows and BLTVA phytoplasmas have been associated with carrot disease symptoms, these phytoplasmas also cause significant economic losses to other crops such as radish, potato (potato purple top disease), and dry bean in the United States (Lee *et al*., 2004; Munyaneza *et al*., 2007).

Spiroplasmas also cause diseases across a variety of different plant species, including carrot, by way of phloem feeding insects that transmit the bacterium in a persistent manner (Liu *et al*., 1983). Among the spiroplasmas, *Spiroplasma citri* is transmitted to various plant species by the leafhopper vector, *Circulifer tenellus*, in the United States (Liu *et al*., 1983), and by the related leafhopper, *Circulifer haematoceps* Mulsant and Rey, in the Mediterranean region (Fos *et al*., 1986). Several insect-transmissible strains of *S. citri* have been isolated, but the propagation of specific strains by grafting or growth in liquid medium has generated non-insect-transmissible strains (Wayadande *et al*., 1995). *S. citri* has naturally been found in warm to hot semiarid areas and deserts and, in these favorable temperatures, can cause infected herbaceous plants to die within the months following infection (Calavan and Bové, 1989). *S. citri* has been linked to citrus stubborn disease of citrus in California, Arizona, and the Mediterranean region, brittle root disease of horseradish in the United States, carrot purple leaf disease in Washington and California, and has recently been identified in celery in Spain (El Shafy *et al*., 1972; Raju *et al*., 1981; Lee *et al*., 2006; Mello *et al*., 2008; Alfaro-Fernández *et al*., 2015).

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Table 1. Symptoms and *arp1* and spiralin PCR results for 5 asymptomatic and 31 symptomatic carrot samples analyzed in this study.

Plant No.	Symptoms	*arp1*	spiralin
1	asymptomatic	–	–
2	asymptomatic	–	–
3	asymptomatic	–	–
4	asymptomatic	–	–
5	asymptomatic	–	–
6	hairy root; yellow leaves	+	+
7	hairy root; yellow, curled leaves	+	+
8	deformed root; brown leaves	+	+
9	hairy root; chlorotic leaves	+	–
10	hairy root; chlorotic leaves	+	–
11	hairy root	+	–
12	chlorotic/brown leaves	–	–
13	deformed, hairy root	–	–
14	hairy root; brown/purple leaves	+	+
15	rootlets on the root; brown leaves	+	+
16	small root; brown leaves	+	+
17	brown leaves	+	–
18	brown leaves	+	+
19	small, slightly hairy root; brown, small leaves	+	–
20	green, small leaves	+	–
21	small, slightly hairy root; brown leaves	+	–
22	deformed root ; brown leaves	+	+
23	small root; very small leaves	+	–
24	hairy, deformed root; yellow/brown leaves	+	+
25	deformed root; brown leaves	+	+
26	deformed, whitish root with purple spots	+	–
27	brown leaves	+	+
28	brown leaves	+	–
29	hairy, small root; brown leaves	+	+
30	hairy, small root; brown/purple leaves	+	+
31	hairy root; yellow/purple leaves	+	+
32	deformed root; yellow leaves	+	+
33	hairy root; green leaves	+	+
34	slightly hairy root; yellow/brown leaves	+	+
35	slightly hairy root; brown leaves	+	–
36	slightly hairy root; green leaves	+	+

In November of 2014, carrot plants exhibiting symptoms similar to those previously described for BLTVA phytoplasma and *S. citri*-infected carrots in Washington State (Lee *et al*., 2006), as well as aster yellows phytoplasma-infected carrots in Texas (Lee *et al*., 2003), were observed in several commercial fields in the state of Zacatecas, Mexico. The symptoms in carrots included hairy, deformed, and occasionally small roots, yellow, brown, and/or purple leaves, and small, rolled leaves (Table 1). The infection rate was about 1% per field. A total of 5 asymptomatic and 31 symptomatic carrot samples (roots and foliage) were collected near Chaparrosa, in the state of Zacatecas, Mexico. Nucleic acids were extracted from the mature root of all carrot samples using a modified cetyl trimethyl ammonium bromide (CTAB) DNA extraction protocol for plants as described previously (Munyaneza *et al*., 2010).

Carrot samples were subjected to molecular analyses to detect *S. citri* using the Spiralin-f/Spiralin-r and P89-f/P89-r primer pairs (Yokomi *et al*., 2008). All PCR reactions were set-up in a 25 µl volume consisting of 5 µl Go *Taq* Green PCR buffer (Promega, USA), 0.5 µl of 10 mM dNTP (each) mix, 1 µl each 20 µM primer, 17.65 µl H_2O, 0.1 µl Go *Taq* Polymerase (Promega, USA), and 1 µl DNA. A DNA sample containing *S. citri* (extracted from carrot; received from Dr. James Crosslin, United States Department of Agriculture, Prosser, WA) was used as a positive control, and H_2O was used as the no-template negative control. The following amplification cycle was used for primers Spiralin-f/Spiralin-r: 95°C for 3 min, 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 45 s, followed by 72°C for 5 min. For primers P89-f/P89-r, the following amplification cycle was used: 95°C for 3 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s, followed by 72°C for 3 min. A 1.5% agarose gel stained with ethidium bromide was used to visualize the 675- and 707-bp PCR products for spiralin and *arp1*, respectively.

Eighteen of the 31 (58%) symptomatic carrot samples from Chaparrosa were positive for *S. citri* using primers Spiralin-f/Spiralin-r, which target the *S. citri* spiralin gene (Table 1). All 5 asymptomatic plants were negative for *S. citri* with the primer pair Spiralin-f/Spiralin-r. Of the 31 symptomatic carrot samples, 29 (94%) were positive for *S. citri* using the primer pair P89-f/P89-r that targets the adhesion-related protein 1 (*arp1*) (Table 1). All 5 asymptomatic plants were negative for *S. citri* with primer pair, P89-f/P89-r. Yokomi *et al.* (2008) previously reported that primer pair P89-f/P89-r (targeting *arp1*) is three orders of magnitude more sensitive than primer pair Spiralin-f/Spiralin-r. Therefore, it is not surprising that 29 plants were positive for *arp1* and only 18 were positive for spiralin. No carrot samples tested positive for phytoplasma infection using the universal nested primer pairs of P1/P7 and fU5/rU3 as described in Crosslin *et al.* (2006).

A representative sampling of *S. citri* PCR products were purified using a QIAquick PCR purification kit (Qiagen, USA) and cloned into Top-10 cells using a TOPO TA cloning kit with vector pCR 2.1-TOPO (Invitrogen, USA) for sequencing. QIAprep spin miniprep kits (Qiagen, USA) were used to isolate plasmid DNA from Top-10 cultures grown from individual colonies picked from imMedia Growth Medium agar (Life Technologies, USA). For the spiralin amplicon, clones were submitted for DNA sequencing analysis in triplicate for five carrot samples and duplicate for one sample. A single consensus sequence was identified from 14 clones from five different carrot samples (GenBank accession No. KT377369). The amino acid sequence showed 100% identity and 100% similarity to the *S. citri* Spiralin protein (AF012877.1, Le Dantec *et al*., 1998). An additional consensus sequence was identified

Table 2. The amino acid sequence of all adhesion-related proteins identified from the Mexican carrot samples were compared to known Arps using the Sequence Manipulation Suite (Stothard, 2000). Each set of numbers indicates the % identity / % similarity.

	Sarp1	Scarp2a	Scarp2b	Scarp3a	Scarp3b	Scarp3c	Scarp3d	Scarp4a	Scarp5a
KT377371	**85.44/87.36**	79.39/82.82	82.43/87.03	83.08/85.38	81.92/84.62	81.30/85.11	80.38/83.08	40.22/52.77	38.55/53.41
KT377372	**88.89/89.66**	83.59/85.11	86.19/88.70	80.15/83.59	84.29/86.21	85.50/87.40	76.72/81.30	39.34/51.84	39.20/54.00
KT377373	**84.29/86.21**	79.01/82.44	82.85/87.45	82.69/85.00	81.54/84.23	80.92/84.73	80.77/83.46	40.59/52.77	38.96/53.41
KT377374	**83.52/86.21**	78.24/82.44	82.01/87.45	81.92/85.00	80.77/84.23	80.15/84.73	80.00/83.46	40.22/52.77	38.55/53.41
KT377375	**87.74/88.12**	82.44/84.35	86.61/89.54	80.08/83.52	85.38/86.15	84.35/86.64	77.39/81.99	40.59/52.77	38.96/53.41
KT377376	**87.74/88.12**	81.68/83.59	86.61/89.54	79.31/82.76	86.15/86.92	83.59/85.88	76.63/81.23	40.22/52.40	38.55/53.01
KT377377	**83.52/86.97**	79.77/82.44	82.01/87.45	83.46/85.00	81.54/84.23	81.68/84.73	81.54/83.46	41.33/52.77	39.76/53.41
KT377378	**83.91/85.82**	78.63/82.06	82.43/87.03	82.31/84.62	81.15/83.85	80.53/84.35	80.38/83.08	40.59/52.77	38.96/53.41
KT377379	81.99/83.91	81.68/83.97	**89.96/91.63**	78.16/80.84	83.08/85.00	81.30/83.97	80.84/83.91	39.85/51.66	38.15/52.21
KT377380	77.78/80.08	77.48/80.15	**87.45/88.70**	73.95/77.01	78.85/81.15	77.10/80.15	76.63/80.08	36.16/48.34	36.14/49.80
KT377381	42.49/52.75	42.64/52.19	45.53/56.18	39.78/50.73	42.86/52.38	42.34/53.65	40.51/51.46	**67.10/70.33**	66.93/75.70
KT377382	**88.51/88.89**	81.68/83.59	88.28/90.38	78.24/82.06	83.14/84.67	84.35/86.64	77.86/82.06	38.94/52.21	38.40/53.60
KT377383	**87.36/88.89**	84.35/85.11	85.36/88.70	80.92/83.59	85.06/86.21	86.26/87.40	78.24/82.06	40.44/51.47	41.20/54.40
KT377384	**88.12/89.27**	84.35/84.73	87.03/89.96	80.92/83.21	84.29/85.06	86.26/87.02	78.24/81.68	41.18/52.57	40.40/54.00
KT377385	**88.12/88.51**	82.82/84.73	87.03/89.96	79.39/83.21	83.52/85.06	84.73/87.02	76.72/81.68	40.44/52.57	38.80/53.20
KT377386	**84.29/86.21**	79.01/82.44	82.85/87.45	82.69/85.00	81.54/84.23	80.92/84.73	80.77/83.46	40.59/52.77	38.96/53.41
KT377387	**86.59/87.36**	81.68/83.59	85.77/88.70	81.23/84.29	84.62/85.38	83.59/85.88	78.54/82.76	40.59/52.03	38.96/52.61
KT377388	**87.74/88.12**	82.44/84.35	86.61/89.54	80.08/83.52	85.38/86.15	84.35/86.64	77.39/81.99	40.59/52.77	38.96/53.41
KT377389	**87.74/88.89**	84.73/85.11	85.77/88.70	81.30/83.59	85.44/86.21	86.64/87.40	78.63/82.06	40.07/51.47	40.80/54.40
KT377390	82.38/83.91	81.30/83.97	**89.54/91.63**	77.78/80.84	83.46/85.00	80.92/83.97	80.46/83.91	39.48/51.66	37.75/52.21
KT377391	82.76/84.29	82.06/84.35	**90.38/92.05**	76.63/79.69	83.46/85.38	81.68/84.35	79.31/82.76	39.85/52.40	38.15/53.01
KT377392	81.61/83.91	81.30/83.97	**89.54/91.63**	77.78/80.84	82.69/85.00	80.92/83.97	80.46/83.91	39.85/51.66	38.15/52.21
KT377393	83.91/86.59	83.21/85.11	84.10/88.70	80.46/83.91	85.38/86.92	**85.88/87.40**	79.31/83.14	40.59/52.03	41.37/54.22

from 3 clones from a single carrot (KT377370), containing a single nucleotide polymorphism (SNP) from KT377369 (c.515g > c), and this SNP resulted in a single amino acid change (data not shown).

For *arp1*, clones were submitted for DNA sequencing analysis in triplicate for four carrot samples, as well as singularly for three samples. Sequencing results of *S. citri* identified 11 unique sequences (GenBank accession Nos. KT377371–KT377381). All *arp* DNA sequences were translated to amino acid sequences using the ExPASy Translate Tool (SIB bioinformatics resource portal, Artimo *et al*., 2012), and compared to known adhesion-related proteins including, Sarp1 (AJ972409.1), Scarp2a (AJ969072.1), Scarp2b (AJ969073.1), Scarp3a (AJ969069.1), Scarp3b (AJ969073.1), Scarp3c (AJ969071.1), Scarp3d (AJ969070.1), Scarp4a (AJ969072.1), and Scarp5a (AJ969073.1) using the % identity and similarity function within the Sequence Manipulation Suite (Stothard, 2000; Table 2). ClustalW2 (Larkin *et al*., 2007; Goujon *et al*., 2010) and the ExPASy Boxshade Tool (SIB bioinformatics resource portal, Artimo *et al*., 2012) were used for aligning amino acid sequences.

Ten clones from five different carrot samples (GenBank accession Nos. KT377371–KT377378) had highest homology to an *S. citri* pBJS-O plasmid (AJ972409.1, Joshi *et al*., 2005). The deduced amino acid sequences had a range of 83.5-88.9% identity and 85.8-89.7% similarity to the Sarp1 protein encoded on the plasmid pBJS-O, which was identified in the BR3-3X *S. citri* strain, originally isolated from horseradish (Joshi *et al*., 2005; Fletcher *et al*., 1981). Two clones from different carrot samples (GenBank accession Nos. KT377379 and KT377380) were also identified with high homology to an *S. citri* pSci5 plasmid (AJ969073.1, Berho *et al*., 2005). The amino acid sequences showed 87.5 and 90.0% identity and 88.7 and 91.6% similarity, respectively, to adhesion-related protein 2b (Scarp2b) encoded on the plasmid. Plasmid pSci5 is among seven plasmids described in *S. citri* strain GII3-3X, originally isolated in a leafhopper, *Circulifer haematoceps* in Morocco (Saillard *et al*., 2008). The same 2 clones also showed high homology to an *S. citri* partial P89 gene for adhesin, isolate Giza-Man (GenBank accession No. HE617172.1).

It is interesting to note that primer pair P89-f/P89-r amplified an adhesion-related protein gene other than *arp1*. A closer look at the primer sequences indicates that they are conserved across many *arp* genes, although this was not explored in Yokomi *et al*. (2008). Primer P89-f is conserved in *arp3a* (plasmid, pSci1, GenBank accession No. AJ969069.1), *arp3c* (pSci3, AJ969071.1), *arp2a* (pSci4, AJ969072.1), *arp2b* (pSci5, AJ969073.1), and *arp3b* (pSci5, AJ969073.1), and contains a SNP in *arp3d* (pSci2, AJ969070.1). Likewise, primer P89-r is conserved in *arp3a* (pSci1, AJ969069.1), *arp3d* (pSci2, AJ969070.1), *arp3c* (pSci3, AJ969071.1), *arp2a* (pSci4, AJ969072.1), *arp4a* (pSci4, AJ969072.1), and *arp3b* (pSci5, AJ969073.1).

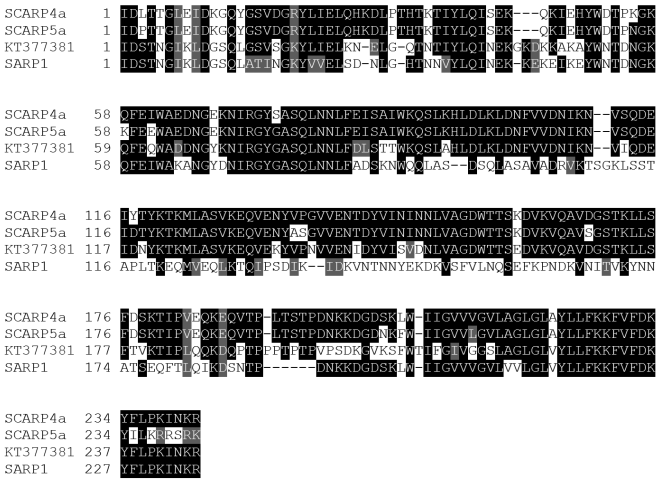

Fig. 1. Amino acid sequence alignment of the adhesion-related protein obtained from a Mexican carrot sample, GenBank accession No. KT377381, to Sarp1, Scarp4a, and Scarp5a. The *S. citri* adhesion-related protein isolated from the Mexican carrot sample was more closely related to Scarp4a and Scarp5a, as compared to Sarp1.

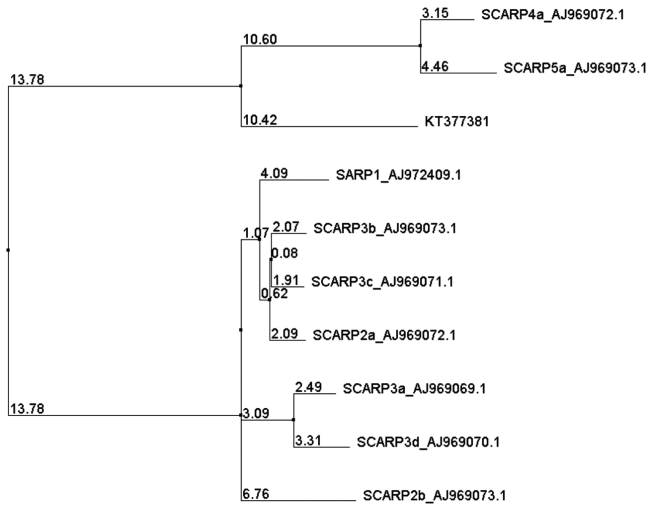

Fig. 2. A neighbor joining tree using percent identity was generated from a multiple sequence alignment of nine adhesion-related protein amino acid sequences with the Mexican carrot sample from this study, GenBank accession No. KT377381. KT377381 showed a close relationship to Scarp4a and Scarp5a.

Therefore, identification of *arp1* and *arp2b* in this study is not surprising, and confirms the presence of *S. citri* in the symptomatic carrot samples from Chaparrosa, Mexico.

Sequence analysis of P89-f/P89-r PCR products identified 3 clones from one carrot sample that generated a single consensus sequence (GenBank accession No. KT377381) with highest homology to an *S. citri* pSci4 plasmid (AJ969072.1, Berho *et al*., 2005) showing 67.1% identity and 70.3% similarity of the amino acid sequence to adhesion-related protein 4a (Scarp4a) encoded on the plasmid. This consensus sequence showed only 42.5% identity and 52.8% similarity to Sarp1 (Fig. 1). Generation of a neighbor joining tree using percent identity (ClustalW2, Larkin *et al*., 2007; Goujon *et al*., 2010; Jalview, Waterhouse *et al*., 2009), revealed that the arp consensus sequence obtained from this carrot sample has a close relationship to Scarp4a and Scarp5a as compared to seven other previously-reported arp proteins (Fig. 2). This consensus sequence may belong to a new, previously unidentified gene encoding an additional adhesion-related protein. Although the amino acid sequence generated was only 42.5% identical to Sarp1, Scarp4a was previously shown to have only 40% identity to Sarp1, and was considered an adhesion-related protein (Saillard *et al*., 2008). Therefore, a novel protein was likely identified in this study belonging to the Arp family.

It has been reported that both the Sarp1 and Spiralin proteins have a role in the insect transmission of *S. citri*, as they are directly involved in the binding interaction between *S. citri* and the leafhopper vector (Yu *et al*., 2000; Killiny *et al*., 2005; Béven *et al*., 2012; Duret *et al*., 2014). A previous study also found that *arp* genes are absent in non-insect transmissible strains of *S. citri* (Berho *et al*., 2005). Therefore, the finding of *S. citri* in carrots collected from Chaparrosa, Mexico, by identification of both *arp* and spiralin genes suggests that the *S. citri* strain(s) present were insect-transmitted. In light of this, 52 *Circulifer tenellus* samples were collected in Zacatecas, Mexico, between September and November 2014. Of the 52 *C. tenellus*, 4 were collected from pepper (*Capsicum annuum* L.) fields, while the remaining 48 were collected near Chupaderos and El Saladillo in Zacatecas, on wild plants located near pepper fields, including *Eruca sativa* Miller (arugula) and *Solanum elaeagnifolium* Cavanilles (silverleaf nightshade) plants. Nucleic acids were extracted from whole insects using a CTAB protocol (Zhang *et al*., 1998) with modifications made by Crosslin *et al.* (2006) for insect samples.

Of the leafhoppers tested, 36.5% were positive for *S. citri* with the P89-f/P89-r primers. All positive leafhopper samples were collected from *E. sativa* plants. Sequencing results of *S. citri* in the leafhopper vector identified 12 unique sequences (GenBank accession Nos. KT377382–KT377393). Eleven clones from five different leafhopper samples (KT377382–KT377389) had highest homology to Sarp1 encoded on *S. citri* pBJS-O plasmid (AJ972409.1, Joshi *et al*., 2005). The deduced amino acid sequences had a range of 90.6-100% identity and 94.9-100% similarity to the Sarp1 sequences identified from carrot in this study. Three clones from two different leafhopper samples (GenBank accession Nos. KT377390–KT377392) were identified with high homology to Scarp2b encoded on the *S. citri* pSci5 plasmid (AJ969073.1, Berho *et al*., 2005), and to *S. citri* partial P89 gene for adhesin, isolate Giza-Man (HE617172.1). The deduced amino acid sequences had a range of 93.6–99.6% identity and 95.3–100% similarity to the Sarp2b sequences identified from carrot in this study.

One clone (KT377393) was found to have high homology to Scarp3c encoded on *S. citri* pSci3 plasmid (AJ969071.1, Berho *et al*., 2005).

The finding of *S. citri* in carrots in Mexico is the first report of this pathogen in this plant species in Mexico. *S. citri* was initially associated with carrot purple leaf disease of carrot plants in Washington and California in the United States, two regions with hot temperatures during the growing season (Lee *et al*., 2006; Mello *et al*., 2008). *S. citri* has since also been reported in the Mediterranean climate of Spain and Israel (Cebrián *et al*., 2010; Gera *et al*., 2011). The climate in Zacatecas, Mexico, is cooler than Washington and California, but *S. citri* can cause infected plants to die within the months following infection in warm semiarid areas as well as hot (Calavan and Bové, 1989). The beet leafhopper has been shown to transmit *S. citri* to carrots (Mello *et al*., 2009), and the presence of *S. citri*- infected insect hosts in this region of Mexico provides additional evidence that the climate in Zacatecas is suitable for the pathogen.

S. citri is capable of causing economic losses in various host crops, and the occurrence of this pathogen in a previously unreported area may raise concern for growers. Furthermore, the presence of the leafhopper, *C. tenellus*, in the same Mexican state, suggests that *S. citri* could be rapidly transmitted among herbaceous crops in this area. Methods to eliminate the insect vector may be necessary to prevent the spread of *S. citri* in this area of Mexico.

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