Silverleaf Nightshade (*Solanum elaeagnifolium*), a Reservoir Host for 'Candidatus Liberibacter solanacearum', the Putative Causal Agent of Zebra Chip Disease of Potato

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Abstract

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Zebra chip disease of potato is caused by the bacterial pathogen 'Candidatus Liberibacter solanacearum' and is a growing concern for commercial potato production in several countries in North and Central America and New Zealand. 'Ca. L. solanacearum' is vectored by the potato psyllid *Bactericera cockerelli*, which transmits the pathogen to several cultivated and wild solanaceous host plants. Silverleaf nightshade (SLN), *Solanum elaeagnifolium*, is a common weed in the Lower Rio Grande Valley of Texas and a host for both the potato psyllid and 'Ca. L. solanacearum'. SLN plants were successfully inoculated with 'Ca. L. solanacearum' under laboratory conditions. Retention studies demonstrated that 'Ca. L. solanacearum'-infected SLN planted in the field in January 2013, concurrent with commercial potato planting, retained the pathogen under field conditions throughout the year despite extensive dieback during summer. The

presence of 'Ca. L. solanacearum' was confirmed in leaves, roots, and stolons of SLN plants collected the following year using polymerase chain reaction. Acquisition assays using *B. cockerelli* adults also revealed that SLN retained the pathogen. Transmission studies determined that *B. cockerelli* can acquire 'Ca. L. solanacearum' within a 2-week acquisition access period on 'Ca. L. solanacearum'-infected SLN and subsequently transmit the pathogen to potato. These results demonstrate that SLN plants can serve as a reservoir for 'Ca. L. solanacearum', providing a source of inoculum for *B. cockerelli* adults colonizing potato the next season. The presence of SLN plants all year round in the LRGV makes the weed an epidemiologically important host. These findings underscore the importance of eradicating or managing SLN plants growing in the vicinity of potato fields to prevent spread of 'Ca. L. solanacearum' and damage caused by zebra chip.

Zebra chip (ZC) disease is an economically important disease of potatoes impacting potato production in the United States, Central America, and New Zealand (Crosslin et al. 2010, 2011, 2012a,b; Liefting et al. 2008; Munyaneza et al. 2007a; Secor and Rivera-Varas 2004). ZC-infected potato plants exhibit yellow to purple discoloration of the foliage, proliferation of auxiliary buds with shortened internodes, swollen nodes, and aerial tubers accompanied by sudden death of plants. A diagnostic symptom of ZC is the appearance of a brown-striped discoloration along the medullary rays of tubers, which becomes highly pronounced after they are fried (Munyaneza et al. 2007a,b). ZC is caused by the bacterium 'Candidatus Liberibacter solanacearum' (Hansen et al. 2008; Liefting et al. 2008, 2009a,b). Thus far, *Bactericera cockerelli* is the only known vector of this pathogen to potato and other solanaceous species (Hansen et al. 2008; Secor et al. 2009). 'Ca. L. solanacearum' also may be transmitted transovarially from *B. cockerelli* females to offspring (Hansen et al. 2008). *B. cockerelli* adults are able to acquire 'Ca. L. solanacearum' within 6 h of an acquisition access period on 'Ca. L. solanacearum'-infected potato plants (Munyaneza 2012) and may be influenced by the host plant from which they acquired the pathogen (Sengoda et al. 2013). Furthermore, *B. cockerelli* adults can transmit 'Ca. L. solanacearum' more efficiently than nymphs (Buchman et al. 2011b) and require

a latent period of about 2 weeks before they can transmit the pathogen to healthy plants (Sengoda et al. 2013). Previous work demonstrated that, in addition to potato, *B. cockerelli* readily transmits 'Ca. L. solanacearum' to other cultivated solanaceous crops, including tomato and pepper (Hansen et al. 2008; Liefting et al. 2008, 2009a,b). Moreover, it has been shown that the adults may transmit 'Ca. L. solanacearum' in as little as 1 h after settling (Buchman et al. 2011a). Although much emphasis has been placed on the ability of *B. cockerelli* to acquire and transmit 'Ca. L. solanacearum' among cultivated solanaceous hosts, relatively little is known regarding its use of wild alternative host plants.

Alternate hosts, especially solanaceous weeds, may contribute significantly to the epidemiology of ZC by serving as refuges for the insect pest and reservoirs for the pathogen (Henne et al. 2010). Very little is known about the role of solanaceous weeds in facilitating 'Ca. L. solanacearum' persistence in the absence of cultivated hosts. Several wild solanaceous species have been reported to serve as breeding and overwintering hosts for potato psyllids. For example, Romney (1939) reported that migrating psyllid populations breed on *Lycium* spp. for several hundred miles along the Rio Grande drainage above Laredo, TX. *B. cockerelli* also has been reported on a number of other wild and cultivated solanaceous hosts that co-occur with the psyllid throughout its range (Crawford 1914; Essig 1917; Janes 1939; Knowlton and Thomas 1934; Pletcher 1947; Wallis 1955). More recently several noncultivated wild solanaceous plants were reported to serve as alternative hosts of both *B. cockerelli* and 'Ca. L. solanacearum' (Henne et al. 2010; Wen et al. 2009).

In this study, we focus on the potential for silverleaf nightshade (SLN) to serve as a pathogen reservoir for 'Ca. L. solanacearum' in inoculum between potato crops in the Lower Rio Grande Valley (LRGV) of Texas. SLN is a broadleaved herbaceous and woody perennial solanaceous weed that is native to northeast Mexico and the southwestern United States, where it is widespread and abundant

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(Robinson et al. 1978). Because SLN is so abundant and difficult to eradicate, the possibility of SLN serving as a significant source of primary inoculum would have important implication for ZC disease management. The main objectives of the study were to assess (i) whether SLN can be infected with '*Ca. L. solanacearum*' by *B. cockerelli* adults; (ii) whether SLN plants infected with '*Ca. L. solanacearum*' during one planting season can retain the pathogen and serve as an '*Ca. L. solanacearum*' reservoir, contributing viable inoculum to the next planting season; and (iii) whether *B. cockerelli* adults can acquire '*Ca. L. solanacearum*' from infected SLN and transmit it to healthy potato

Materials and Methods

Source of plants. SLN seeds were collected from fully mature fruits on SLN plants growing along roadsides close to the Texas A&M AgriLife experiment station in Weslaco, TX. Seeds were planted individually in cone-shaped pots measuring 3 by 3 by 4 cm filled with Metro-Mix 360 growth medium (Sungro Horticultural Distribution, Bellevue, WA) and maintained in a greenhouse at 28 to 30°C under natural light. When seedlings were 1 to 2 weeks old, they were transplanted into 10-cm-diameter square, black plastic pots filled with the same potting mix. SLN plants used in the experiments were 5 to 6 weeks old. Potato seed tubers ('Atlantic') obtained from J. W. Farms (Edinburg, TX) were cut in pieces, allowed to suberize, and planted in square 10-cm-diameter black plastic pots filled with the same potting mix as mentioned above. Potato plants used in the experiments were 3 to 4 weeks old. All plants (potato and SLN) were fertilized once a week and watered three times a week or as needed.

Source of insects. '*Ca. L. solanacearum*'-infected colony. *B. cockerelli* adults were originally collected from a potato field at the Texas A&M AgriLife Experiment Station in Weslaco, TX in 2009 and were continuously reared over several generations on '*Ca. L. solanacearum*'-infected potato plants within BugDorm insect cages (60 by 60 by 60 cm, catalog number BD2120F; MegaView Science Co., Taichung, Taiwan). The cages were kept inside an insectary maintained at 25 to 27°C, 65 to 70% relative humidity, and a photoperiod of 16 h of light and 8 h of darkness. The colonies were tested for '*Ca. L. solanacearum*' once every 2 months using conventional polymerase chain reaction (cPCR) (Buchman et al. 2011b), and 90 to 100% of the insects always tested positive for '*Ca. L. solanacearum*'.

'Ca. L. solanacearum'-free colony. *B. cockerelli* adults originally collected and isolated from Texas fields in 2011 were obtained from the United States Department of Agriculture (USDA) Agricultural Research Service, Wapato, WA and were confirmed to be '*Ca. L. solanacearum*'-free via PCR testing. The '*Ca. L. solanacearum*'-free insects were maintained on potato plants inside BugDorm cages under conditions identical to the '*Ca. L. solanacearum*'-infected colony but in an insectary separated from the '*Ca. L. solanacearum*'-infected colony room. '*Ca. L. solanacearum*'-free *B. cockerelli* colonies were tested once every month for '*Ca. L. solanacearum*' using cPCR to verify they were free of the bacterium.

'*Ca. L. solanacearum*' inoculation to SLN plants using *B. cockerelli* adults. SLN (100 plants, 5 to 6 weeks old) grown under greenhouse conditions within cages (to prevent accidental infection) were selected for the study. Seven *B. cockerelli* adults from the '*Ca. L. solanacearum*'-infected colony were restrained within white organza pouches (7.5- by 10-cm organza drawstring pouches, handmade, supplied from various sources) tied to a lower-tier leaf on each of the 100 plants. The netted bags ensured that insects had room to feed and move about on the leaves but not escape. After a 1-week inoculation access period (IAP), the entire bag containing *B. cockerelli* adults along with the leaf was clipped off the plant, as demonstrated for potato and tomato (Levy et al. 2011). Three weeks after initial inoculation, the plants were tested for the presence of '*Ca. L. solanacearum*' via cPCR using DNA extracted from leaf midrib samples collected from upper- and middle-tier leaves (Buchman et al. 2011b).

Retention of '*Ca. L. solanacearum*' in field-planted SLN. Thirty-six SLN plants that tested positive for '*Ca. L. solanacearum*' in the inoculation study described above were planted in the field in January 2013 inside field cages (soil emergence trap-headless insect

rearing tent, 60 by 60 by 60 cm, catalog number BT2007; BugDorm store, MegaView Science Co.) to avoid incidental infestation from resident *B. cockerelli* (Fig. 1). The plants remained completely caged throughout the entire period of study, except for collection of samples (e.g., leaves, roots, or stolons) and exposure of *B. cockerelli* adults for the acquisition study, which were done via the zippered opening on the side of the cages (Fig. 1, insert). One year later, several of the SLN plants lost their leaves due to extensive dieback and were gradually refoiling. Leaves from the upper and middle tier were also collected and DNA was extracted and tested for '*Ca. L. solanacearum*' using quantitative real-time PCR (qPCR). Roots and stolons were sampled by removing the top soil to a depth of 15 cm. Thin, fibrous roots and stolons close to the stem were collected from 20 plants. DNA samples from the roots and stolons were extracted and pooled as one sample per plant and tested for '*Ca. L. solanacearum*' using qPCR, as described below. Psyllid acquisition assays were performed to confirm retention of the pathogen in field-planted '*Ca. L. solanacearum*'-infected SLN. Seven '*Ca. L. solanacearum*'-free adults contained in 1,000- μ l pipette tips were released within white organza pouches tied to a middle- and lower-tier branch of each plant; each pouch surrounded at least three leaves. Acquisition assays were performed in March 2014 on 20 SLN plants that were inoculated with '*Ca. L. solanacearum*' the previous year and retained under field conditions. At 3 and 5 weeks after initial *B. cockerelli* exposure, the pouches were clipped off from the plants, adults were collected, and DNA was extracted and tested for the presence of '*Ca. L. solanacearum*' using qPCR.

***B. cockerelli* acquisition of '*Ca. L. solanacearum*' from SLN and transmission to potato under laboratory conditions.** Twenty of the SLN plants that tested positive for '*Ca. L. solanacearum*' in the inoculation study described above were placed into two BugDorm insect cages (75 by 75 by 115 cm, catalog number BD2400; MegaView Science Co.) with 10 plants each. Five hundred *B. cockerelli* adults from a '*Ca. L. solanacearum*'-free colony were released inside each cage for acquiring '*Ca. L. solanacearum*' and allowed to remain on the '*Ca. L. solanacearum*'-infected SLN plants in both cages. Periodically, starting 5 days after release, 10 to 15 *B. cockerelli* adults were randomly drawn from each cage and tested for the presence of '*Ca. L. solanacearum*' using cPCR. Reliable detection of '*Ca. L. solanacearum*' in *B. cockerelli* has been reported by Crosslin et al. (2011) using cPCR and qPCR. After detecting '*Ca. L. solanacearum*' in the adults, three 3- to 4-week-old potato plants were placed within both cages to allow the adults to feed on potato. After 3 and 4 weeks of IAP, potato leaf samples were collected (three from top- and three

Fig. 1. '*Candidatus Liberibacter solanacearum*'-infected silverleaf nightshade (SLN) plants in the field covered with soil emergence cages to avoid incidental psyllid infestation. Picture in insert shows white organza pouches with *Bactericera cockerelli* adults for insect acquisition studies.

from middle-tier leaves) and pooled by plant (e.g., six plants = six samples) prior to being tested for '*Ca. L. solanacearum*' by cPCR, using DNA extracted from leaf midribs and petioles.

DNA extraction and PCR. *Plant DNA extraction.* Total genomic DNA was extracted from plant tissues using the cetyltrimethylammonium bromide (CTAB) method, as described previously by Buchman et al. (2011b), with modifications. A portion of the leaf midrib (or root or stolon) measuring approximately 0.3g constituted a sample. Individual plant samples were washed thoroughly with water and dried on paper towels to ensure that they were free of *B. cockerelli* eggs or nymphs. Each sample was cut into small pieces, placed in a 2-ml tube (Lysing Matrix A; MP Biomedicals, Santa Ana, CA) with 1,000 μ l of plant extraction buffer, and pulverized for 4 min using a Mini Beadbeater-96 according to the manufacturer's specifications (BIOSPEC Products Inc., Bartlesville, OK). Tubes were centrifuged at 12,000 rpm for 2 min, and 300 μ l of the supernatant was pipetted into 1.5-ml microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA) containing 80 μ l of lysozyme and incubated in a dry bath for 30 min at 37°C. CTAB buffer (500 μ l) was added to the samples and incubated for 30 min at 65°C. The samples were vortexed and centrifuged at 12,000 rpm for 10 min after adding 500 μ l of chloroform. The supernatant was transferred to another 1.5-ml microcentrifuge tube containing 1.3 μ l of glycogen and 500 μ l of isopropanol and placed on ice for 30 min. The precipitated DNA pellet was rinsed with 70% ethanol and centrifuged at 12,000 rpm for 2 min. The ethanol was drained and the pellet was air dried at 37°C free of ethanol. The DNA pellet was then resuspended in 50 μ l of nuclease-free water.

Psyllid DNA extraction. Total genomic DNA was extracted from individual psyllids using the CTAB extraction method described by Buchman et al. (2011b). Individual psyllids were crushed in 500 μ l of CTAB buffer in a 1.5-ml microcentrifuge tubes (Thermo Fisher Scientific) using an Eppendorf micropestle (number 2317314; Sigma-Aldrich, St. Louis). Samples were incubated for 30 min at 65°C, and 500 μ l of chloroform was added to samples at room temperature. The samples were vortexed and centrifuged for 3 min at 12,000 rpm. The supernatant was transferred to a new 1.5-ml microcentrifuge tube containing 1.3 μ l of glycogen and 500 μ l of isopropanol and placed on ice for 30 min. The precipitated DNA pellet was rinsed with ice-cold 70% ethanol and centrifuged at 12,000 rpm for 2 min. The ethanol was drained and the pellet was air dried, then resuspended in 100 μ l of nuclease-free water.

PCR assay for 'Ca. L. solanacearum' detection in plant and insect hosts. At the start of experiments during the year 2013, cPCR was

used to detect '*Ca. L. solanacearum*' in insect and plant samples. One year later, '*Ca. L. solanacearum*' was detected using qPCR methods, as described below, due to increasing volume of samples tested.

cPCR. '*Ca. L. solanacearum*' detection was performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) in 21- μ l reactions containing 9 μ l of 2 $\times$ Phusion flash high-fidelity-PCR master mix (Thermo Fisher Scientific), 1 μ l each of OA2/OI2c primers (Liefting et al. 2009a) (10 pmol concentration), 9 μ l of water, and 1 μ l of template DNA. Amplification of the β -tubulin gene Btub1F 5'-TGATTTCGAAGGTAAGGGAGGA-3' and Btub1R 5'-CATGTTGCTCTCGGCTTCAG-3' (Ravindran et al. 2011) indicated quality of DNA extracted from potato and SLN plant samples. Quality of *B. cockerelli* DNA extractions was confirmed using BC 28S F 5'-GCGAGGACTCAGTTTCGTGT-3' and BC 28S R 5'-AGAGCTCGACTCGGATTGTC-3' (Hansen et al. 2008). PCR conditions for the above primer sets were 98°C for 10 s, followed by 35 cycles of 98°C for 1 s, 60°C for 5 s, 72°C for 15 s, and 72°C for 1 min. The PCR products were visualized by electrophoresis on 1% agarose gels with ethidium bromide staining. The presence of the predicted 1,168-bp 16S ribosomal DNA band for the OA2/OI2c F/R primer pair was indicative of samples being '*Ca. L. solanacearum*' positive.

qPCR. qPCR reactions were performed using optical-grade 0.2-ml thin-walled tube strips with individually attached optically clear flat caps (Temp Assure PCR 8-strips; USA Scientific, Inc., Ocala, FL). Detection and amplification of '*Ca. L. solanacearum*' were performed by simplex and multiplex PCR assays using the ZC primer-probe set (Li et al. 2009). The 25- μ l reaction mix consisted of 2 μ l of template DNA extract, 9 μ l of Phusion high-fidelity PCR Master Mix (Thermo Scientific, Pittsburg), 3 μ l of 0.1 μ M ZC probe, 3 μ l of 0.25 μ M of sense and antisense primers, and 8 μ l of nuclease-free water. The amplification was carried out using a C1000 Touch thermal Cycler (CFX96 Real-Time System; Bio-Rad Laboratories). A plant mitochondrial cytochrome oxidase-based primer-probe set (Li et al. 2006) was used as internal quality control to verify quality of plant DNA extractions. Each qPCR consisted of 96 sample tubes and included the following controls: (i) known '*Ca. L. solanacearum*'-positive control DNA, (ii) '*Ca. L. solanacearum*'-free plant or psyllid DNA, and (iii) nontemplate water control. The control reactions were run on separate eight-tube strips to avoid possible contamination of test samples. PCR cycling conditions were 98°C for 10 s followed by 35 cycles of 98°C for 1 s and 58°C for 10 s. The cycle threshold (Ct) value was determined by the Bio-Rad CFX manager software and a cutoff value of 35 was established for samples to test positive for '*Ca. L. solanacearum*'.

Results

'*Ca. L. solanacearum*' inoculation to SLN plants. Out of the 100 SLN plants inoculated with '*Ca. L. solanacearum*'-infected *B. cockerelli* adults under laboratory conditions, 56 plants were confirmed to be '*Ca. L. solanacearum*' positive (Fig. 2) and, of those, 36 were used in subsequent retention and 20 in acquisition studies. The quality of DNA extractions were confirmed using Btub-F/R primers (Fig. 3).

Retention of '*Ca. L. solanacearum*' in field-planted SLN. Of the 36 of 56 '*Ca. L. solanacearum*'-positive SLN planted in the field in January 2013, many of the SLN plants had shed their foliage during the summer but most had re-foliated by the following year. Twenty-eight plants had leaves for sampling in January 2014. Testing of leaf samples revealed that only 1 of 28 plant samples was found to be '*Ca. L. solanacearum*' positive, with a Ct value < 35. Similar results of poor diagnosis of leaf samples have been previously reported (Levy et al. 2011; Wen et al. 2009). Further testing of lateral stolons and roots showed that 4 of 20 samples were '*Ca. L. solanacearum*' positive. Results of insect acquisition assays conducted on field-planted SLN that were inoculated with '*Ca. L. solanacearum*' a year ago are summarized and presented in Table 1. DNA extracted from seven *B. cockerelli* adults within each organza pouch (pooled sample) was tested for the presence of '*Ca. L. solanacearum*' and results showed that 15 of 17 samples tested positive for '*Ca. L.*

Fig. 2. Silverleaf nightshade (SLN) plant DNA tested for '*Candidatus Liberibacter solanacearum*' using OA2/OI2c F/R primers. Lanes 1–10 indicate DNA extracted from individual SLN plants; + indicates positive control (e.g., an '*Ca. L. solanacearum*'-infected plant sample) whereas – indicates no-template DNA (e.g., a water sample). Lane M = 1-kb DNA ladder (Invitrogen).

Fig. 3. Silverleaf nightshade (SLN) plant DNA tested for quality of the DNA extraction using Btub1F/R primers. Lanes 1–10 indicate individual SLN plants; + indicates positive control (e.g., an '*Candidatus Liberibacter solanacearum*'-infected plant DNA sample) whereas – indicates no-template DNA (e.g., a water sample). Lane M = 1-kb DNA ladder (Invitrogen).

Table 1. ‘*Candidatus Liberibacter solanacearum*’ acquisition by *Bactericera cockerelli* adults from field-planted, ‘*Ca. L. solanacearum*’-infected SLN plants^a

Sample	<i>B. cockerelli</i> release (March 2014)	
	Ct value (3 WAE)	Ct value (5 WAE)
1	18.18	23.94
2	15.87	17.68
3	17.86	18.85
4	25.78	28.01
5	16.66	20.71
6	33.91	22.79
7	19.95	25.89
8	18.54	21.61
9	29.03	19.35
10	22.49	21.60
11	18.51	18.99
12	22.13	25.71
13	17.89	23.97
14	ND	21.03
15	33.93	ND
16	24.32	25.21
17	(38.24)	(37.02)
Controls		
Infected ^b	14.2	...
Uninfected	ND	...
Water	ND	...

^a Number of polymerase chain reaction (PCR) cycles required to get fluorescence signal is represented by cycle threshold (Ct) values obtained from real-time quantitative PCR performed on DNA samples of *Bactericera cockerelli* adults 3 and 5 weeks after initial exposure (WAE; collection I and II, respectively). Ct values ≤ 35 are considered positive for ‘*Ca. L. solanacearum*’. CT values > 35 are considered negative for ‘*Ca. L. solanacearum*’ and are shown in parentheses. ND = not detected and indicates that ‘*Ca. L. solanacearum*’ could not be detected in these samples.

^b ‘*Ca. L. solanacearum*’-infected.

solanacearum’ both 3 and 5 weeks after initial exposure on ‘*Ca. L. solanacearum*’-infected SLN plants. In all assays, the Ct values ranged from 15.87 to 33.93 (Table 1).

***B. cockerelli* acquisition of ‘*Ca. L. solanacearum*’ from ‘*Ca. L. solanacearum*’-infected SLN under laboratory conditions.** In all, 20 of 56 ‘*Ca. L. solanacearum*’-positive plants generated earlier were used in this experiment. At 5 days after release of ‘*Ca. L. solanacearum*’-free *B. cockerelli* into cages of ‘*Ca. L. solanacearum*’-infected SLN, 10 to 15 adults were randomly collected and tested for ‘*Ca. L. solanacearum*’. No psyllids tested ‘*Ca. L. solanacearum*’ positive 5 days after release (Fig. 4). PCR amplification using the BC28S F/R primers confirmed that quality of the DNA extraction was good (Fig. 5). In total, 1 of 10 adults tested positive 2 weeks later, and 3 of 14 and 4 of 15 adults tested positive 4 and 6 weeks later, respectively (Fig. 4). Thus, we report that *B. cockerelli* adults can acquire ‘*Ca. L. solanacearum*’ within 2 weeks of exposure to ‘*Ca. L. solanacearum*’-infected SLN plants. Following ‘*Ca. L. solanacearum*’ acquisition by psyllid adults from SLN, three 3- to 4-week-old potato plants were placed in each of the two cages, allowing the psyllids access to the potato plants for 3 weeks. No leaves from the six plants sampled 3 weeks after exposure to the psyllids tested positive for ‘*Ca. L. solanacearum*’. However, 4 weeks after IAP, one of six plants tested positive for ‘*Ca. L. solanacearum*’ (i.e., 4 weeks after exposure to *B. cockerelli*) This result shows that psyllids acquiring ‘*Ca. L. solanacearum*’ from ‘*Ca. L. solanacearum*’-infected SLN plants are able to transmit the pathogen to potato plants.

Discussion

Preliminary studies were conducted by Henne et al. (2010), inoculating wild solanaceous hosts with ‘*Ca. L. solanacearum*’ using ‘*Ca. L. solanacearum*’-infected *B. cockerelli* adults. Test results revealed that, of the three wild hosts—SLN, buffalobur nightshade (*Solanum rostrum*), and Berlandier wolfberry (*Lycium berlandierii*)—buffalobur nightshade plants died quickly after being infected with ‘*Ca. L.*

5 days after release –(0/10)

2 weeks after release- (1/10)

4 weeks after release- (3/15)

6 weeks after release – (4/15)

Fig. 4. ‘*Candidatus Liberibacter solanacearum*’ acquisition by *Bactericera cockerelli* adults collected 5 days and 2, 4, and 6 weeks after release on ‘*Ca. L. solanacearum*’-infected silverleaf nightshade in the laboratory. Lanes 1–15 indicate individual *B. cockerelli* adults; + indicates positive control (e.g., a known ‘*Ca. L. solanacearum*’-infected *B. cockerelli* sample) whereas ‘H₂O’ indicates no-template DNA (e.g., a water sample; ‘cold psyllid’ indicates negative control (e.g., a known ‘*Ca. L. solanacearum*’-free *B. cockerelli* DNA sample). Lane M = 1-kb DNA ladder (Invitrogen).

solanacearum' whereas SLN harbored 'Ca. L. solanacearum' but was not killed by the pathogen and motivated the present study.

Studies to evaluate SLN as a ZC pathogen reservoir host were initiated by inoculating 'Ca. L. solanacearum' in SLN plants using 'Ca. L. solanacearum'-infected *B. cockerelli* adults to determine acquisition, retention, and transmission of the pathogen from and to SLN. Although nymphs are reported to harbor the pathogen (Rush et al. 2010), adults are more efficient in transmitting 'Ca. L. solanacearum' (Buchman et al. 2011b). Results of the present study confirm that 'Ca. L. solanacearum' is transmitted to SLN through feeding by *B. cockerelli* adults and demonstrate for the first time that 'Ca. L. solanacearum'-free *B. cockerelli* adults can acquire the pathogen from 'Ca. L. solanacearum'-infected SLN and then infect potato. The present study further demonstrated that 'Ca. L. solanacearum'-infected SLN in the field can retain the bacterium from one potato planting season to the next. This was confirmed through testing of above- and belowground plant samples and also through acquisition assays by 'Ca. L. solanacearum'-free *B. cockerelli* adults. Taken together, these findings demonstrate that SLN can serve as a reservoir host, carrying the pathogen over the summer and into the next field season. Given the abundance of this weedy species in the LRGV, SLN may be an overlooked source of primary inoculum bridging potato cropping cycles.

As previously reported, 'Ca. L. solanacearum' detection in leaves of potentially infected plants using PCR-based methods is relatively unreliable (Wen et al. 2009) and could possibly be attributed to low frequency of 'Ca. L. solanacearum' infection in SLN and potato (Thinakaran et al. 2013). For example, Levy et al. (2011) demonstrated that, although plants may become infected by 'Ca. L. solanacearum' after only 1 week of insect feeding, 'Ca. L. solanacearum' may not be detected in leaf midrib or petiole samples until 2 to 3 weeks after infestation, and then most frequently in younger leaves (e.g., upper- and middle-tier leaves). One of six samples from potato plants tested 4 weeks after exposure to 'Ca. L. solanacearum'-infected *B. cockerelli* adults (e.g., having acquired 'Ca. L. solanacearum' from infected SLN) was positive for 'Ca. L. solanacearum'. Based on this, we report that *B. cockerelli* insects acquiring 'Ca. L. solanacearum' from 'Ca. L. solanacearum'-infected SLN plants are able to transfer the pathogen to potato plants but at a low frequency, as determined by PCR. Again, it is likely that the actual rate of plant infection may have been higher. The emphasis of this study was to demonstrate whether SLN plants are epidemiologically important reservoir hosts of the pathogen. Assessing rate or magnitude of 'Ca. L. solanacearum' transmission is beyond the scope of this study and will form part of a separate study designed for the purpose. Amplification of the β -tubulin gene was used as a positive control to verify the quality of DNA extractions (Ravindran et al. 2011). The primer pairs OA2/OI2c were used for 'Ca. L. solanacearum' detection; these are currently widely used and shown to provide relatively reliable detection (Liefing et al. 2009b). However, Crosslin et al. (2011) clearly demonstrated unambiguous detection of 'Ca. L. solanacearum' in DNA samples of *B. cockerelli* adults using conventional and quantitative real-time PCR methods. This was also evident in our observations from insect acquisition assays (Table 1; Fig. 4).

Interestingly, 'Ca. L. solanacearum' was detected more frequently in roots and stolons of field-planted SLN compared with the leaf samples. Consistent with the hypothesis that bacterial titers may be higher in roots and/or stolons, 1/5 of the surviving plants tested positive for 'Ca. L. solanacearum' in the pooled stolon or root samples but only 1/28 tested positive for 'Ca. L. solanacearum' in the leaf

midrib samples. Furthermore, *B. cockerelli* adults that fed on 'Ca. L. solanacearum'-infected SLN acquired the pathogen. These results confirmed that 'Ca. L. solanacearum' was present in SLN at titers high enough to be acquired by insects but perhaps too low for PCR detection in the leaves sampled. Similar reports have been made of more reliable detection of 'Ca. Liberibacter' strains in the roots as compared with leaves of citrus (Johnson et al. 2014; Kunta et al. 2014) and other solanaceous hosts (Wen et al. 2009). These results demonstrated that 'Ca. L. solanacearum' was retained within SLN plants from one cropping season to the next despite dieback of above-ground tissues during the summer months.

If bacterial translocation patterns follow source-to-sink dynamics within the phloem, it can be hypothesized that the highest 'Ca. L. solanacearum' titers might be within the most significant resource sinks, including metabolically active roots or storage tissues such as stolons and tubers. Under natural conditions, SLN grows as a shrub, putting forth stolons that give rise to new daughter plants following death or separation from the mother plant (Fig. 6). This growth habit is somewhat similar to potato in that both SLN and potato reproduce vegetatively via underground stems (stolons versus tubers in the case of SLN and potato, respectively) and sexually by producing seed. The distribution of SLN plants is often observed as patches (J. Thinakaran, personal observation), suggesting that reproduction through vegetative means is common. The plants also have a very deep taproot system that can grow up to 1 m long, which makes the plant hardy and difficult to eradicate. A longstanding question for understanding the epidemiology of ZC has been identifying the source of the 'Ca. L. solanacearum' primary inoculum. A central hypothesis has been that 'Ca. L. solanacearum' is conveyed to the LRGV annually by migrations of 'Ca. L. solanacearum'-infected *B. cockerelli* adults, rather than via population surges of 'Ca. L. solanacearum'-infected *B. cockerelli* residents. In support of this hypothesis, previous research suggests that, at the end of the potato cropping season, *B. cockerelli* numbers in the LRGV are greatly diminished (Thinakaran 2014), with potato psyllids migrating northward from their breeding areas in South Texas as reported by Romney (1939) and Janes (1939). Furthermore, remaining psyllids may not retain 'Ca. L. solanacearum' because it has been reported that 'Ca. L. solanacearum' is sensitive to temperatures exceeding 35°C (Munyanza et al. 2012), temperatures that commonly occur during summers in the LRGV. In the absence of 'Ca. L. solanacearum'-infected *B. cockerelli* adults, primary inoculum would need to come in with 'Ca. L. solanacearum'-infected *B. cockerelli* or be acquired locally by arriving 'Ca. L. solanacearum'-free migrant adults. Although migration of 'Ca. L. solanacearum'-infected adults may be the principal source of primary inoculum, our findings indicate that SLN may serve as a reservoir of the pathogen that can be acquired by 'Ca. L. solanacearum'-free resident and migrant *B. cockerelli* populations, thus increasing the probability of ZC incidence.

The importance of alternative host plants, especially solanaceous weeds that may contribute to epidemiology of ZC by serving as refuges for the insect pest and reservoirs for the pathogen in the absence of cultivated species, is emphasized. The results presented here

Fig. 5. DNA quality of *Bactericera cockerelli* samples (collected after 5 days) tested for 'Candidatus Liberibacter solanacearum' using BC 28S F/R primers. Lanes 1–10 indicate individual *B. cockerelli* samples; + indicates positive control (e.g., a known 'Ca. L. solanacearum'-infected *B. cockerelli* sample) whereas – indicates no-template DNA (e.g., a water sample). Lane M = 1-kb DNA ladder (Invitrogen).

Fig. 6. Growth of silverleaf nightshade in the field. Mother plants die, giving rise to daughter plants from stolons.

demonstrate that SLN can serve as a pathogen reservoir bridging crop cycles. Future work is necessary to determine whether ‘*Ca. L. solanacearum*’ infection can be transferred to and retained within daughter plants and whether, once infected, ‘*Ca. L. solanacearum*’ can persist within SLN for more than one growing season. Given the abundance of SLN in the LRGV, these findings, coupled with the potential for long-term maintenance of the pathogen in clonal populations, clearly indicate the importance of eradicating SLN plants to prevent them from serving as a source of primary inoculum for vector transmission of ‘*Ca. L. solanacearum*’ to potato fields. Identification of potential pathogen reservoirs in other potato-growing regions may lead to a better understanding of the ZC epidemiology, especially as the disease continues to spread to new potato-growing regions.

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