Dynamics of ‘*Candidatus Liberibacter asiaticus*’ Colonization of New Growth of Citrus

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ABSTRACT

‘*Candidatus Liberibacter asiaticus*’ is a phloem-colonizing intracellular bacterial pathogen of citrus associated with the disease huanglongbing. A study of patterns of colonization and bacterial population growth in new growth of different citrus types was conducted by pruning infected citron, sweet orange, sour orange, mandarin, citrange, and *Citrus macrophylla* trees to force the growth of axillary and adventitious shoots. The first three leaves on newly emerged shoots were collected at 30, 60, and 90 days to assess colonization and population growth of ‘*Ca. L. asiaticus*’ using real time PCR (qPCR). Single trials were conducted with mandarin and citron, two trials each for citrange, sour orange and sweet orange, and four trials for *C. macrophylla*. In citron the proportion of colonized leaves increased significantly over time, with 67, 85, and 96% of leaves colonized at 30, 60, and 90 days, respectively. For the other

citrus types, the exact proportion of colonized leaves differed, but colonization exceeded 60% in mandarin, sour orange, and citrange, and exceeded 80% at 30 days in two trials with sweet orange and three trials with *C. macrophylla*, but there was no significant increase in the proportion of colonized leaves at 60 and 90 days. Bacteria were readily detected by 30 days in new leaves of all citrus types. Differences in the growth of the bacterial population between citrus types and at different times of the year were noted, but common trends were apparent. In general, bacterial titers peaked at 60 days, except in leaves of *C. macrophylla* where bacterial titers peaked at 30 days. The early and consistently high proportion of leaf colonization observed for new growth of sweet orange during two trials and for *C. macrophylla* during three trials indicates a near synchronous colonization of new leaves by 30 days.

Huanglongbing (HLB) is a disease of citrus associated primarily with infection by the alphaproteobacteria ‘*Candidatus Liberibacter asiaticus*’. In Brazil, both ‘*Ca. L. asiaticus*’ (Coletta-Filho et al. 2004) and another species, ‘*Ca. L. americanus*’, have been associated with HLB (Teixeira et al. 2005a, b). In the United States, the three major citrus producing states are Florida, California, and Texas. The disease has become widespread and destructive in Florida, with, by 2016, the decline in fruit production nearing 50% (Singerman and Useche 2016; Spreen and Zansler 2016) since the confirmation of the disease and pathogen in the state in 2005 (Halbert 2005). To a more limited extent, the pathogen and disease are now spreading in Texas where both were confirmed present in residential trees and in commercial citrus plantings in 2012 (da Graça et al. 2015). The pathogen and disease have been confirmed in trees in suburban areas in southern California (Kumagai et al. 2013), but to date neither has been found in trees in the major areas of commercial citrus production.

In the field, feeding by the Asian citrus psyllid (*Diaphorina citri* Kuwayama) is the likely predominant mode of dissemination and transmission of the pathogen (Halbert and Manjunath 2004; Inoue et al. 2009; Pelz-Stelinski et al. 2010). Feeding by adult psyllids injects bacteria into phloem sieve elements and the bacteria colonize these cells as intracellular pathogens and use the interconnected nature of the phloem cells to colonize multiple tissues in the tree (Folimonova and Achor 2010; Tatineni et al. 2008). Experimentally, insects (Inoue et al. 2009; McCollum et al. 2016; Pelz-Stelinski et al. 2010), grafting with infected bud wood or leaves (Coletta-Filho et al. 2010; Hilft and Lewis 2016; Lopes et al. 2009) and the parasitic plant dodder (*Cuscuta* spp.) (Garnier and

Bové 1983; Hartung et al. 2010) have been used to transmit ‘*Ca. L. asiaticus*’.

The confirmation of the presence of ‘*Ca. L. asiaticus*’ in plant and insect tissues is by detection of bacterial DNA by PCR, primarily quantitative real-time PCR (qPCR) (Li et al. 2006). For diagnosis and confirmation in samples from field trees, leaves showing a symptom known as blotchy-mottle, considered the hallmark of infection with ‘*Ca. L. asiaticus*’ and of the HLB disease are preferred for testing. Bacterial DNA has been detected in many tissues, including bark, flower petals, pistils, stamens, fruit peduncles and columellas, seed coats, bark (Li et al. 2009; Tatineni et al. 2008), and roots (Johnson et al. 2014; Li et al. 2009; Tatineni et al. 2008).

Despite reasonable effort, ‘*Ca. L. asiaticus*’ has not been established in a continuous laboratory culture so currently it is considered noncultivable (Davis et al. 2008; Parker et al. 2014; Sechler et al. 2009). Its metabolic capabilities and requirements can be deduced from its genomic sequence (Duan et al. 2009; Tyler et al. 2009) but not verified by growth in a laboratory batch culture. Nothing is known in detail about its growth dynamics.

Whether bacteria are introduced into a plant by grafting or insect inoculation, they enter at a specific point of inoculation and from that point disperse to establish a systemic infection throughout the plant. The perspective on the movement of ‘*Ca. L. asiaticus*’ in infected trees is that the bacteria move from infected tissues that function as sources of photosynthate production to noninfected tissues, which are sinks for photosynthate movement and uptake (Wang et al. 2017). Although genes for flagellin are coded by the ‘*Ca. L. asiaticus*’ genome (Duan et al. 2009; Tyler et al. 2009; Zou et al. 2012), flagella have not been observed on bacterial cells imaged by microscopy, so it is not clear if movement along this path is active or passive.

As part of a study on the colonization of new tissues by ‘*Ca. L. asiaticus*’, infected trees were pruned to induce new growth (sink tissues) and leaves from new shoots were collected at 30, 60, and 90 days after their emergence to assess the presence and titer of ‘*Ca.*

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L. asiaticus'. We present information from this study which shows that in the citrus types studied the majority of the new growth forced by pruning was near-synchronously infected within the first 30 days after emergence and that bacterial populations in this tissue developed to an easily detectable level by 30 days, followed by significant increases in bacterial titer in this new growth during the remaining trial period. We discuss the potential application of this behavior to studying basic aspects of '*Ca. L. asiaticus*' biology in planta and we discuss how this behavior can be used in a small tree system for evaluating compounds for antimicrobial activity against '*Ca. L. asiaticus*'.

MATERIALS AND METHODS

Propagation and care of plants. Six citrus types were chosen for this study. Named cultivars studied were Cleopatra mandarin (*Citrus reticulata* Blanco), Ridge Pineapple sweet orange (*C. sinensis* (L.), and Carrizo citrange (*C. sinensis* (L.) × *Poncirus trifoliata* (L.) Raf.). In addition, seedlings of a standard selection of sour orange (*C. aurantium* L.), citron (*C. medica* L.), and *C. macrophylla* Wester (aka Alemow) were studied. Seeds of Ridge Pineapple, sour orange, and Cleopatra were obtained from trees maintained on the U.S. Department of Agriculture-Agricultural Research Service A.H. Whitmore Foundation Farm in Leesburg, FL. Seeds of *C. macrophylla* and Carrizo were obtained from Lyn Citrus Seed, Inc., Arvin, CA. Citron seeds were obtained either from trees maintained in the greenhouse at the U.S. Horticultural Research Laboratory, Fort Pierce, FL or from the University of California Lindcove Research and Extension Center, Exeter, CA. During the study trees were grown in Pro Mix B soil mix (Premier Tech Horticulture, Rivière-du-Loup, Quebec, Canada) using D40H Deepots (0.66 liters) obtained from Stuewe & Sons, Inc., Tangent, OR. Seedlings were fertilized weekly with a general 20-10-20 liquid fertilizer and watered as needed. During the study, trees were maintained in a greenhouse where temperatures were moderated by evaporative cooling and by supplemental heating in the winter when needed.

Inoculation of plants with '*Ca. L. asiaticus*'. All trees in this study were individual seedlings which had been infected with '*Ca. L. asiaticus*' prior to the start of the experiment. Trees were infected with '*Ca. L. asiaticus*' by graft inoculation with a single, HLB-symptomatic leaf as described previously (Hilf and Lewis 2016). Approximate tree ages in months at the time of inoculation were 16, 7, 17, 8, 12 or 16, and 9 months for Cleopatra, citron, Carrizo, sour orange, Ridge Pineapple, and *C. macrophylla*, respectively. Approximate age of trees at the start of these trials were 32, 19, 30, 20, 28 or 32, and 18 months for Cleopatra, citron, Carrizo, sour orange, Ridge Pineapple and *C. macrophylla*, respectively.

Prior to this study, qPCR, as outlined in Materials and Methods, was used to verify infection by '*Ca. L. asiaticus*' in each tree.

Nucleic acid extraction and assaying for bacterial DNA in plant tissue. Plant tissue (0.18 g) was pulverized in API buffer (Qiagen, Valencia, CA) in a 2.0-ml microcentrifuge tube using a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK). The DNA was extracted from pulverized tissue with the DNeasy Plant Mini Kit (Qiagen). qPCR to detect the '*Ca. L. asiaticus*' 16S rRNA gene sequence was performed as described by Li and Hartung (2006), in 20 µl with 25 ng of DNA, 0.25 µM each of the forward and reverse primers and 0.15 µM probe, and 2× TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) with one cycle of denaturation at 95°C for 20 s followed by 40 cycles of denaturation for 3 s at 95°C and annealing/extension at 60°C for 30 s using an Applied Biosystems 7500 Fast Real-Time PCR System. For each DNA sample three equivalent volumes were analyzed. The number of bacterial cells per sample was calculated using the following regression equation: $\log \text{ copy number} = (\text{Ct} - 36.3) / -3.16$, where 'Ct' is the derived cycle threshold value. The final

numbers of bacteria per sample were calculated taking into consideration the sample volume and the sample tissue mass and presented as $\log_{10}$ colony forming units (CFU) per gram.

Data analysis. Statistical processing and presentation of the data generated by the project was carried out using Excel and R Statistical software (R Core Team 2015). Prior to statistical analysis, preliminary data exploration was carried out to demonstrate that no further transformation was required to satisfy the assumptions of the proposed statistical comparison methods. For both the '*Ca. L. asiaticus*' colonization proportion and population measurements, data presented were the mean values (mean ± SE) across replicates at 30, 60, and 90 days postpruning. Analysis of variance (ANOVA) with Tukey's honest significant difference test was used to compare '*Ca. L. asiaticus*' development across different citrus type and assessment time point. The significance level for all tests was set at 0.05.

RESULTS

Selection of branches and leaves for analysis. Infected trees were pruned to remove the apex and all side branches to encourage growth of new adventitious and axillary shoots (Fig. 1). At time zero (defined as when the length of a new shoot was approximately 1 cm), a branch was tagged to be analyzed at either 30, 60, or 90 days from time zero. For the purpose of discussing the data, these times will be referred to as days postpruning. Within a population of trees, an individual branch was arbitrarily assigned a time for analysis to reduce bias in selection but care was taken to have an equivalent number of branches for each assay time. At the indicated time, the first three leaves in succession, moving from the base to the tip of the branch, were collected and analyzed individually for colonization by '*Ca. L. asiaticus*' using qPCR. A branch was rated as infected when at least one harvested leaf was colonized based on the presence of '*Ca. L. asiaticus*' DNA. Branches from 13 *C. macrophylla* for trials 1, 2, and 3, and eight trees for trial 4, 20 Ridge Pineapple, 20 Carrizo, 13 sour orange, 14 Cleopatra, and 14 citron trees were analyzed in this study. In general, for a given citrus type, new shoots emerged as a population within a relatively short window of time, and the leaves from a portion of these branches were collected at each sampling time (Fig. 1).

Trees used in multiple trials were allowed to grow for a period of time after the conclusion of a trial before pruning was done for the next trial. This was done to allow for the accrual of adequate carbohydrate stores in the roots to assure robust new growth after the next pruning.

General effects of time and citrus type on leaf colonization. Single trials were conducted with Cleopatra and citron and multiple trials were conducted with Carrizo, sour orange, Ridge Pineapple, and *C. macrophylla*. Trials were conducted at different times of the year in a greenhouse environment (Table 1). Temperatures were moderated during the warmer summer months by an evaporative cooling system, and by heating in the winter months as needed, but in general temperatures were warmer during the spring, summer months and early fall months than in the late fall and winter months.

Single trials each for Cleopatra and citron were conducted from September to December and August to November, respectively (Table 1). For Cleopatra, the proportions of colonized leaves at the three times were not significantly different, indicating that the majority of leaves in the test population was colonized by 30 days, with no significant increase in this proportion over time (Table 2). In contrast to Cleopatra, the proportion of infected citron leaves was significantly higher at each successive sampling (Table 2).

Two trials were conducted with the Carrizo trees. In the July to October trial, the proportion of infected leaves was not significantly different at 30 and 60 days, but the proportion at 90 days was significantly larger (Table 2).

In the November to February trial, the proportion of colonized leaves was 64% at 30 days and there was no significant increase in this proportion at 60 and 90 days (Table 2). This is similar to the pattern of colonization observed with Cleopatra, but different from the July to October trial with Carrizo. Comparing the two trials, there was no significant difference in the proportion of leaves colonized at 30 and 60 days, but the proportion at 90 days was significantly less in the November to February trial (Table 2). Since the population of Carrizo trees was the same for both trials, this

Fig. 1. Pruned tree showing new adventitious and axillary branches that have been tagged for analysis at 30, 60, or 90 days postpruning.

difference suggested a change in conditions occurred which reduced the final proportion of leaves colonized by 90 days in the November to February trial.

Two trials were conducted with sour orange (Table 1). In the April to August trial the proportion of infected leaves at the different sampling times was variable, with no consistent trend (Table 2). The proportion of infected leaves was more consistent during the October to January trial with no significant difference in the proportion of colonized leaves at the three sampling times, indicating that a majority of leaves was colonized by 30 days postpruning (Table 2). The behavior of '*Ca. L. asiaticus*' in the October to January trial was similar to that found for Cleopatra and Carrizo, in that a majority of leaves was colonized by 30 days, with no significant change in this proportion during the remainder of the trial.

Two trials were conducted with Ridge Pineapple from September to December and from February to May (Table 1). In both trials, there was no significant difference in the proportion of colonized leaves at the three sampling times within a trial or at equivalent times between trials (Table 2). For both trials, the data indicated there was a rapid and near synchronous colonization of the majority of the sampled leaves during the first 30-day period postpruning, and that this pattern was similar in both trials irrespective of the different times of the year when the two trials were conducted.

Four trials were conducted with trees of *C. macrophylla* (Table 1). In the March to June trial the proportion of colonized leaves was high at all samplings, with 91, 90, and 82% colonization at 30, 60, and 90 days, respectively, with no significant difference in the proportion at any sampling time (Table 2). As was observed with Ridge Pineapple, leaf colonization in these *C. macrophylla* trees reached a maximal level by 30 days postpruning.

Two subsequent trials were conducted at USHRL from August to November and from January to April (Table 1). In the August to November trial, the proportion of colonized leaves was 87, 85, and 88% at 30, 60, and 90 days, respectively, and there were no significant differences in the proportion of colonized leaves at any sampling time. Comparing trials, there were no significant differences in colonization between equivalent sampling times in the March to June and August to November trials (Table 2).

The trial conducted from January to April gave somewhat different results with proportions of colonized leaves of 52, 69, and 81% found at 30, 60, and 90 days postpruning; the proportion of colonized leaves was significantly larger at 90 than at 30 days, but there was no significant difference between 90 and 60 days (Table 2). The proportion of colonized leaves at 30 days was significantly less in this trial than in the two previous trials, and this was true for the 60 day sampling time between this trial and the March to June trial. No significant difference in leaf colonization was found at the 90 day time between any these three trials (Table 2).

A fourth trial with *C. macrophylla* was conducted in a screened facility on site at the University of Florida, Citrus Research and Experiment Station, Lake Alfred, Florida from November thru

TABLE 1. Citrus type, number of trials and trees, and trial periods for studying colonization of new branches

Citrus type	Trial	Number of trees	Trial period
Cleopatra	1	14	September to December
Citron	1	14	August to November
Carrizo	1	20	July to October
	2	20	November to February
Sour orange	1	13	April to August
	2	13	October to January
Ridge Pineapple	1	20	September to December
	2	20	February to May
<i>C. macrophylla</i>	1	13	March to June
	2	13	August to November
	3	13	January to April
	4	8	November to February

February (Table 1). The trees in this study were grown and infected independently of the trees in the first three trials at USHRL. In this trial, leaf colonization was 88, 88, and 47% at 30, 60, and 90 days postpruning, with no significant difference in colonization at 30 or 60 days, but with significantly less colonization at 90 days (Table 2). In the January to April trial, the lowest incidence of colonization was at 30 days, which covered the period of early January to early February, and the lowest incidence of colonization in the November to February trial was at 90 days, which covered a period of late January to late February, suggesting a possible similar environmental effect on leaf colonization in these two trials. Comparing sampling times between trials, the January to April trial showed significant differences in colonization from one or more of the other trials at 30 and 60 days, and the November to February trial showed significantly less colonization at 90 days when compared with the March to June and August to November trials (Table 2).

In general, the data for these *C. macrophylla* trials suggests a colonization pattern similar to that observed for Ridge Pineapple, in which a maximal portion of the target population of leaves is colonized in a near synchronous manner by 30 days postemergence.

'Ca. L. asiaticus' population development in leaves of new growth. The growth of bacterial populations in new leaves was evaluated by assessing the titer in the first three successive leaves on new growth, as outlined in the previous section. Evaluation by ANOVA (analysis not presented) indicated no significant differences in mean bacterial titers in these leaves for a given assessment time, a given citrus type or a given trial period. Based on this result, the pooled means for all three leaves were subject to further analysis.

For the single trial of Cleopatra conducted September to December, the titers at 30 and 60 days were not significantly different from one another, but were significantly less than the titer at 90 days, indicating that population growth was static between 30 and 60 days then increased significantly between 60 and 90 days (Table 3). The changing population is in contrast to the proportion of colonized leaves, which was similar at all samplings (Table 2).

During the single trial with citron conducted August to November, there was no significant increase in bacterial titer from 30 to 60 days, while the titer at 90 days was significantly higher than at 30 days (Table 3).

During the July to October trial with Carrizo, the mean titers at 30, 60, and 90 days were significantly different, showing a progressive increase in titer over time (Table 3). In the November to February trial, the bacterial titers found at 60 and 90 days were both significantly higher than the titer at 30 days, but were not significantly different from one another. This slowing of growth of

the bacterial population in the last 30 days contrasts with the significant increase in the population found during the last 30 days of the July to October trial. Comparing the two trials, bacterial titers were significantly different between trials at all three sampling times, indicating a possible environmental effect on population growth.

During the April to August trial with sour orange, bacterial growth reached a maximum level by 60 days, as mean titers at 60 and 90 days were significantly higher than at 30 days, but not significantly different from one another (Table 3). During the October to January trial, mean bacterial titers at 30 and 60 days were not significantly different, but there was a significant increase in titer at 90 days (Table 3). Comparing sampling times between the two trials, there was a significantly higher titer at 30 days in the October to January trial, but no significant differences at 60 or 90 days.

In both the September to December and February to May trials for Ridge Pineapple, titers at the 60 and 90 days were both significantly higher than at 30 days, but were not significantly different from one another (Fig. 1), indicating that in both trials populations peaked by 60 days.

The pattern of bacterial growth in *C. macrophylla* leaves was distinct among the six citrus varieties in this study. For three of four trials, August to November, January to April, and November to

TABLE 3. '*Candidatus Liberibacter asiaticus*' population development in new leaves of the indicated citrus types at 30, 60, and 90 days postemergence

Type	Trial	Bacterial titer (log CFU/g) ^z		
		30	60	90
Cleopatra	1	3.89 ± 0.12 ^A _{abc}	4.04 ± 0.14 ^A _{bc}	4.61 ± 0.12 ^B _{ab}
Citron	1	3.81 ± 0.20 ^A _{abc}	4.22 ± 0.13 ^{AB} _{abc}	4.68 ± 0.15 ^B _{ab}
Carrizo	1	3.40 ± 0.18 ^A _{cd}	4.32 ± 0.14 ^B _{abc}	4.99 ± 0.14 ^C _{ab}
	2	2.82 ± 0.11 ^A _d	3.79 ± 0.16 ^B _c	3.70 ± 0.16 ^B _{cd}
Sour orange	1	3.28 ± 0.14 ^A _{cd}	4.59 ± 0.58 ^B _{abc}	5.07 ± 0.40 ^B _{ab}
	2	3.87 ± 0.13 ^A _{abc}	4.22 ± 0.28 ^A _{abc}	5.06 ± 0.18 ^B _{ab}
Ridge Pineapple	1	3.59 ± 0.15 ^A _{bc}	4.54 ± 0.19 ^B _{abc}	4.74 ± 0.14 ^B _{ab}
	2	3.52 ± 0.18 ^A _{bc}	4.82 ± 0.12 ^B _{abc}	5.37 ± 0.18 ^B _{ab}
<i>Citrus macrophylla</i>	1	3.47 ± 0.14 ^A _{bc}	4.69 ± 0.09 ^B _{ab}	4.33 ± 0.12 ^B _{bc}
	2	4.54 ± 0.14 ^A _a	4.61 ± 0.08 ^A _{ab}	4.12 ± 0.10 ^B _{bcd}
	3	4.17 ± 0.21 ^A _{ab}	4.42 ± 0.17 ^A _{abc}	4.53 ± 0.22 ^A _b
	4	4.13 ± 0.12 ^A _{abc}	3.99 ± 0.20 ^A _{bc}	3.14 ± 0.28 ^B _d

^z Values are the mean log₁₀ of colony forming units per gram of tissue ± standard error. Values in a row not followed by the same superscript uppercase letter in bold are significantly different (*P* < 0.05). Values in a column not followed by the same subscript lowercase letters are significantly different (*P* < 0.05).

TABLE 2. '*Candidatus Liberibacter asiaticus*' colonization of leaves on new branches on trees with an established infection at 30, 60, and 90 days postemergence

Type	Trial	Number of branches ^y	Leaf colonization ^z		
			30	60	90
Cleopatra	1	22	0.62 ± 0.15 ^A _{abc}	0.72 ± 0.16 ^A _{abc}	0.61 ± 0.15 ^A _{bc}
Citron	1	26, 25, 24	0.67 ± 0.11 ^A _{abc}	0.85 ± 0.07 ^B _{bc}	0.96 ± 0.03 ^C _a
Carrizo	1	24	0.43 ± 0.08 ^A _a	0.61 ± 0.10 ^A _{ab}	0.85 ± 0.04 ^B _{ab}
	2	24	0.64 ± 0.04 ^A _{abc}	0.57 ± 0.08 ^A _a	0.60 ± 0.10 ^A _c
Sour orange	1	12, 12, 11	0.40 ± 0.11 ^{AB} _a	0.18 ± 0.11 ^d	0.67 ± 0.16 ^B _c
	2	16	0.69 ± 0.09 ^A _{abcd}	0.57 ± 0.13 ^A _{abd}	0.81 ± 0.09 ^A _{abc}
Ridge Pineapple	1	24, 24, 20	0.85 ± 0.07 ^A _{bc}	0.67 ± 0.12 ^A _{ab}	0.79 ± 0.07 ^A _{abc}
	2	24, 22, 21	0.75 ± 0.10 ^A _{bcd}	0.79 ± 0.08 ^A _{abc}	0.77 ± 0.12 ^A _{abc}
<i>Citrus macrophylla</i>	1	24, 20, 21	0.91 ± 0.07 ^A _{abc}	0.90 ± 0.06 ^A _c	0.82 ± 0.16 ^A _a
	2	18	0.87 ± 0.07 ^A _{bd}	0.85 ± 0.08 ^A _{abc}	0.88 ± 0.07 ^A _{ab}
	3	20	0.52 ± 0.09 ^A _{ac}	0.69 ± 0.13 ^{AB} _{ab}	0.81 ± 0.07 ^B _{abc}
	4	14, 13, 11	0.88 ± 0.05 ^A _{bd}	0.88 ± 0.08 ^A _{abc}	0.47 ± 0.16 ^B _c

^y Values indicate the number of branches analyzed for leaf colonization in the indicated trials. A single number indicates that value applies to all sampling times. Multiple numbers are branch numbers analyzed at 30, 60, and 90 days postemergence, respectively.

^z Values are the mean proportion of infected leaves ± standard error. Values in a row not followed by the same uppercase superscript letter in bold are significantly different (*P* < 0.05). Values in a column not followed by the same lowercase subscript letters are significantly different (*P* < 0.05).

February, there was no significant increase in bacterial titer after 30 days (Table 3). For the March to June trial, titers at 60 and 90 days were significantly higher than those at 30 days, but the 60 and 90 day titers were not significantly different from each other. Interestingly, at 90 days in the August to November and the November to February trials, the titers at 90 days actually were significantly less than at 30 and 60 days (Table 3). Except for the March to June trial, comparison of titers at 30 days in the different trials showed no significant differences and at 60 days there was no significant difference in titers between the four trials. Similar to what was observed for trees of Ridge Pineapple, new growth on *C. macrophylla* trees is colonized rapidly and extensively, but in contrast to Ridge Pineapple, bacterial populations reached a maximum level by 30 days.

In all trials, the blotchy mottle symptom that is the diagnostic symptom for HLB was not observed on leaves collected at 30 days. A small portion of leaves of several citrus types used in this study showed mild HLB-like blotchy mottle at 60 days, but this was not common. A larger number of leaves showed this symptom at 90 days, but the majority of leaves generally were still asymptomatic at 90 days.

DISCUSSION

Movement of '*Ca. L. asiaticus*' into new growth is the path by which citrus trees with an established population of bacteria become and remain systemically infected. In commercial groves pruning trees to reduce old and induce new growth is a process known colloquially as hedging and topping (Phillips 1978). The new growth resulting from this process functions as new habitat for colonization and continued proliferation of the bacterial population. In this study, we pruned small trees of six distinct citrus types to mimic hedging and topping on a limited scale to force new growth from adventitious and axillary buds to study the patterns of bacterial colonization and bacterial growth over a 90-day period.

The expected pattern of leaf colonization was a progressive increase in colonization over time. It was surprising that this pattern was observed only for trees in the single trial with citron, where the proportion of leaves colonized was significantly higher at each sampling date. The most common pattern observed was that the incidence of leaf colonization reached a near maximum value by 30 days postemergence, as was observed for trees of Cleopatra, Carrizo, sour orange, Ridge Pineapple and *C. macrophylla*. One explanation for this unexpectedly rapid colonization is that there may be a "window of susceptibility" for individual leaves, so colonization must occur within that window of time. It should be remembered that even though different populations of leaves were sampled at the different times, the new branches emerged after pruning in a near contemporaneous manner and developed temporally as a population, so there would have been an equivalent window of susceptibility for a majority of the leaves in that population.

A general perception is that the movement of '*Ca. L. asiaticus*' in plants follows the direction of phloem sap translocation, from photosynthetic source tissues such as mature leaves into photosynthetic sink tissues, such as young expanding leaves, roots, and fruits (Wang et al. 2017). In this study, the new shoots forced into development by pruning would be considered sink tissues during their early growth, and bacteria found in phloem sieve elements in the mature vascular tissues of the tree trunk would be expected to move into and colonize the expanding leaves. The significant colonization of leaves on new growth which occurred within 30 days postpruning can be explained by this concept of source to sink movement of bacteria. Since overall colonization of newly emerging and expanding leaves (sinks) did not reach 100% for any citrus type during this study, it is possible that the natural transition from sink to source had begun for some leaves and this precluded their colonization during the course of the study.

Environmental conditions, in particular temperature influence the sink to source transition (Lemoine et al. 2013) and this in turn could influence the pattern and extent of colonization, as shown by the lesser degree of leaf colonization in the January to April trial compared with the March to June trial with *C. macrophylla*. The mean bacterial populations in leaves from both trials were significantly different only at 30 days, so temperature appears to have had a limited effect on population growth. With Carrizo, proportions of leaves colonized and bacterial titers differed significantly between the two trials which were conducted at different times of the year. Carrizo is an intergeneric hybrid of *P. trifoliata* × *C. sinensis*, and the *P. trifoliata* parent is fully deciduous and enters full dormancy during winter. While Carrizo is not deciduous, it is recognized as growing more slowly during winter than *Citrus* species grown under similar greenhouse conditions. Populations were lower during the cooler months (November to February) so it seems that in Carrizo the temperature likely affected bacterial population growth.

The trials for Cleopatra and citron were conducted at similar times in a similar environment of ambient temperature and light. Leaf colonization for Cleopatra did not exceed 72% during the trial period, whereas it reached 96% for citron, suggesting there also may be host-specific temporal aspects to the transition from sink to source for individual leaves and their susceptibility to colonization.

Despite reasonable efforts to do so by different research groups, a continuous laboratory culture of '*Ca. L. asiaticus*' has not been established (Davis et al. 2008; Parker et al. 2014; Sechler et al. 2009). Aspects of the basic biology of '*Ca. L. asiaticus*', such as potential nutritional requirements are surmised only from genomic data (Duan et al. 2009; Tyler et al. 2009) and population growth dynamics, are unknown.

Both Ridge Pineapple and *C. macrophylla* plants showed equivalently high levels of leaf colonization by 30 days, with no significant change in the proportion of infected leaves at subsequent assay times. For both citrus types, these data suggest there is a near synchronous colonization of the population of susceptible leaves within the first 30 days postpruning. Populations of bacteria did not increase over time in *C. macrophylla* leaves, but they did in Ridge Pineapple leaves, so for Ridge Pineapple it seems reasonable to view this population of leaves as supporting a near-synchronously replicating in planta culture of '*Ca. L. asiaticus*'. The qPCR-based confirmation of '*Ca. L. asiaticus*' in citrus leaves is a destructive process (Li et al. 2006), so changes in bacterial populations cannot be monitored over time in a given set of leaves. The synchronous nature of colonization observed in Ridge Pineapple leaves allows the larger populations observed in leaves sampled at 60 and 90 days to be viewed as how populations found in leaves at 30 days would have developed if multiple samplings from those leaves were feasible. This small tree system then functions as a dynamic in planta culture which can be used to study aspects of bacterial populations as they change over time. If sampling these populations at different times can indicate when populations are entering the widely recognized phases (lag, exponential, stationary, and death) of bacteria grown in a defined laboratory culture, then this small tree system represents a means of studying physiological and genetic changes associated with these phases of population growth of what currently is a noncultivable species. Currently, information on host anatomical, physiological and genetic changes in response to infection have been studied without knowledge of the growth status of the bacteria in the tissues that were examined (Albrecht and Bowman 2008, 2012; Aritua et al. 2013; Fan et al. 2011; Folimonova and Achor 2010; Kim et al. 2009). This small tree system could be used to study changes in both the host and bacteria in a temporal manner and host responses to infection could be correlated with changes in the bacterial population.

Studies using DNA intercalating agents to inhibit PCR-based amplification of DNA from dead bacterial cells indicated that a

majority of '*Ca. L. asiaticus*' DNA from infected citrus tissues was derived from dead cells, in particular in DNA extracted from highly symptomatic leaves (Trivedi et al. 2009). Although no similar assessments were done in this current study, it is expected that some of the '*Ca. L. asiaticus*' cells in the populations characterized were dead as a result of the natural maturation of the population. Based on the descriptions in the Trivedi et al. (2009) study, both the leaves and bacterial populations contained therein in that study were much older than those in this current study, and it is expected that the percentage of viable cells in the populations analyzed in this current study was significantly higher.

Continued production losses due to HLB have prompted research to find antimicrobial compounds, including clinical antibiotics, which kill or suppress growth of bacteria in infected trees. Published studies include drenching the soil for uptake into the plant through the roots (Zhang et al. 2010), application of foliar sprays for absorption through the leaves, trunks, and branches (Zhang et al. 2011), soaking infected bud wood to evaluate inhibition of transmission of '*Ca. L. asiaticus*' via grafting (Zhang et al. 2012, 2014) and trunk injections (Hu and Wang 2016; Puttamuk and Thaveechai 2013; Zhang et al. 2011). In these studies, the time from application to a final determination of efficacy was many months and relied on both testing for the pathogen and observing the development of symptoms.

The results from this study suggest how a small tree system can be applied to evaluate antimicrobial compounds in a more expedient manner. Using *C. macrophylla* as a model for this proposed assay system, antimicrobial compounds can be applied via the various methods. Following the application of the compound, trees are pruned to force new flush. Since *C. macrophylla* showed high levels of leaf colonization by 30 days at multiple times of the year, leaves on new flush can be sampled for bacterial DNA. If any method of application effectively delivers an inhibitory compound, an observed reduction or delay in the extent of colonization of new leaves or a reduction or delay in microbial growth when comparing treated and nontreated trees would be evidence of a direct antimicrobial effect on '*Ca. L. asiaticus*' which would be evident at approximately 30 days, a much shorter evaluation period than has been reported in previous studies.

The study described in this paper provides new information on the in planta behavior of '*Ca. L. asiaticus*', the noncultivable causal agent of an economically devastating disease of citrus. The data derived from this study suggest how the bacterial colonization of new growth forced by the pruning of infected small trees can provide an in planta version of a continuous culture of '*Ca. L. asiaticus*' that can be used to study aspects of population growth that normally would be derived from a laboratory culture growing in a defined medium. This behavior of '*Ca. L. asiaticus*' also could be exploited to develop a faster and more effective system to screen antimicrobial compounds for efficacy against '*Ca. L. asiaticus*'. We recognize that further research is needed to demonstrate the utility of such a screening system.

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