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Research Article

Dynamics of Huanglongbing-associated Bacterium *Candidatus* Liberibacter asiaticus in *Citrus aurantifolia* Swingle (Mexican Lime)

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Abstract

Background: The bacterial disease citrus huanglongbing (HLB), associated with "*Candidatus* Liberibacter asiaticus" (*C*.Las) has severely impacted the citrus industry, causing a significant reduction in production and fruit quality. In the present study, it was monitored the *C*.Las population dynamics in symptomatic, HLB-positive Mexican lime trees (*Citrus aurantifolia* Swingle) in a tropical, citrus-producing area of Mexico. The objective of this study was to identify the dynamics of the population of huanglongbing-associated bacterium *Candidatus* Liberibacter asiaticus and its insect vector in *Citrus aurantifolia* Swingle (Mexican lime). **Materials and Methods:** Leaf samples were collected every 2 months over a period of 26 months for quantification of bacterial titers and young and mature leaves were collected in each season to determine preferential sites of bacterial accumulation. The proportion of living and dead bacterial cells could be determined through the use of quantitative real-time PCR in the presence of ethidium monoazide (EMA-qPCR). **Results:** It was observed a lower bacterial titer at high temperatures in the infected trees relative to titers in mild weather, despite a higher accumulation of the insect vector *Diaphorina citri* in these conditions. This study also revealed seasonal fluctuations in the titers of bacteria in mature leaves when compared to young leaves. No statistically significant correlation between any meteorological variable, *C*.Las concentration and *D. citri* population could be drawn. **Conclusion:** Although, HLB management strategies have focused on vector control, host tree phenology may be important. The evaluation of citrus phenology, *C*.Las concentration, ACP population and environmental conditions provides insights into the cyclical, seasonal variations of both the HLB pathogen and its vector. These findings should help in the design of integrative HLB control strategies that take into account the accumulation of the pathogen and the presence of its vector.

Key words: *Candidatus* Liberibacter asiaticus, huanglongbing, *Citrus aurantifolia*, population dynamics, plant management, epidemiology, bacterial quantification, environment influence

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Citrus huanglongbing (HLB) is one of the most devastating diseases of citrus worldwide. This disease is associated with four phloem-restricted α-proteobacteria, "*Candidatus* Liberibacter" spp.,[1] which prevent the translocation of photoassimilates from source to sink plant tissues. The four reported bacterial species associated with HLB are "*Candidatus* Liberibacter asiaticus" (*C*.Las), which is the most widely distributed, infecting citrus crops in Asia, Africa and the America, "*Candidatus* Liberibacter africanus" (*C*.Laf) found only in Africa, "*Candidatus* Liberibacter americanus" (*C*.Lam) present only in Brazil[1] and "*Candidatus* Liberibacter caribbeanus" (*C*.Lca) recently reported in Colombia[2,3]. The bacteria are transmitted by at least two insect vectors. The Asian citrus psyllid (ACP), *Diaphorina citri*, transmits three of the four species: *C*.Las, *C*.Lam and *C*.Lca[1-4], while *Trioza erytreae* only transmits *C*.Laf[1].

Citrus fruits are a high value crop in Mexico. The Pacific region of Mexico is renowned for its production of Mexican limes, with the state of Colima as one of the principal producers of this particular citrus fruit. According to data from the Food and Agriculture Organization, Mexico produces over two million tons of limes and lemons, making it the third largest producer globally, preceded only by India and China[5]. The total contribution of the citrus industry to the Mexican economy is valued at 567 million dollars[6]. The first reported cases of HLB in Mexico were detected in Mexican lime trees from the Tizimín municipality in Yucatán[7]. The HLB has the potential to greatly disrupt the total production of not just Mexican lemons and limes but all Mexican citrus fruits. Unfortunately, as of yet, there is no cure for HLB. So far in Mexico, HLB management strategies are based on vector control with chemical insecticides. Monitoring the ACP population using yellow sticky traps is an important phytosanitary measure used to decide which strategies for insecticide application to pursue[6]. Thus far, this has been the most effective way to mitigate the rapid spread of HLB in orchards when used in conjunction with disposal of infected trees and the use of healthy bud wood.

Attempts to correlate seasonal fluctuations in *C*.Las population with ACP populations and their effects on the spread and severity of HLB have yielded mixed results. Measurement of bacterial viability by propidium monoazide qPCR (PMA-qPCR) in graft-infected, greenhouse grown sweet orange and *Severinia buxifolia*, a non-host plant, across 20 months (August, 2010-April, 2012) demonstrated that the populations of live *C*.Las were lower during the winter months[8,9]. In Brazil, the population dynamics of *C*.Las in naturally infected sweet orange trees were monitored for 2 years (April, 2010-April, 2012) using qPCR, revealing that bacterial concentrations tended to be highest in autumn and lowest in spring and summer[9]. A similar study carried out in Florida reported large numbers of HLB-positive trees diagnosed between August and January, though the highest bacterial concentrations were found in December[10]. Conversely, in Pakistan, the number of trees diagnosed with HLB was largest during the summer, inspite of fewer *C*.Las circulating in the ACP vector population[11].

Epidemiological models developed for HLB have assumed a linear relationship between infection and manifestation of symptoms because as of yet, it has not been possible to precisely determine the point where infection begins[12]. Attempts to characterize the epidemiology of HLB are also hampered by the lack of studies on the variation of *C*.Las concentration in both plant tissues and the ACP vector[13], again stemming from the difficulty in defining a starting point of infection. Under controlled conditions, the incubation period of HLB is estimated to range from 3-12 months. However, the incubation periods seen in the field can last $\geq$5 years[14,15]. This discrepancy illustrates the importance of understanding how a plant pathogen progresses throughout its pathology and how that relates to crop management practices. So far, information on the behavior of *C*.Las in the field has been scarce since the complexity of its pathology makes it a difficult disease to observe.

It is worth noting that various technical reports have indicated a weak correlation between the concentration of the bacteria and the severity of the HLB symptoms. It seems as though the expression of symptoms in an infected tree depends on host factors such as citrus variety, age and nutritional status, among others[13,16]. As such, visual quantification of the characteristic diffuse mottle in individual trees is not indicative of *C*.Las concentration in the plant[16].

In this study, it was monitored HLB-infected Mexican lime trees in Tecomán, Colima, Mexico for over 2 years in order to track bacterial titer fluctuations and potentially reveal any seasonal patterns. Both living and dead *C*.Las concentrations were quantified in leaf midrib tissues using EMA-qPCR. Young and mature leaves were also sampled and analyzed separately to determine how the changes in *C*.Las titer vary across tissues in distinct stages of development. Counts of adult ACP within the orchard and meteorological data were also collected throughout the experimental period. While there were some slight seasonal variations in *C*.Las concentration observed, statistical analyses failed to uncover any meaningful relationships between the bacterial titers and the environmental variables considered in our study. To our knowledge, the data herein constitutes the first study on *C*.Las population dynamics in Mexican lime in this country. It will

serve as a useful complement to existing ACP control strategies; this will, in turn, produce more effective, integrated disease management protocols and yield further insight into the epidemiology of HLB.

MATERIALS AND METHODS

Tree selection: The state of Colima was chosen for the experiment because HLB is endemic to the area and it is one of the largest producers of Mexican lime. The trial was run in a citrus orchard called "Rancho el Pandelo y Las Animas" in the town of Tecomán, Colima State with the following geographical reference coordinates: 18.93487°-103.92576°. In June, 2013, leaf samples were collected from 3 years old trees, exhibiting the diffuse mottling typical of HLB for molecular diagnosis of HLB in the laboratory.

Total DNA purification and HLB detection: Finely minced leaf midrib tissue (100 mg) was transferred to a 1.5 mL microcentrifuge tube containing a porcelain pellet. About 550 µL of PBS (50 mM KH_2PO_4, 150 mM NaCl, pH 7.2) was added and the tissue was macerated using a TissueLyser LT homogenizer (Qiagen; Hilden, Germany). The DNA purification was performed with the commercial AxyPrep Multisource Genomic DNA Miniprep kit (Axygen Biosciences) according to the manufacturer's instructions. The DNA was resuspended in 100 µL of elution buffer before quantifying concentration and purity with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Scientific; Waltham, MA). All samples were stored at -20°C for further use.

Molecular detection of *C*.Las by PCR: End-point PCR was employed for *C*.Las detection using a previously described primer pair[17]. A T100 Thermal Cycler (Bio-Rad; Hercules, CA) was used to carry out all the 25 µL PCR reactions containing 2 µL of sample DNA (50 ng μL^{-1}) and reagents at the following concentrations: 1x buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 0.16 mM of each dNTP (Invitrogen), 0.5 µM forward primer OI1 (5'-GCGCAAATGCGTGAGCGGTACCA-3'), 0.5 µM reverse primer OI2c (5'-TCGGCCGCCCTTCGAAACC CAT-3') and 1 U of platinum Taq (Invitrogen). The amplification program consisted of an initial denaturing step at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 60 sec with a final extension at 72°C for 10 min. Positive HLB infection was confirmed by resolution of the 1160 bp PCR product on a 1% (w/v) agarose gel.

Twenty four HLB-positive trees, averaging 1.8 m in height, were selected. The trees were planted with an 8 m spacing between rows and 4 m between columns. The trees were isolated from the orchard with anti-aphid mesh tunnels measuring 6×36×3 m high (12 trees $structure^{-1}$). Crop management was performed according to the regional practices. Leaf samples were taken every 2 months, starting in October, 2013.

Quantification of live and dead bacteria: Leaf samples for the determination of live HLB titers were collected in March (spring), May (summer), September (autumn) and December (winter). Seven young and mature leaves from four branches per tree were collected; all seven leaves from the same branch. Non-infected trees were similarly sampled to serve as a control. Young leaves were identified by their apical position on the selected branch while mature, photosynthetically active leaves were collected from the basal part of the same branch. Young and mature leaves were processed separately. The plant material was prepared for DNA extraction as follows: Leaf midribs (100 mg) were finely minced using disposable blades, weighed and transferred to a 1.5 mL microcentrifuge tube for homogenization with the TissueLyser LT (Qiagen). Duplicate samples were prepared: One for standard genomic DNA extraction and the other for extraction in the presence of Ethidium MonoAzide (EMA) to assess live/dead bacterial concentration. The homogenized tissue samples were stored at -80°C pending further processing.

DNA extraction from live bacteria: The DNA extraction from live bacteria was performed according to the previously described EMA method[18] with the following modifications: About 300 µL of PBS buffer (50 mM KH_2PO_4, 150 mM NaCl, pH 7.2) was added to a 1.5 mL microcentrifuge tube containing plant tissue and the sample was agitated for 5 min. The EMA was added to the samples to a final concentration of 100 µg mL^{-1} and gently shaken for 5 min in the dark. The EMA was bound to free DNA by exposing the samples to a 650 W halogen bulb for 2 min while the samples were on ice. The samples were centrifuged at 10,000 rpm for 5 min, the supernatant was discarded and finally the tissue was macerated with a TissueLyser LT (Qiagen). The DNA extraction proceeded using the previously mentioned method. The DNA concentration and purity was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

***C*.Las 16S recombinant plasmid construct:** A *C*.Las 16S rDNA fragment was amplified using previously described primers OI1/OI2[19] yielding a PCR product of 1160 bp, which was then cloned into the pGEM®-T-Easy vector (Promega; Madison, WI) by following the manufacturer's instructions. Chemically competent Mach1-T1 *E. coli* cells were transformed by heat shock and plated on LB agar with 100 µg mL^{-1} ampicillin. The bacterial colonies containing the insert were identified by

α-complementation. Plasmid purification was performed using the PureLink Quick Miniprep Plasmid Kit (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). To calculate a quantification curve, the plasmid copy number was determined as follows:

$$\text{Copies of 16S plasmid} = \frac{\text{Concentration (ng)}\times\text{Avogadro's number}}{\text{Length (bp)}\times 1\times 10^{9}\times 650}$$

where, 650 is the mass of each base pair in daltons and Avogadro's number is 6.022×10^{23}. Ten-fold serial dilutions of plasmid DNA at 10^8, 10^7, 10^6 and 10^5 copies μL^{-1} were prepared.

Detection of *C*.Las by quantitative real time PCR: The real-time PCR reactions for detecting *C*.Las used previously reported primers and probes[17,19]. All qPCR amplifications were run on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each 25 µL reaction contained 2 µL of sample DNA and the following reagents: 1x PCR buffer (Invitrogen), 2.4 mM $MgCl_2$ (Invitrogen), 0.24 mM of each dNTP (Invitrogen), 0.12 mM forward HLBas (5'-AGCTCGGCGGCATATATACG-3'), 0.12 mM reverse HLB (5'-TCCGCGTTACGTAGAAGGAAATAG-3'), 0.12 µM HLB probe (5'-6-FAM-AGACGGAGTGTGGCCAAC-3'BHQ-1), 0.12 mM forward COX (5'-GTATGCGCACACGTCTT CCAGA-3'), 0.12 mM reverse COX (5'-GCCAAAAAGACTGCTGG CATTC-3'), 0.12 µM COX probe (5'-TET-ATCATGCAGGCACTTCT GG-3' BHQ-2) and 1 U Taq Platinum (Invitrogen). Each DNA sample and the standard curve reactions were assayed in triplicate. The qPCR assays were run at 94°C for 20 sec, followed by 40 cycles of 94°C for 1 sec then 58°C for 40 sec. Fluorescent signal capture was set for each 58°C step.

***C*.Las quantification:** Cycle threshold values, Ct, obtained from the standard curve reactions were used to draw a linear regression plot, from which the resulting equation was calculated: $y = -3.4282x+46.596$, with an $R^2 = 0.9997$. Based on that equation, following formula was obtained for determining bacterial titer:

$$\text{Concentration} = 10^{\left(\frac{ct-46.596}{-3.4282}\right)}$$

The *C*.Las concentrations in the experimental samples were determined by interpolating their Ct values.

Asian citrus psyllid quantification: Two sticky 24×18 cm yellow traps were placed at the center of each tunnel of Mexican lime trees. The old traps were collected and new yellow traps were installed on the same dates as leaf sampling. Positive ACP identification and count was performed at the Laboratory of Entomology and Acarology of the National Phytosanitary Reference Centre.

Weather information: Meteorological data were obtained from the National Agricultural Meteorological Station Network of the National Institute of Forestry, Agriculture and Fisheries (INIFAP), from the Tecomán station in Colima, located at 18.96683°-103.84227°. Average temperature, minimum temperature, maximum temperature, average relative humidity and rainfall were all monitored during the trial period.

Statistical analysis: The database generated from the quantification of *C*.Las in the various samples obtained from distinct places on 24 Mexican lime trees was compiled in Microsoft Excel and then transferred to SAS[20] for an analysis of variation and identification of statistically significant differences between aggregation factors (evaluation dates, trees, branches, young and mature leaves) using the Fisher 'F' ratio of variances (PROC GLM in SAS). Having identified the significant relationship of the above factors, a multiple comparison of averages was applied with the Tukey test ($\alpha = 0.05$). Average values grouped in the same letter are not statistically different. The identification of possible statistical correlations between atmospheric variables, ACP counts from the yellow traps and concentration of *C*.Las was performed using the SAS PROC CORR procedure to generate the estimations of Pearson's "r" with a probability of $p<0.05$. Calculated standard deviation is representd in graphs as error bars.

RESULTS

***C*.Las population dynamics in Mexican lime trees:** During the 2 years trial period, the total *C*.Las titer peaked numerous times (Fig. 1). The first peak, in December, 2013 shows an increase in the concentration of living bacteria. The next peak in total bacterial titer, in May, 2014, actually has a greater proportion of dead cells. The ratio of live/dead *C*.Las skewed towards live cells again in November, 2014. Interestingly, the total bacterial titer after the winter of 2014 never returns to the same levels, though there are two peaks in 2015, during July and December. In July, 2015, there was a majority of dead *C*.Las, while in December, 2015, there was a return to a majority of live cells. The ratio of live/dead bacteria was largest during the autumn and winter peaks (December, 2013, November, 2014 and December, 2015), while a higher proportion of dead *C*.Las was seen during the hot summer

months. These fluctuations followed a particular pattern, with highest total titers of bacteria present in months with mild temperatures and decreasing during those with high tropical temperatures. Indeed, these high titers also coincided with the periods of new shoot emergence.

The concentrations of living *C*.Las display a slightly different behavior. After the first peak concentration of live *C*.Las in December, 2013, there was a fairly long period-from July, 2014 to November, 2015, where the ratio of live/dead bacteria remained elevated. Thereafter, a steady reduction was observed until July, 2015. Finally, the last recorded peak in live *C*.Las concentration occurred in December, 2015. Throughout the evaluation period, the trees exhibited typical HLB-associated symptoms even as the bacterial titers fluctuated between each assessment date. Live *C*.Las concentrations varied across samples from 9.93×10^3 to 5.93×10^6 *C*.Las g^{-1} tissue, while the titer of total bacteria ranged from 5.38×10^5 to 1.98×10^7 *C*.Las g^{-1} tissue.

A comparison between average concentrations obtained during the different assay dates revealed that the largest titers of live bacteria were present in December, 2013, September, 2014 and November, 2014, while the lowest titers of live *C*.Las corresponded to October, 2013, February, 2014, May, 2015 and September, 2015 (Table 1). As concentrations of live *C*.Las decreased, the proportion of dead cells increased.

Table 1: Average log concentrations of live and dead *C*.Las bacteria recorded in Mexican lime

Date of evaluation	Total samples	Average log live HLB	Tukey (p<0.05)	Average log dead HLB	Tukey (p<0.05)
November, 2014	96	6.570	A	0.519	F
September, 2014	96	6.392	A	0.305	F
December, 2013	96	6.270	AB	0.472	F
January, 2015	96	5.965	BC	0.593	EF
July, 2014	96	5.840	CD	0.632	EF
December, 2015	96	5.680	CD	0.596	EF
July, 2015	96	5.551	D	1.197	D
March, 2015	576	5.006	E	0.939	DE
May, 2014	96	4.720	EF	2.375	AB
October, 2013	96	4.434	FG	2.223	B
September, 2015	576	4.377	G	1.653	C
May, 2015	450	4.284	G	2.091	B
February, 2014	96	4.234	G	2.657	A
		DMS = 0.343		DMS = 0.351	

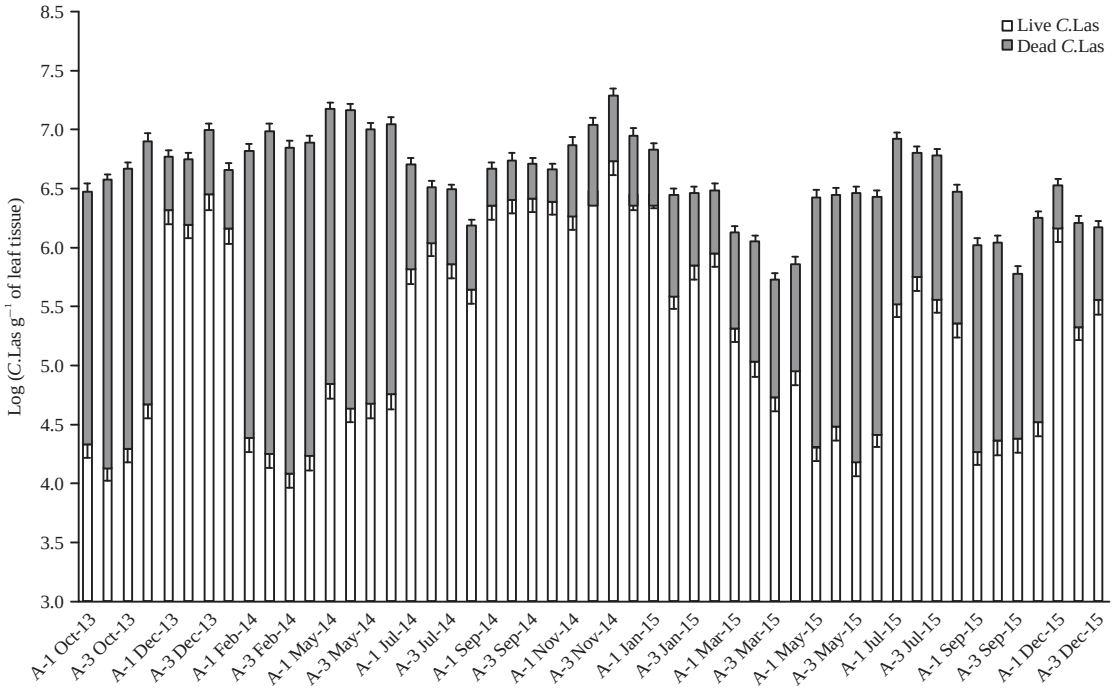

Fig. 1: Fluctuation of dead and live *Candidatus* Liberibacter asiaticus (*C*.Las). Bacteria was detected in the open field trial from October, 2013 to December, 2015. Bacterial quantification was performed by real-time PCR and normalized by tissue leaf weight

Differences in *C*.Las titers between young and mature leaves: The quantification of live and dead *C*.Las in both developing and photosynthetic leaves indicated that a higher total concentration of bacteria is present in mature leaves (Fig. 2). No disease symptoms were observed in the young leaves sampled, even though they contained high

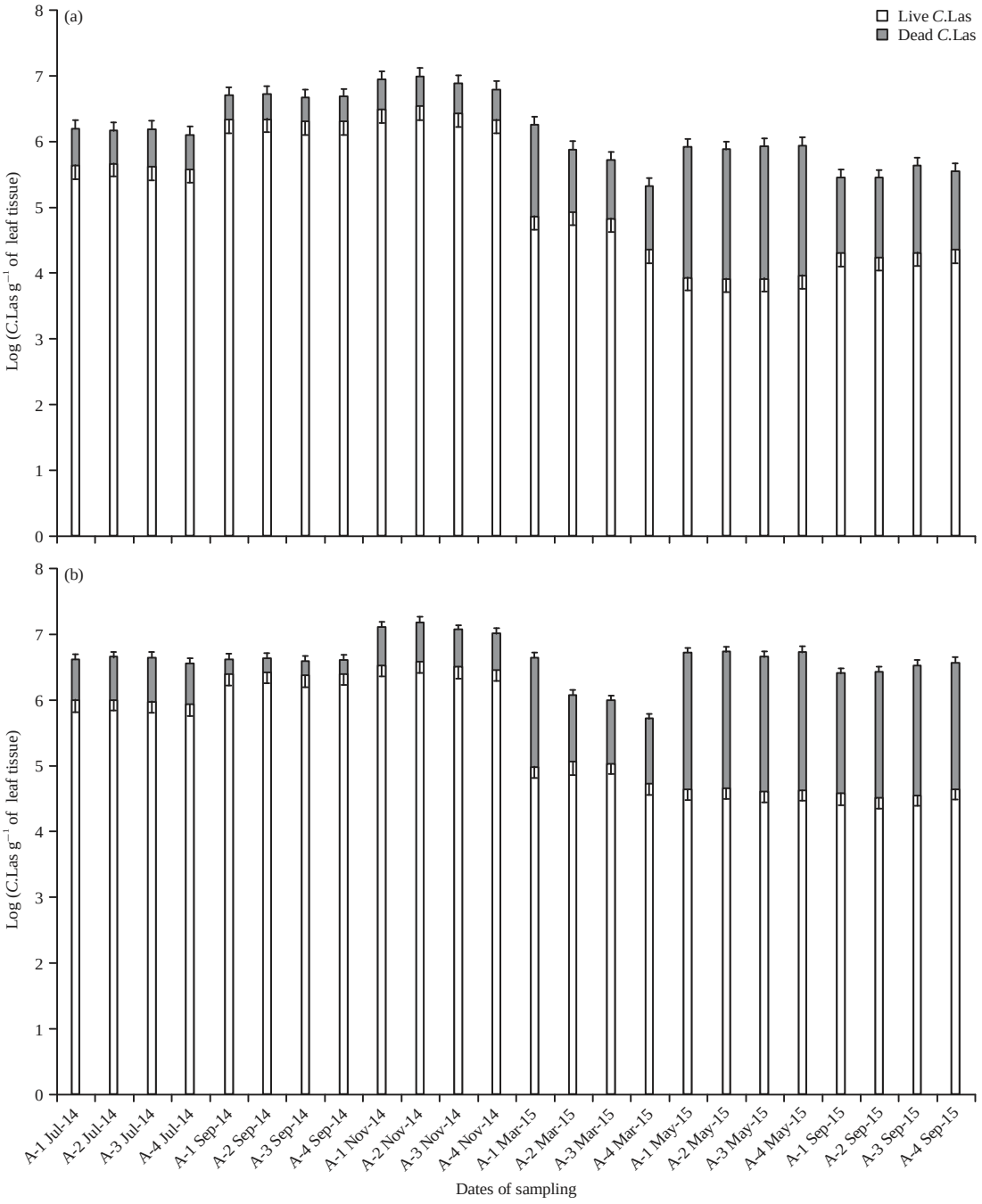

Fig. 2(a-b): Differences in *C*.Las titers between young and mature photosynthetic leaves. The quantification of live and dead *C*.Las in both developing and photosynthetic leaves showed a higher bacterial concentration is present in mature leaves. (a) Bacteria present in young leaves, grey: Dead *C*.Las, white: Live *C*.Las and (b) Bacteria present in mature leaves, grey: Dead *C*.Las, white: Live *C*.Las. A-1 to A-4 represents pooled samples from 6 trees, while the collect month and year are indicated

concentrations of *C*.Las, which strongly supports the idea that the bacterial titer is not proportional to the severity of HLB symptoms.

Significant variation in bacterial concentration was observed within the same tree, ranging from 8.79×10^7 to 1.76×10^4 *C*.Las g^{-1} of tissue. Despite the fact that this tendency was similar for all sampled trees, in some cases the differences in concentration was very drastic. It was noted that, generally, the mature leaves contained a higher total concentration of bacteria, as well as a greater proportion of live *C*.Las, this is probably due to their high photoassimilate content, which can be employed as a carbon source by *C*.Las ($F = 12.45$, $p<0.0001$). Yet, in the last two evaluations (September and December, 2015), the titers of dead bacteria were also higher in mature leaves than in developing leaves (Table 2). Other studies reported that young leaves harbor greater numbers of bacteria compared to mature leaves. Supposedly, this distribution takes advantage of the ACP feeding preference for younger leaves[21] to bolster *C*.Las transmission, when in actuality the ACP seeks out new growth for deposition of eggs. Consequently, it is possible that the high concentrations seen in mature leaves are indicative of the bacterium attempting to evade high ambient temperatures by mobilizing towards the basal part of the branches[11].

Dynamics of *C*.Las population in 2015: The dynamics of living and dead *C*.Las were monitored in both young and mature leaves sampled during four different evaluation periods in 2015 (spring, summer, autumn and winter) (Fig. 3). Highest concentration of total bacteria occurred in spring, with dead bacteria present in low concentrations and mature leaves exhibiting higher titers (Fig. 3a, b). In summer, a drastic increase was observed in the concentration of dead bacteria (Fig. 3c, d). The trend in autumn showed a pattern similar to the one in summer. The concentration of dead bacteria exhibited an increase compared to the dynamics seen in the spring. However, dead bacteria were present at low concentrations in young leaves (Fig. 3e, f). In winter, dead bacteria declined overall, while in young leaves the total concentration of bacteria was noticeably lower than in mature leaves (Fig. 3g, h). The total concentration of *C*.Las was greater in older leaves in all seasons (Fig. 3b, d, f, h). It is pertinent to mention that the major periods of citrus shoot emergence in Tecomán take place during the summer and autumn months. That period overlaps with spring and summer: The seasons with the greatest total concentrations of *C*.Las (Table 3). Nevertheless, the results of the statistical analyses indicate that the largest numbers of dead bacteria are present in these seasons, while, in autumn and winter, there is a recovery of living bacteria.

Weather conditions during the study: Tecomán, Colima State has a dry tropical climate: The average annual maximum and minimum temperatures were 32.65 and 20.78°C, respectively, with an average annual temperature of 26°C (Fig. 4), this tropical municipality is considered an isothermal area since the temperatures do not vary much during the four seasons. It is known that in tropical regions, the low temperatures during the nights induce new shoot growth in citrus[22]. For the state of Colima there are two periods of shoot emergence reported. The first and most important, is from November to March, while the second is from June until September, when the rains begin. During this study, new shoot growth was observed when the temperatures increased and the rains arrived. In fact, some slight new growth was recorded between the months of August and December.

Associations between *C*.Las population and external variables: An inverse, highly significant relationship ($r = -0934$ Pearson, $p<0.0001$) between the concentrations of live and dead bacteria emerged from the analyses of the Mexican lime tissues during the experimental period (Table 4). Based on the strength of this inverse relationship, it is suggested that the concentration of live bacteria correlates with a concomitant reduction of dead bacteria in Mexican lime, this could be caused by a compromised defense response on behalf of the plant, which could be affected by the temperature or other environmental variables. As a

Table 2: Average log concentration of *C*.Las found in young and mature leaves of Mexican lime

Leaf age	Total No. of samples	Average log *C*.Las	Tukey ($p<0.05$)
Mature	1329	5.109	A
Young	1137	4.804	B
		DMS = 0.085	

Table 3: Comparison of the average concentrations of live and dead *C*.Las in Mexican lime, based on tissue type and season

Parameters	Total young	Live young	Dead young	Total mature	Live mature	Dead mature
Summer	5.8676^{A}	3.9311^{B}	1.9267^{A}	6.7149^{A}	4.7150^{B}	1.9999^{A}
Spring	5.8507^{A}	4.9189^{A}	0.9318^{BC}	6.0414^{B}	5.0503^{B}	0.9911^{B}
Autumn	5.6848^{A}	5.0035^{A}	0.6813^{C}	6.8318^{A}	6.2547^{A}	0.5771^{B}
Winter	5.5889^{A}	4.2477^{B}	1.3412^{B}	6.4861^{A}	4.5187^{B}	1.9788^{A}
Mean value	5.7480 (0.697)	4.5253 (0.6601)	1.2202 (0.680)	6.5185 (0.5312)	5.1346 (0.7157)	1.3867 (0.5604)
DMS	0.527	0.4987	0.5139	0.4013	0.5406	0.4233

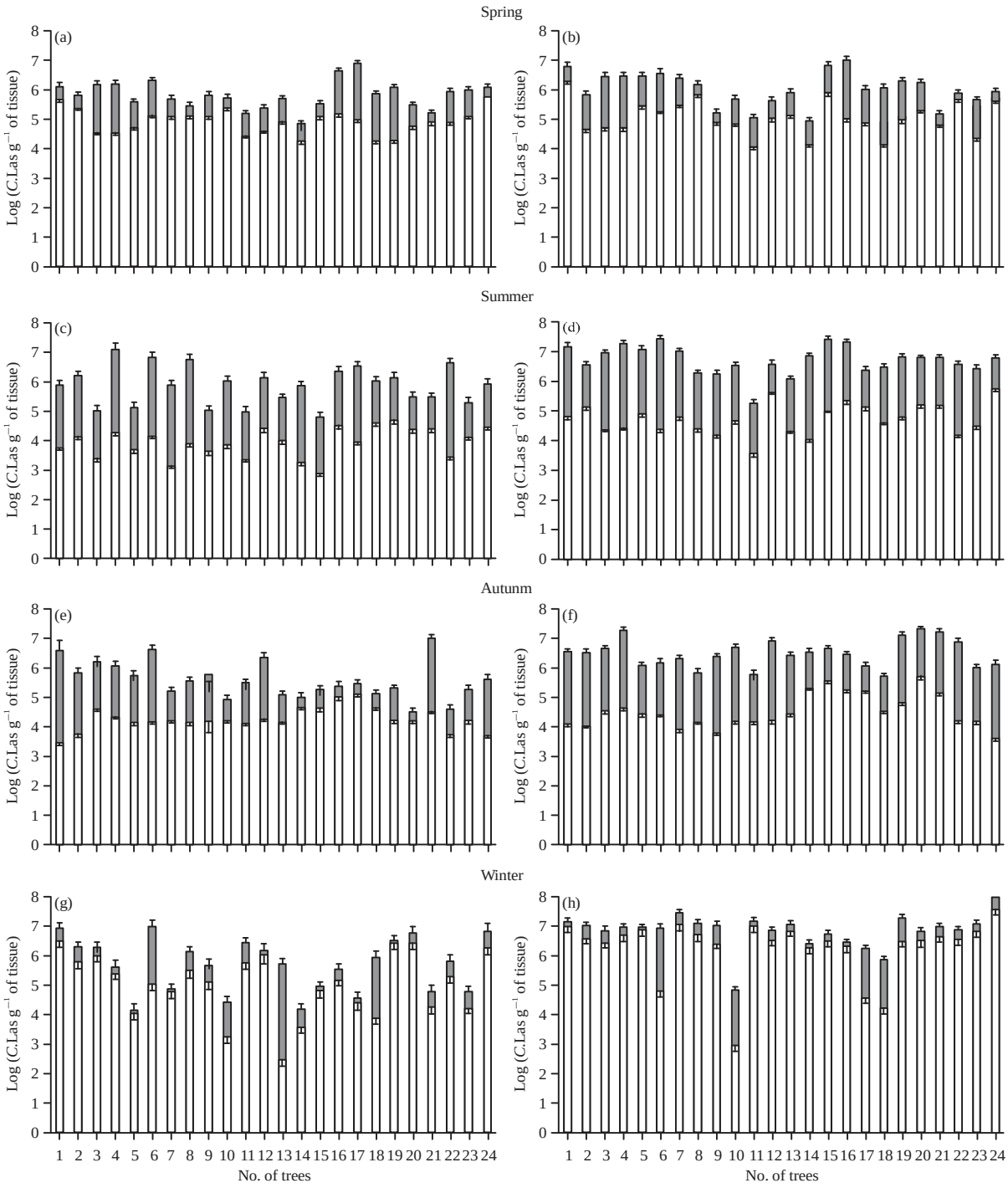

Fig. 3(a-h): Dynamics of *C*.Las population in 2015. Representation of living and dead *C*.Las in both young and mature leaves sampled during four different evaluation periods in 2015. (a, c, e, g) Young leaves and (b, d, f, h) Mature, photosynthetic leaves. Grey bars are dead bacteria, while white are alive. A-1 to A-24 are measurements of each tested tree

speculative note, t is also possible that quorum sensing may regulate oscillations in the bacteria population[23]. There was no correlation between live bacterial concentration and the citrus Asian psyllid density, according to statistical analysis [Corr test, parameters (r = -0.137, p = 0.686)]. Hence, this represents the first evidence from the Tecomán region, Colima, Mexico. In addition, there was a period of elevated bacterial concentrations between July and November, 2014 (Fig. 1),

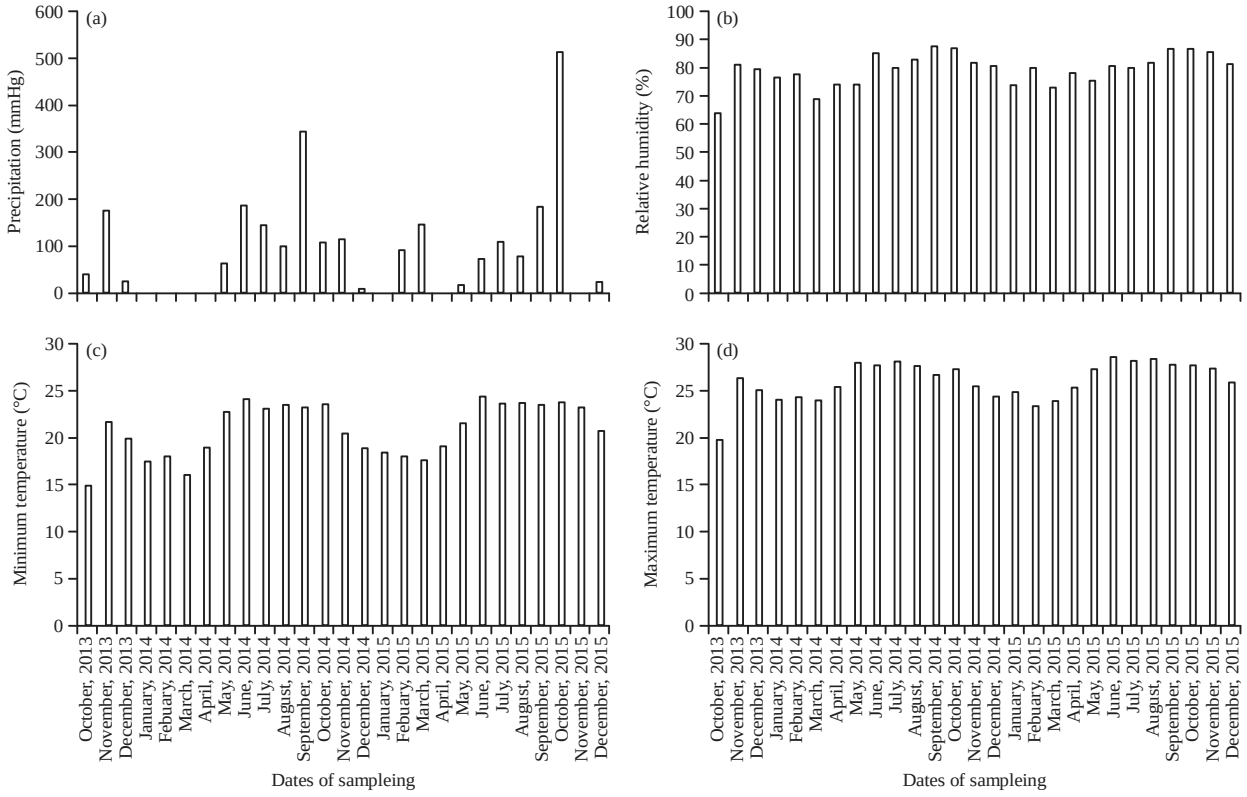

Fig. 4(a-d): Weather conditions during the study, (a) Precipitation (mmHg), (b) Relative humidity (%), (c) Minimum temperature (°C) and (d) Maximum temperature (°C)

Table 4: Statistical correlations between *C*.Las titers and environmental variables

Parameters	Live	Dead	Temperature ($°C_{med}$)	Relative humidity	Temperature ($°C_{min}$)	Precipitation	ACP count
Live	1.000	-0.909<0.0001**	0.0065 (0.938)^{ns}	0.0945 (0.758)^{ns}	0.138 (0.651)^{ns}	-0.153 (0.616)^{ns}	-0.137 (0.686)^{ns}
Dead		1.000	0.061 (0.841)^{ns}	0.0118 (0.969)^{ns}	-0.028 (0.925)^{ns}	0.085 (0.781)^{ns}	0.231 (0.494)^{ns}
Temperature ($°C_{med}$)			1.000	0.439 (0.132)^{ns}	0.922 <0.0001)**	0.500 (0.081)^{ns}	-0.094 (0.782)^{ns}
Relative humidity				1.000	0.594 (0.032)**	0.5192 (0.073)^{ns}	0.166 (0.625)^{ns}
Temperature ($°C_{min}$)					1.000	0.524 (0.065)^{ns}	-0.076 (0.822)^{ns}
Precipitation						1.000	0.500 (0.117)^{ns}
ACP count							1.000

occurring simultaneously with the period of new shoot emergence reported for the region. These sustained levels of *C*.Las were somehow related to the emergence of new growth, although our statistical analyses failed to establish a concrete correlation. Interestingly, it appears that the months with low nighttime temperatures are the same in which the highest concentrations of live bacteria are detected; conversely, there is an increase in the number of dead bacteria in the warmest months. Moreover, no relationship between bacterial titer and severity of HLB symptoms were observed.

DISCUSSION

The population dynamics of *C*.Las in Mexican lime were elucidated by monitoring the concentrations of both live and dead bacteria throughout 2014 and 2015, identifying a similar pattern of cyclical behavior as previously[9]. This variation coincides with reports from Florida, USA and Paraná, Brazil and supports the hypothesis that the bacterial distribution changes throughout the year, depending on the host tree phenology and the environmental conditions[8,9]. This cyclic variation should now be factored into the rationale of HLB management and ACP control strategies, since diagnostic results from sampling both plant material and psyllid vector can be skewed, depending on whether the *C*.Las population is in decline or on the rise.

The trends in variation of *C*.Las population in Mexican lime were observed over a period of a full year (January to December, 2014), similar to the trial that has been reported for *C*.Las in sweet orange[8]. On a speculative note, this annual

variation in the concentration of *C*.Las could be more closely associated with the phenomenon of Quorum Sensing (QS), in addition to the meteorological variables or ACP populations found in infected orchards. Laboratory tests with *C*.Las, grown in culture media supplemented with citrus juices, found that the viability of the bacteria showed cyclical growth patterns, with increasing and decreasing phases throughout the experimental period. Cyclic or oscillatory development is a feature of bacterial populations attributable to internal or external fluctuations in the system, mainly related to the presence of nutrients. According to some reports, QS phenomena are related to the physiological and nutritional state of the host. By knowing the distribution of viable and non-viable bacteria in infected trees, it is possible to infer the periods when ACP transmission of *C*.Las is most probable. In turn, the probability of transmission is also most likely related to the distribution of photoassimilates within the trees throughout the year. Therefore, it is necessary to characterize these physiological aspects of Mexican lime varieties, since the presence of photoassimilates contributes to the carbon sources available for *C*.Las growth and development.

The finding of higher *C*.Las concentrations in mature leaves, compared to young leaves, runs contrary to the trend reported by several research groups[24]. In fact, the presence of major carbohydrate stores in the more photosynthetically active mature leaves would make them a more favorable environment for *C*.Las and could explain the difference in reported distributions[11,25].

This study demonstrated a lack of statistically significant relationships between environmental variables and *C*.Las concentration. The correlation values between the concentration of live bacteria and rainfall demonstrated an inversely proportional relationship (Pearson $r = -0.153$, $p = 0.616$), indicating that the number of living cteria is reduced when precipitation increases in the region, albeit not in a statistically significant manner. Alternatively, a similar phenomenon occurred with ACP count and rainfall (Pearson's $r = -0.137$, $p = 0.686$), where the amount of ACP found in the yellow traps decreased with increasing rainfall in the region, although it was also not statistically significant (Table 4). This lack of correlations or presence of weak correlations between atmospheric variables, *C*.Las concentration and ACP populations has been reported in different citrus-growing areas of the world[16,26]. While the evidence gathered thus far supports the notion of a dynamic *C*.Las population within an infected citrus tree, the research community has been unable to decisively associate any one environmental variable to bacterial titer.

These were time points when the average log concentrations of dead bacteria greatly surpassed the average log concentrations of live *C*.Las. The first instance was October, 2013, when the titer of live bacteria only reached log 4.3 *C*.Las g^{-1} of tissue, compared to the log 6.7 *C*.Las g^{-1} of tissue recorded for the dead *C*.Las. This represents a 100 fold increase in dead bacterial cells over living *C*.Las. The same was seen again in February, 2014, May, 2015 and September, 2015. This distinct proportion of dead to live cells could be associated with the formation of a biofilm. The dead cells quantified during these periods would be those that makeup the biofilm and cover their living relatives. As the behavior is seen during warmer months, this could be another defense mechanism used by *C*.Las to protect itself from high temperatures. There have been recent reports of genes which encode components of bacterial communication systems found via quorum sensing in the *C*.Las genome[26,27], which could be a strategy used by these bacteria to regulate its population.

CONCLUSION

The evaluation of citrus phenology, *C*.Las concentration, ACP population and environmental conditions provides insights into the cyclical, seasonal variations of both the HLB pathogen and its vector. Depending on the season, bacterial population fluctuates, representing the first report on the seasonal variation of *C*.Las concentration in Mexican lime. The understanding of the fluctuation of HLB-associated bacteria and its insect vector populations will help in the design of integrative control strategies. This information should be consolidated into existing HLB agronomic management practices and decision-making policies.

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