

# Quantitative Distribution of *Candidatus Liberibacter asiaticus* in the Aerial Parts of the Huanglongbing-infected Citrus Trees in Texas

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**Abstract.** The Asian citrus psyllid, *Diaphorina citri* Kuwayama, one of the known vectors for citrus greening disease or Huanglongbing (HLB) pathogens, has been present in Texas for over a decade, but the detection of the disease is recent. HLB has been confirmed in only two adjacent commercial citrus groves of grapefruit and sweet orange. A study was conducted to compare the population of *Candidatus Liberibacter asiaticus* (CLAs) cells in different plant parts including peduncle, columella, leaves, seeds, young shoots, flower buds, flowers, and bark of 6-year-old known infected grapefruit and sweet orange trees. The bacterial population was estimated using a previously described grand universal regression equation  $Y = 13.82 - 0.2866X$ , where  $Y$  is the log of the target copy number and  $X$  is the Ct (threshold cycle) of the assay. Except for bark tissue, there was no significant difference in the concentration of CLAs cells in other plant parts between the two cultivars. Within the cultivar, the bacterial concentration also varied with the plant part, with peduncle, columella, midrib having significantly higher titer of CLAs compared with other plant parts. The obtained results here are in agreement with previous studies conducted on Florida samples, but the consistently lowest bacterial titer recorded in young shoots, leaf blade, and especially leaf margins relative to the midrib has never been previously reported.

Huanglongbing is considered to be the most destructive citrus disease worldwide, having caused devastating economic damage to citrus in Asia and Africa for more than 100 years (Aubert, 1992) and more recently in Brazil and Florida (Bové, 2006; da Graça and Korsten, 2004).

HLB is associated with phloem-inhabiting Gram-negative  $\alpha$ -Proteobacteria (Jagoueix et al., 1994), specifically, CLAs, ‘*Ca. L. africanus*’ (CLaf), and ‘*Ca. L. americanus*’ (CLam) (Bové, 2006). CLAs and CLam are naturally vectored by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Capoor et al., 1967) and CLaf is vectored by the African citrus psyllid, *Trioza erytrea* Del Guercio (McClean

and Oberholzer, 1965). *D. citri* was first found in Florida in 1998 (Knapp et al., 1998) and was detected in Texas in 2001 (French et al., 2001). HLB was confirmed in Florida in 2005 (Halbert, 2005) and subsequently in Georgia, Louisiana, South Carolina, Texas, and California (Gottwald, 2010; Kumagai et al., 2013; Kunta et al., 2012). It was estimated that HLB may reduce the value of Texas citrus production (\$140 million) by 20% after 2 years of infestation and up to 60% after 5 years (Niemeyer et al., 2007).

Thus far, *Candidatus Liberibacter* spp. have not been isolated or cultured on artificial media; hence, traditional bacterial quantification through colony-forming units plate counts cannot be used (Li et al., 2008). An estimation of CLAs quantitative distribution in different plant parts of five citrus species (sweet orange, pumelo, sweet lime, lemon, and sour orange) revealed that different plant tissue contain varied levels of bacterial populations, which were not consistent across different species, although they were consistent in leaf blade, midrib, or petiole in symptomatic field trees (Li et al., 2009). This study pointed out

that there is a possibility of overestimation of bacterial cells because quantitative polymerase chain reaction (qPCR) cannot differentiate live from dead cells.

A similar study using both qPCR and conventional PCR (cPCR) to amplify 16S rDNA showed that CLAs is unevenly distributed in infected bark, leaf midribs, roots, and floral and fruit parts, ranging from 14 to 137,031 cells/ $\mu$ g of the tissue, with relatively high populations in fruit peduncles, but no bacteria were detected in endosperm and embryo of the seed (Tatineni et al., 2008). However, Hilf (2011) has detected bacteria in seedcoats. Furthermore, Tatineni et al. (2008) suggested that knowledge of pathogen distribution is essential to choose appropriate samples for diagnosis, understand virulence mechanisms, and develop disease management strategies.

Teixeira et al. (2008) reported that bacterial concentrations of CLam were highest in leaves with blotchy mottle, ranging from  $1.1 \times 10^6$  to  $2.8 \times 10^7$ . Higher concentrations were observed in leaves at the distal ends of branches compared with those at the proximal ends, close to the trunk. Moreover, 84% of the leaves with zinc deficiency symptoms were PCR-positive. They observed that when genuine zinc deficiency is present, it affects practically all of the trees in an orchard but when HLB first begins to affect an orchard, the deficiency-like symptoms will affect few trees and will be widely scattered.

A reliable, rapid, and sensitive qPCR test to detect and identify *Ca. Liberibacter* spp. in HLB-infected citrus trees, which is 100-fold more sensitive than cPCR, was developed by Li et al. (2006). *Ca. Liberibacter* spp. genome equivalents were estimated in citrus using a grand regression equation that incorporate all of the sample effects such as host tissue type, host species, geographic location, tissue storage, DNA quality, inhibitors, and PCR amplification efficiency (Li et al., 2008). However, its limitation for pathogen detection is that the 16S rDNA copy number must be greater than  $2.27 \times 10^5$ /g of host tissue, which is below the threshold concentration for symptom development. Furthermore, qPCR of root samples using 16S primers amplifies non-specific target (Kunta et al., unpublished data).

HLB disease symptoms may be confused with nutrient deficiencies or other disease symptoms (Bové, 2006). Moreover, CLAs is unevenly distributed in the phloem of the infected citrus plants (Garnier and Bové, 1993), and consistent detection of the disease depends on plant tissue with high bacterial titers. Gottwald et al. (2008) showed that in the field-grown citrus trees, often irregular distribution of HLB symptoms is seen in individual branches as symptoms develop, and CLAs is readily PCR-detectable from several tissue samples throughout the tree. They concluded that CLAs infection is systemic or nearly completely systemic in the trees; however, the bacterial concentrations in different portions of the tree may be below detectable levels. Furthermore, a recent study

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showed that temperature influences infection and establishment of CLAs and CLam in citrus plants where the highest CLAs titers were observed in citrus plants maintained at 22/27 °C and CLAs concentrations were negatively affected by a daily temperature regime of 27/32 °C (Gasparoto et al., 2012). Additionally, it was reported that continuous exposure to 40 to 42 °C for a minimum of 48 h significantly reduced CLAs titers or even eliminated CLAs from infected citrus seedling (Hoffman et al., 2013). In Texas, HLB is a recent introduction, and it is confirmed present only in two adjacent groves in San Juan, TX, and a residential grapefruit tree in Mission, TX; therefore, it is very important to establish CLAs distribution in citrus tree parts under field conditions where the temperatures are consistently high and different from Florida or elsewhere. According to 1981–2010 temperature data for June to August from the National Weather Service (<http://www.weather.gov>), the average and high temperatures were on average higher by 4.4 and 5.7 °F, respectively, in south Texas compared with central Florida. Moreover, along with temperature, factors such as nutrient status of the trees, psyllid populations, soil conditions, irrigation regime, and presence of *Phytophthora* may influence the distribution of CLAs in the field trees. This is the first study that was conducted to estimate the CLAs concentrations in different tree parts of field-grown grapefruit and sweet orange trees in Texas.

## Materials and Methods

**Plant material.** HLB-infected tissue samples (Table 1) were collected during June to July 2012 from six trees each of 6-year-old naturally infected field-grown ‘Valencia’ sweet orange and ‘Rio Red’ grapefruit trees on sour orange rootstock with symptomatic branches in a commercial citrus grove in San Juan, TX. Fruit, young leaves, young shoots, and flower buds were collected from symptomatic branches with classic leaf mottle that were previously confirmed positive by qPCR for the presence of CLAs. A total of 11 and five peduncle, 33 and 19 columella, 10 and 13 leaf midrib, 10 and 22 bark, nine and seven seed, nine and eight leaf blade middle parts,

and 10 and 10 leaf blade edge tissue samples from grapefruit and sweet orange, respectively, were used in CLAs quantitative distribution analysis. In sweet orange, no young plant parts were available at the time of collection and were not collected from all six trees and only two young tissue samples per each of leaf, flower bud, and shoot were collected from two grapefruit trees. Six flower samples were collected only from sweet orange trees because they were not available from grapefruit trees. Samples were collected because citrus trees tested positive for CLAs in the groves and because citrus phenology varies with time, all plant developmental stages were not present for sampling at a given time. Therefore, the number of samples of each type from the cultivars was random and a completely randomized design was used in the analysis.

**DNA extraction.** Total DNA was isolated using the Qiagen DNeasy Plant Mini Kit from 200 mg chopped tissue of different plant parts. The tissue was placed in a 2-mL lysing matrix A tube (MP Biomedicals, Santa Ana, CA) with extraction buffer and pulverized for 3 min using a Mini-Beadbeater-96 (Biospec Products Inc., Bartlesville, OK). The extract of total DNA was eluted in 100 µL nuclease-free water.

**Estimation of *Candidatus Liberibacter asiaticus* population in different tissues.** For the detection and quantification of CLAs, multiplex qPCR assays (Li et al., 2006) were performed using a HLBfpr primer-probe set on 2 µL total DNA extract in a 25 µL reaction using a SmartCycler II (Cepheid, Sunnyvale, CA) or ABI 7500 Fast thermocycler (Life Technologies). A citrus mitochondrial cytochrome oxidase (COX)-based primer probe set COXfpr (Li et al., 2006) was used as a positive internal control. All reactions contained known positive control DNA, healthy plant DNA, and non-template water control. The presence of the target sequences in the DNA extracts were confirmed based on the Ct values obtained. The Ct values obtained were incorporated into the universal regression equation as described by Li et al. (2008):  $Y = 13.82 - 0.2866X$  (where  $Y$  is the log of the target copy number and  $X$  is the Ct value) to calculate the CLAs genome equivalents present in the DNA extracts from different tissue

parts and the number obtained was converted into CLAs genome equivalents per gram of the tissue. Data were subjected to a two-way analysis of variance (ANOVA) to evaluate the effect of cultivar, plant part from which the tissue was collected, and their interaction on bacterial concentration using PROC GLM of SAS (SAS Institute, Cary, NC). Before analysis, data were tested for normality and homogeneity of variances. In case of violation of ANOVA assumption, data were log-transformed before analysis. Whenever significant  $F$  values were obtained, treatment means were separated using the Student Newman Keuls test (Zar, 1999). Data were subsequently back-transformed and reported as actual concentration.

## Results

Analysis of variance showed that *Ca. L. asiaticus* concentrations per gram of fresh tissue was significantly affected by the interaction between cultivar and plant part ( $F = 3.46$ ;  $df = 6, 187$ ,  $P = 0.0029$ ). Thus, the effects of plant part on CLAs concentrations were evaluated separately for each host cultivar. Similarly, the comparison of bacterial titers between grapefruit and sweet orange was conducted separately for each plant part.

***Ca. L. asiaticus* in different tissues of grapefruit.** The genome equivalents of CLAs per gram of fresh tissue varied from  $1.18 \times 10^6$  in the young shoot to  $2.95 \times 10^9$  in the peduncle. Tissue samples including peduncle, columella, leaf midrib with petiole, bark, and seeds consistently produced lower Ct values containing higher CLAs genome equivalents (Table 1). The young tissue samples including leaves, flower buds, and shoots consistently showed lower CLAs concentrations than mature tissue. Additionally, there was no significant difference between leaf blade edges and leaf blade middle part tissues, and the CLAs concentrations were much lower compared with the leaf midrib. The distribution of CLAs in grapefruit tree aboveground parts is graphically represented in Fig. 1.

***Ca. L. asiaticus* in different tissues of sweet orange.** *Ca. L. asiaticus* was readily detected in different aboveground tree parts and the bacterial titers ranged from  $4.40 \times 10^9$

Table 1. Different plant parts used in the quantification of *Candidatus Liberibacter asiaticus* by quantitative polymerase chain reaction assay from Huanglongbing-infected field-grown ‘Valencia’ sweet orange and ‘Rio Red’ grapefruit trees in Texas

Grapefruit		Sweet orange		
Tissue	No. samples tested	<i>Ca. L. asiaticus</i> /g of fresh tissue <sup>z</sup>	No. of samples tested	<i>Ca. L. asiaticus</i> /g of fresh tissue <sup>z</sup>
Peduncle	11	$2.95 \times 10^9$ a A	5	$4.40 \times 10^9$ a A
Columella	33	$1.68 \times 10^9$ a A	19	$1.58 \times 10^9$ ab A
Leaf midrib	10	$1.11 \times 10^9$ ab A	13	$1.09 \times 10^9$ ab A
Bark	10	$2.13 \times 10^8$ abc A	22	$2.80 \times 10^6$ c B
Seed	9	$2.08 \times 10^8$ abc A	7	$3.72 \times 10^7$ bc A
Leaf blade middle part	9	$5.82 \times 10^7$ bcd A	8	$6.41 \times 10^6$ c A
Leaf blade edge	10	$2.93 \times 10^7$ cd A	10	$8.36 \times 10^6$ c A
Flower	0	.	6	$7.18 \times 10^6$ c
Young leaf	2	$4.72 \times 10^6$ de	0	.
Flower bud	2	$1.93 \times 10^6$ e	0	.
Young shoot	2	$1.18 \times 10^6$ e	0	.

<sup>z</sup>Means followed by the same small upper case letter within each column are not significantly different at  $P = 0.05$  [Student Newman Keuls (SNK) test]; for each plant part, means followed by the same capital letter are not significantly different at  $P = 0.05$  (SNK test).

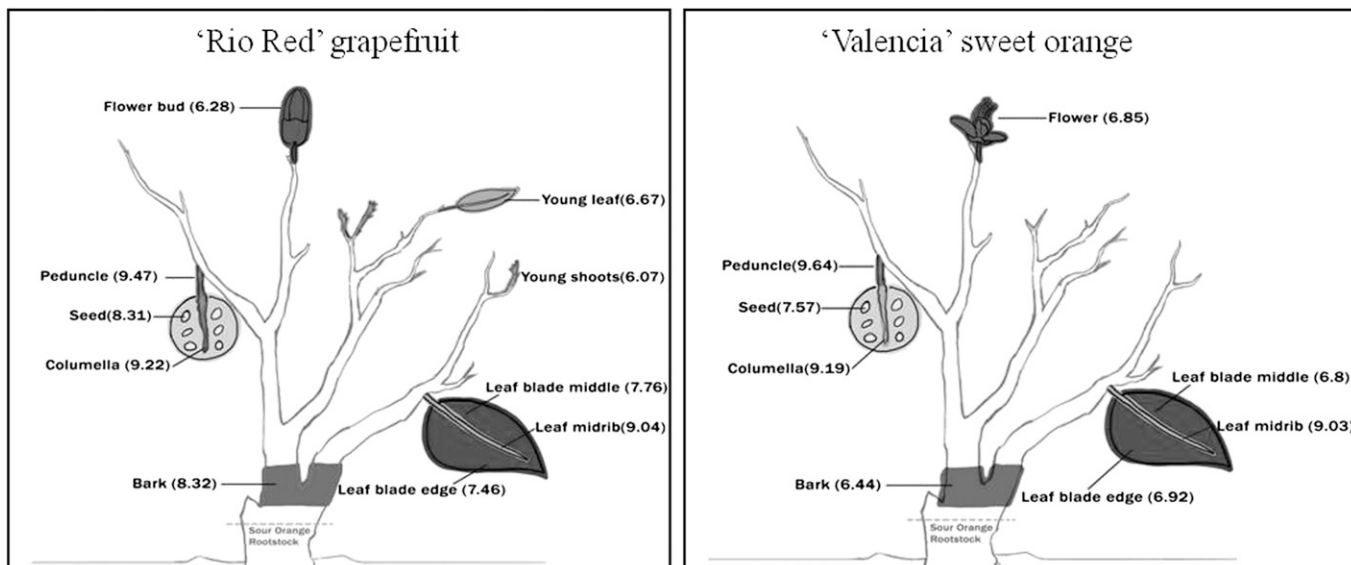


Fig. 1. Distribution of *Ca. L. asiaticus* in the aboveground tree part tissues of field-grown 6-year-old naturally infected 'Rio Red' grapefruit and 'Valencia' sweet orange trees in Texas. Values in parentheses represent log(CLAS) of various plant parts.

to  $7.18 \times 10^6$  with the highest titer recorded for peduncle (Table 1). In general, peduncle, columella, and leaf midrib with petiole produced lower Ct values with higher CLAs genome equivalents. The tissue samples including bark, leaf blade middle part, leaf blade edges, and flowers showed lower CLAs populations, whereas seeds showed intermediate levels of CLAs concentrations. A graphic representation of CLAs populations in sweet orange aerial plant parts is shown in Fig. 1.

*Comparison of Ca. L. asiaticus titers in grapefruit and sweet orange for different plant parts.* With the exception of bark tissue in which significantly higher bacterial titers were recorded for grapefruit compared with sweet orange ( $F = 17.49$ ;  $df = 1, 30$ ,  $P = 0.0002$ ) and juvenile tissues (not sampled from sweet orange), no significant differences were observed for other plant parts between the two host plants.

## Discussion

With the exception of bark in which higher *Ca. L. asiaticus* concentration was recorded in HLB-infected sweet orange trees relative to their grapefruit counterparts and juvenile tissue (not sampled from sweet orange), we found no significant differences in bacterial titer between the two cultivars for the other plant tissues. It is important to note that a recent study reported that there is a significantly lower acquisition of CLAs from grapefruit by *D. citri* compared with other citrus cultivars (Pelz-Stelinski and Stelinski, 2013). Furthermore, previous studies have shown that the transmission of CLAs by adult *D. citri* is lower if the pathogen was acquired as adults compared with acquire as nymphs (Stelinski et al., 2010). Recently, it was reported that different infected scions of cultivars shows varied levels of CLAs concentrations suggesting that there is a potential for using

scions with increased tolerance to effectively manage the disease (Stover and McCollum, 2011).

Current protocols to detect CLAs predominantly use DNA extracts from mature symptomatic leaf midrib. We found that both in sweet orange and grapefruit, leaf midribs harbor very high CLAs concentrations and there is no significant difference in CLAs populations among leaf midrib, columella, and peduncle tissues. Moreover, the leaf blade middle parts and leaf blade edges contained lower bacterial concentrations than midrib. Based on these results, we conclude that our current practices of using leaf midrib tissue to detect CLAs is appropriate and leaf midribs along with petiole are a good source of aboveground tree tissue to detect CLAs. Furthermore, it is important to note that young tissue including leaves, shoots, and flower buds contain the lowest bacterial concentrations.

Understanding the movement of the bacterium inside the tree and its distribution in different plant parts is critical to understanding virulence mechanism and to manage the spread of the disease (Tatineni et al., 2008). It is important to note that on average there were lower CLAs populations in Texas citrus tree tissues compared with an average of  $10^{10}$  CLAs genomes per gram of tissue (Li et al., 2009) that were reported in Florida tissue samples. However, we do not know whether fluctuations of environmental conditions during different seasons in a year will affect this finding because the study was only conducted during June to July 2012 and was not done at different times during the year.

Since its first detection, in Texas, in Jan. 2012 (Kunta et al., 2012), we did not detect the disease outside of the quarantine zone except in a residential grapefruit tree in downtown Mission, TX. We speculate that general low-level acquisition of CLAs from grapefruit by *D. citri*, environmental conditions in Texas

such as high temperatures, proactive *D. citri* control strategies, and early detection of CLAs and tree removal might have contributed to prevent the spread of the disease. The findings of this study provide a useful tool for the Texas citrus industry for using appropriate tissue for efficient CLAs detection so one could take necessary actions to prevent further spread of the disease.

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