Evidence That ‘*Candidatus Liberibacter asiaticus*’ Moves Predominantly Toward New Tissue Growth in Citrus Plants

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Abstract

‘*Candidatus Liberibacter asiaticus*’ (Las) is an unculturable, phloem-limited, insect-transmitted bacterium associated with the Asiatic form of huanglongbing (HLB), the most destructive citrus disease. In Asia and the Americas, it is transmitted by the Asian citrus psyllid (*Diaphorina citri* Kuwamura). Despite considerable research, little is known about the processes involved in plant infection and colonization by Las. This study was conducted to determine whether the basal portion (below girdling) of the plant is an important route for Las to move laterally from a point of inoculation on a branch to pathogen-free branches elsewhere in the canopy, and to quantify the influence of actively growing tissues on vertical upward (acropetally) or downward (basipetally) movement of Las. Nongirdled and fully or partially girdled stems of potted plants of ‘Pera’ sweet orange, graft-inoculated above or below girdling, were sampled in distinct regions and assessed by qPCR, 6 months postinoculation. Las invaded all regions of partially and nongirdled plants but remained

restricted to the inoculated regions of fully girdled plants, evidence that in planta bacterium movement is limited to the phloem. In fully girdled plants, starch accumulated above the girdling site, probably because of changes in flow of phloem sap. To study the influence of actively growing tissues, inoculated ‘Valencia’ sweet orange plants were kept intact or were top- or root-pruned to force production of new tissues, and sampled at 15-day intervals. Las migrated rapidly and most predominantly toward newly developing root and leaf tissues. The rapid and predominant movement of Las to newly developed shoots and roots would explain failures of canopy heat treatments and pruning to cure HLB-affected trees, and reinforces the need to protect rapidly growing new shoots from feeding by *D. citri* in order to minimize transmission and spread of the pathogen by the vector within and between orchards.

Keywords: huanglongbing, girdling, qPCR, *Liberibacter* movement

‘*Candidatus Liberibacter asiaticus*’ (Las) is a phloem-limited bacterium associated with the severe Asiatic form of huanglongbing (HLB), a highly destructive citrus disease (Bové 2006, 2014; da Graça 1991; Gottwald 2010). Less severe forms of HLB are associated with ‘*Ca. L. africanus*’ (Laf) and ‘*Ca. L. americanus*’ (Lam) (Bové 2006). The three species vary in their adaptation to environmental conditions and in geographic distribution. Laf is heat sensitive and occurs in Africa (Bové 2006; Bové et al. 1974). Lam also is heat sensitive (Gasparoto et al. 2012; Lopes et al. 2009a) and has been found in commercial orchards only in Brazil. Las is heat tolerant and present in Asia, Africa, and the Americas (Bové 2014; da Graça et al. 2016). Laf is transmitted by *Trioza erytreae* del Guercio (Hemiptera: Sternorrhyncha: Triozidae) (McClean and Oberholzer 1965) and Lam and Las by *Diaphorina citri* Kuwayama (Sternorrhyncha: Psylloidea) (Capoor et al. 1967; Yamamoto et al. 2006). Laf, Lam, and Las can also be transmitted by plant tissue grafting (Coletta-Filho et al. 2010; Hilf and Lewis 2016; Lopes et al. 2009b) or parasitically by *Cuscuta* sp. (Solanales: Convolvulaceae) (Garnier and Bové 1983; Hartung et al. 2010). Under experimental conditions, Las transmission through tissue grafting is more efficient than through its natural vector *D. citri*, providing an easier approach to

study plant-pathogen interactions (Albrecht et al. 2014; Cifuentes-Arenas et al. 2019).

Although Las acquisition and transmission by *D. citri* have been the focus of various studies, and despite considerable research (Canale et al. 2017; Inoue et al. 2009; Pelz-Stelinski et al. 2010), little is known about the processes that follow inoculation, namely, infection, establishment, and colonization of host plants by the pathogen. Random localization within the plant (Hilf et al. 2013; Jagoueix et al. 1994; Kim et al. 2009) and inability to grow the pathogen in culture media make such studies very challenging. Microscopy and electrical penetration graph (EPG) studies suggest that successful inoculation of citrus by infectious psyllids can occur within 1 h of feeding, commencing after their stylets reach phloem (Bonani et al. 2010). Once established, the bacterium moves and colonizes unevenly all plant parts, except the seed endosperm (Li et al. 2009; Tatineni et al. 2008) and pollen grains (Etcheberria et al. 2016). In young, stem-graft inoculated potted plants, Las moved upward reaching the leaves that remained free from symptoms for at least 2 months (Lopes et al. 2009b). In graft-inoculated potted plants, the pathogen was found in the first evaluation date, 2 months postinoculation, in both roots and leaves, but in higher proportion in roots than in the canopy (Johnson et al. 2014). The detection of the pathogen in the roots 2 months after grafting suggested that downward bacterium movement is rapid (Johnson et al. 2014). Also, Las movement dynamics seemed to follow that of phloem sap flow (Johnson et al. 2014), which moves predominantly from mature source tissues to actively growing sinks, such as fruit, root tips, and new shoots (Blanke 2009; Fischer et al. 2012; Taiz et al. 2017).

Within the Las genome, genes involved in flagella biosynthesis were found (Duan et al. 2009), but in microscopy images of cells of the bacterium within plant tissues no structures like flagella have been found (Andrade et al. 2019; Hilf et al. 2013). This suggests that

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online.

The author(s) declare no conflict of interest.

Accepted for publication 8 July 2020.

Las would move passively within the phloem, as do vascular viruses and phytoplasmas (Christensen et al. 2004; Dawson et al. 2013; Folimonova et al. 2008; Jiang et al. 2004). With dimensions smaller than those of phloem sieve pores (Achor et al. 2020; Bové 2006; Kim et al. 2009), Las cells would be easily transported with phloem sap, movement of which follows the source/sink relationships (Blanke 2009; De Schepper et al. 2013; Fischer et al. 2012; Goldschmidt 1999; Oparka and Cruz 2000; Patrick 1997; Taiz et al. 2017). In the process of host plant colonization, Las cells must move from the point of inoculation on a branch to reach pathogen-free branches elsewhere in the canopy, as indicated by the progress of visible symptomatic tissues in tree canopies. Where lateral movement of the pathogen occurs in host plants is not known. It could be at point in trunks or roots.

A better understanding and quantification of the main route of Las dispersal within a citrus tree will help in assessing new strategies to control HLB as, for instance, testing of novel antimicrobial molecules against Las. It may also help to assess the tolerance mechanisms to the Asiatic form of HLB observed in some rootstock genotypes (Albrecht and Bowman 2012; Boava et al. 2015; Castle et al. 2015; Stover et al. 2015). This study was carried out with two objectives: (i) to determine whether the basal portion (below girdling) of a plant is an important route for Las to move laterally from a point of inoculation on a branch to pathogen-free branches elsewhere in the canopy, and (ii) to quantify the influence of actively growing tissues (e.g., new shoots or root tips) on vertical, upward (acropetally) or downward (basipetally) movement of the pathogen.

Materials and Methods

In total, 290 healthy potted sweet orange (*Citrus × aurantium* L., syn. *C. sinensis* [L.] Osbeck [Rutaceae: Aurantioideae: Aurantieae]) plants, grown in decomposed pine bark substrate (Plantmax citrus, Eucatex, Paulínia, SP), were used in four greenhouse experiments,

with the replications (plants) within treatments distributed in complete randomized designs. All plants were irrigated three times a week with a solution containing appropriate macro and micronutrients for citrus, and sprayed monthly with insecticides and miticides.

Lateral movement of Las. The influence of the plant basal portion on lateral movement of Las was explored in experiments L-1 and L-2. A group of 1.5-year-old plants of ‘Pera’ sweet orange grafted on ‘Rangpur’ lime (*C. limonia* Osbeck), growing in 12-liter pots, were top pruned 60 cm above soil level to stimulate growth of new shoots. Two shoots on opposite sides of each plant were allowed to grow into two branches in a “Y” configuration (Fig. 1). When the new branches reached approximately 1 cm in diameter, they were pruned 20 cm from their juncture with the main stem. At this point, each scion was wrapped in aluminum foil to stimulate production of new shoots on the rootstock stem and prevent production of new shoots on scions. When primordia of new shoots were visible, ca. 10 days later, all but one were removed from each rootstock. The aluminum foil was removed 25 days later to allow single new shoots to develop on each rootstock (Fig. 1).

To test the hypothesis that the rootstock, including root, is a relevant pathway for Las to move laterally from the inoculated branch to the uninoculated branch on the opposite side of the plant canopy, girdling procedures were used in two experiments. The initial experiment (exp. L-1) involved nongirdled (control) (Fig. 1A) and fully girdled plants (Fig. 1B and C). Girdling was carried out to interrupt phloem connection between the upper and lower parts of the plants. It consisted of removing a 1-cm-wide ring of bark from the entire perimeter of the main stem of the scion, 30 cm above the soil level. To ensure there were no phloem tissues at the girdled site, whitish woody tissue below the bark was fully removed by scratching it with a razor blade immediately after girdling. This procedure was repeated daily for 2 weeks thereafter. The single shoot that developed on each

Fig. 1. Schematic representation of the 1.5-year-old potted plants of ‘Pera’ sweet orange grafted on Rangpur lime rootstock used in the experiments to assess the lateral movement of ‘*Candidatus Liberibacter asiaticus*’ (Las) in the plant canopy. Values indicate the number of Las-positive sites over the total number of sites, and titers of Las cells g^{-1} tissues (see Table 1 for details). The plants remained intact (A) or were fully (B and C) or partially girdled (D to G) as indicated by the dark arrows. Girdling consisted of removing a 1-cm-long bark segment from the stem 30 cm above soil level. A Las-positive budstick (white arrows) was grafted at different positions in relation to that of the girdling side, namely, above or below (B and C); above, at the same or opposite side (D and E); or below, at the same or opposite side (F and G). Samples were collected from the inoculated scion branch (i), scion main stem (ii), noninoculated scion branch (iii), new shoots (only in exp. 2) (iv), rootstock stem (v), rootstock sucker (vi), and fibrous roots (vii). Samples were analyzed by quantitative real time PCR 6 months after inoculation. Treatments A to C were used in a first experiment and A to G in the second experiment.

rootstock was maintained to generate photoassimilates to the roots of the fully girdled plants. A second experiment (exp. L-2) involved fully girdled plants and partially girdled plants from which the 1 cm width of bark was removed from half of the stem perimeter (Fig. 1D to G). This procedure was undertaken to minimize starch accumulation above girdling site.

All plants in both experiments were inoculated by grafting the scion as described (Lopes et al. 2009b). Graft inoculation usually results in higher frequency of Las infection than through insects and, in our view, would be appropriate for the purpose of this work. As inoculum sources, we used stem segments (budsticks) collected from HLB-symptomatic and Las-positive sweet orange trees. In exp. L-1, the budsticks were ca. 2 to 3 cm long and 0.8 cm wide and were grafted onto the plants at two heights: (i) on the upper branch, 10 cm distant from the main stem (top inoculation) and (ii) on the rootstock stem, 20 cm below the girdle or, for the nongirdled plants, 15 cm above the surface of the pine bark substrate (base inoculation). The controls received Las-negative budsticks. In exp. L-2, the budsticks were 2 to 3 cm long by 0.5 cm wide. The nongirdled and fully girdled plants were inoculated on one of the two upper branches, as described in exp. L-1 (Fig. 1A). The partially girdled plants were grafted at the same or opposite side of the stem section in relation to the girdling site, about 40 cm above or 15 cm below the girdle (Fig. 1D to G). Additionally, in exp. L-2, to stimulate development of a new shoot, the upper branch opposite to the one that received the inoculum was pruned about 5 cm distant from the apex, 6 months after inoculation (MAI). As indicated, in exp. L-2, the budstick diameter was ca. 3 mm narrower than those used in exp. L-1. This was done in an attempt to reduce the proportion of the branch perimeter occupied by the inoculum in relation to that of the inoculated stem, and consequently, to minimize the chances that the bacteria eventually detected in other portions of the plant had migrated laterally from the side edge of the inoculum source (budstick). With this decrease in width, the perimeter of the branch or stem occupied by the budstick, which in exp. L-1 was ca. 30 and 15%, respectively, for the upper and lower inoculated sites, was reduced in exp. L-2 to ca. 20 and 10%.

In both experiments, the plants (eight per treatment) were routinely observed. At 6 MAI, the plants were assessed for leaf symptoms of

HLB and for the presence and titer of Las at different portions, namely: (i) inoculated scion branch, just below the basal edge of the budstick; (ii) scion main stem, 10 cm below the stem bifurcation (upper stem); (iii) noninoculated opposite scion branch, at 10 cm above stem bifurcation; (iv) new shoots that developed after pruning on the noninoculated scion branch (only in exp. 2); (v) rootstock stem, just below scion-rootstock junction (lower stem); (vi) rootstock sucker, at 10 cm above its insertion into the rootstock; and (vii) fibrous roots (root) (Fig. 1).

In exp. L-1, one fully girdled and one nongirdled plant were selected for a more detailed analysis of Las distribution. From the inoculated upper branch of these plants, bark tissue of the inoculum budstick was sampled, and bark rings of the branch were taken at distances starting 1, 2, and 3 cm in upward and downward directions from the inoculum budstick edge. Each bark ring was 1 cm wide and was divided into two halves (same side and back side to the inoculum budstick position) that corresponded to the stem semi-perimeter. All samples were processed individually for DNA extraction and qPCR analysis. Except for new shoots and roots, all samples consisted of bark tissues (1-cm ring). Root samples consisted of ca. 0.3 g of the tips of newly formed plus old fibrous tissues collected after removal of the potting medium (or soil). Exps. L-1 and L-2 were carried out from 29 September 2014 to 17 April 2015, and from 26 February to 02 September 2016. Daily temperatures and relative humidity varied from 11.7 to 44.2°C and 13.3 to 97.4% (averages 26.6°C and 75.9%) during exp. L-1, and 4.7 to 37.7°C and 30.9 to 96.8% (averages 22.1°C and 80.8%) during exp. L-2.

Vertical movement of Las. The influence of new growth on vertical upward or downward movement of Las was explored in experiments V-1 and V-2. Three-year-old plants of 'Valencia' sweet orange grafted on 'Volkamer' lemon (*C. limonia*) rootstock, growing in 4-liter pots and trained as whip trees (single main stem), were pruned 60 cm above the substrate surface to force the growth of new shoots. Two months after pruning, the plants were graft inoculated on the stem with 2 to 3 cm long by 0.8 cm wide Las-positive budsticks as described. The inoculum was positioned at 30 cm above the surface of the pine bark substrate (20 cm above the scion-rootstock union). Fifteen days after inoculation, the plants were separated in four groups of 35 plants (Fig. 2). In group A, the plants were maintained intact (non-pruned). In group B, the mature leaves present on the upper 20 cm portion, and all shoots that had developed above 60 cm of the main stem, were removed to force new shoot growth (top-pruned). In group C, the plants were carefully removed from the pots and the root system ball was pruned to reduce most of the root volume to force the emergence of new roots (root-pruned). Then, the plants were returned to the pots. In group D, the pruning procedure and parts removed were as in B, except that pruning was carried out in five plants each, at the same inoculation date and at every sampling date.

For plants in groups A, B, and C, sampling to determine the presence and titer of Las was carried out on mature leaves, newly developed shoots and leaves, stem bark, and fibrous roots, 30, 45, 60, and 75 days after pruning. Five plants per group (treatment) were destructively sampled on each sampling date. Samples consisted of (i) four mature leaves that were present evenly distributed on the stem before the inoculation date, (ii) one young new shoot or six young immature leaves that developed after inoculation, (iii) a 1-cm-long full ring of bark removed from the stem at 10 cm above the inoculation site, and (iv) old fibrous and the newly developed whitish postpruning roots. For the plants in group D, only the 15-day-old new shoots that developed after every top pruning date were sampled. Two experiments were undertaken. The first, exp. V-1, ran from 14 August to 14 December 2015 and the second, exp. V-2, was a repetition of exp. V-1 from 18 April to 17 August 2016. Daily temperatures and relative humidity varied from 13.1 to 38.9°C and 28.6 to 97.1% (averages 23.1°C and 73.3%) during exp. V-1, and 7.6 to 36.9°C and 38.8 to 95.7% (averages 21.9°C and 80.8%) during exp. V-2.

qPCR analysis. Total DNA was extracted from 0.3 g of leaves, bark, young shoots, or fibrous roots per plant using the CTAB

Fig. 2. Schematic representation of the upper and lower parts of nonpruned (A), top-pruned (B), root-pruned (C), and pruning procedure and parts removed were as in B, except that pruning was carried out in five plants each, at the same inoculation date and at every sampling date (D), plants of 'Valencia' sweet orange grafted on 'Volkamer' lemon rootstock used to assess the influence of new tissue growth on the movement direction of '*Candidatus Liberibacter asiaticus*' (Las) in experiments V-1 and V-2. Sampling was carried out 30, 45, 60, and 75 days after top- or root-pruning. In the top-pruned plants, shoots and leaves present in the upper 20 cm of the main stem were removed. In the root-pruned plants over half of the total volume of fibrous roots was removed. Pruning was carried out 15 days after the inoculation date. Inoculum consisted of a 2- to 3-cm long Las-infected budstick, which was grafted at the scion main stem 30 cm above substrate level.

extraction buffer (Murray and Thompson 1980) as described by Teixeira et al. (2005). Quantitative PCR was run in a total volume of 12 µl, containing 6 µl master mix (Invitrogen, Carlsbad, CA), HLBspr primers kit (Li et al. 2006), and 3 µl of DNA samples, in 7500 Real-Time PCR equipment (Applied Biosystems). Cycle threshold (Ct) values lower than or equal to 34.0 were transformed in log number of Las genomes per gram of tissue using the quantification curve proposed by Lopes et al. (2013). Ct values above 34.0 (equivalent to 2.2 Las cells per gram of tissue), and ‘undetermined’ results after qPCR were taken as negative for the presence of Las. As positive and negative controls for qPCR, we used DNA samples extracted from diseased or healthy potted plants maintained constantly inside an insect-proof greenhouse. In all cases, the samples resulted in ‘undetermined’ or in Ct values over 34.1 (avg. 36.4; highest value 39.7) for negative controls, or in Ct values lower than 23.9 (avg. 22.2) for the positive controls.

Data analyses. For the data on experiments (exp. L-1 and exp. L-2) undertaken to determine whether the basal portion of a plant is an important route for Las to move laterally from inoculated branches to uninoculated branches within canopies, no statistical analysis was performed. For the experiments (exp. V-1 and exp. V-2) undertaken to assess the influence of new growth on vertical movement of Las within tree canopies, logistic regression analysis was used on the proportion of Las-positive sites to determine the probabilities of detecting Las in specific tissues sampled, in the top or root-pruned plants in relation to the nonpruned plants (odds ratios), and analysis of variance followed by mean comparison (Tukey, $P \leq 0.05$) was used on titer values. For the average titer of Las cells in new shoots of treatment D in exp. V-1 and exp. V-2, the monomolecular model $y_t = k_u * (1 - e^{(-\beta * (t - \delta))})$ was adjusted, in which y_t is the Las titer at time t , k_u the asymptote of the curve or maximum Las titer, β is a locational parameter related to the initial inoculum, δ is the rate of bacterium growth, and t the time in days (Madden et al. 2007). The model was adjusted separately for exp. V-1 and exp. V-2, and then for the combined data of both experiments. Equation parameters were compared by the t test.

Results

Lateral movement of Las. Lateral movement of Las was studied in two experiments (L-1 and L-2) involving nongirdled and fully and partially girdled plants. Nine budsticks from exp. L-1 and 15 from exp. L-2 died within 3 weeks of grafting, so the plants were discarded. No impact of stem girdling on budstick survival was observed, but stem girdling affected plant development. Six months after the grafting of Las-positive budsticks, the plant

height averaged 73.9 ± 5.18 , 82.1 ± 2.34 , and 107.1 ± 9.11 cm, and the rootstock suckers were 99.9 ± 8.66 , 86.9 ± 5.78 , and 77.9 ± 8.85 cm long in fully girdled, partially girdled, and nongirdled plants, respectively.

In nongirdled plants, which served as a control for the girdling procedure, Las was detected in all sample types 6 MAI, but the distribution within individual plants varied (Table 1, Fig. 1A). In exp. L-1, Las was detected in 10 out of 24 sampled sites. Las was not detected in the upper portion of one plant (#2), and in any portion of another plant (#4). In exp. L-2, Las was detected in most sampled sites (46/56). In three out of eight plants, Las was not detected in the branch and their respective new shoots that developed after pruning, nor in the lower stem (rootstock bark) or in the rootstock sucker. Las titer was lower in the roots (avg. 3.6 ± 0.12 log of Las cells g^{-1}) than in the upper sites (avg. 5.1 ± 0.08 log cells g^{-1}). Typical HLB leaf symptoms were observed on the upper branches of all Las-positive (Supplementary Fig. S1B and C) but not in any of the Las-negative plants (Supplementary Fig. S1A). Symptoms started with a light chlorosis on leaf lamina about 4 MAI, progressing to typical mottling and thickened corky veins associated with HLB about 2 months later. Strong yellowing typical of mineral deficiencies also appeared on some leaves of Las-positive HLB-symptomatic plants (Supplementary Fig. S1D). On the rootstock sucker, leaf symptoms progressed as in the upper sweet orange scion (Supplementary Fig. S1F), but the symptoms of mineral deficiency were lighter (Supplementary Fig. S1G) than those on shoots located in the upper scion.

On plants that were fully girdled, Las could not cross the girdle site in any plant, in either downward or upward directions (Table 1, Fig. 1B and C). In the plants inoculated above the girdle, Las was detected in the upper stem and inoculated branch (six of 47 sites), at avg. 4.2 ± 0.55 log of Las cells g^{-1} , but not in the non-inoculated opposite branch (Fig. 1B). Las was also not detected in new shoots that developed after pruning on the noninoculated opposite branch in exp. 2. In the plants that were inoculated below girdling, Las was found in 35 of 39 sites sampled from lower stem, rootstock sucker, and roots, at avg. 4.9 ± 0.14 log cells g^{-1} (Table 1, Fig. 1C).

In fully girdled plants, symptoms similar to those that developed on the Las-positive nongirdled plants started to appear on leaves of the upper scion of all plants, 1 month after girdling, regardless of inoculation. Also, lower numbers of new shoots developed on the scion of fully girdled plants. On the rootstock sucker of fully girdled plants and graft inoculated below girdling, typical HLB symptoms appeared and progressed as in nongirdled plants (Supplementary Fig. S1F and

Table 1. Frequency of infection by ‘*Candidatus Liberibacter asiaticus*’ (Las) and bacterium titers at different sites of 1.5-year-old potted plants of ‘Pera’ sweet orange growing on ‘Rangpur’ lime rootstock in experiments L-1 and L-2, 6 months after inoculation, in nongirdled (NG), or fully (FG) or partially (PG) stem girdled plants, with inoculum consisting of a Las-infected budstick grafted below or above the girdling site

Plant status, girdling position ^a and side ^b	Exp	Inoculated branch		Upper stem		Opposite branch		New shoots		Lower stem		Rootstock sucker		Roots	
		n/N ^c	Log ^d	n/N	Log	n/N	Log	n/N	Log	n/N	Log	n/N	Log	n/N	Log
NG	1	1/4	6.1	2/4	5.1 ± 1.10	1/4	6.2	ns	ns	2/4	5.8 ± 0.15	2/4	5.2 ± 0.05	2/4	5.4 ± 0.05
	2	8/8	5.5 ± 0.21	8/8	5.3 ± 0.22	5/8	6.0 ± 0.18	5/8	5.6 ± 0.17	8/8	4.6 ± 0.36	5/8	4.6 ± 0.37	7/8	3.8 ± 0.22
FG, below	1	1/5	5.0	2/5	3.0 ± 0.65	0/5	nd	ns ^f	ns	0/5	nd	0/5	nd	0/5	nd
	2	2/8	4.3 ± 1.15	1/8	5.5	0/8	nd	0/8	nd	0/8	nd	0/8	nd	0/8	nd
FG, above	1	0/6	nd ^e	0/6	nd	0/6	nd	ns	ns	5/6	6.0 ± 0.05	6/6	5.1 ± 0.19	5/6	5.3 ± 0.06
	2	0/7	nd	0/7	nd	0/7	nd	0/7	nd	6/7	4.9 ± 0.23	7/7	4.8 ± 0.30	6/7	3.7 ± 0.24
PG, below, same	2	4/8	5.3 ± 0.39	5/8	5.5 ± 0.27	5/8	5.3 ± 0.37	4/8	4.8 ± 0.44	5/8	5.2 ± 0.32	5/8	5.2 ± 0.33	4/8	3.0 ± 0.28
PG, below, opposite	2	5/8	5.4 ± 0.22	6/8	5.8 ± 0.25	5/8	4.8 ± 0.53	5/8	5.1 ± 0.79	6/8	5.2 ± 0.49	4/8	6.2 ± 0.16	4/8	4.1 ± 0.05
PG, above, same	2	4/8	5.0 ± 0.43	5/8	5.1 ± 0.31	4/8	5.3 ± 0.44	4/8	4.6 ± 0.71	5/8	5.1 ± 0.22	4/8	5.4 ± 0.45	4/8	3.1 ± 0.25
PG, above, opposite	2	4/8	5.0 ± 0.55	3/8	5.0 ± 0.65	3/8	5.4 ± 0.34	3/8	5.8 ± 0.15	3/8	5.3 ± 0.11	3/8	5.0 ± 0.35	2/8	3.5 ± 0.20

^a At 30 cm above the soil level (see Fig. 1 for details).

^b Side at which the partially girdled site was in the stem in relation to that of the inoculum.

^c Number of Las-positive samples (n)/total (N) number of samples assessed by qPCR.

^d Log₁₀ ($\pm$ SEM) of Las cells g^{-1} of tissue transformed from Ct values (Lopes et al. 2013).

^e nd: not detected.

^f ns: not sampled.

G), but only on Las-positive shoots. The fast yellowing of leaves of this group of plants suggested that the girdling procedure had induced starch accumulation in the mature leaves (Cimò et al. 2013; Gonzalez et al. 2012). The iodine test (Takushi et al. 2007) was then applied on one leaf sample from all plants. A purple color resulted from all symptomatic leaves of nongirdled and fully girdled plants, regardless of whether the plants had or had not been inoculated and whether they were or were not showing HLB symptoms (results not shown). Although not quantified, the purple color was stronger from the fully girdled plants, indicating a higher starch concentration. Six months after inoculation, most symptomatic leaves had dropped.

In an attempt to minimize the strong accumulation of starch observed in the fully girdled plants of exp. L-1, a group of plants was partially girdled and included in exp. L-2. Overall, partial bark removal did not impair upward or downward movement of Las. The bacterium was detected in 80.3% (118/147), 82.1% (46/56), and

34.9% (22/63) of the sampled sites of partially girdled, nongirdled, and fully girdled plants (Table 1). However, in the partially girdled plants, the extent of bacterium movement seemed to be influenced by the position occupied by the inoculum in relation to the girdle. Las was detected in 89 and 73% of samples where inoculum and girdling sites were at the same or opposite sides, respectively. In partially girdled plants, symptom appearance and progress followed those on nongirdled plants, without any starch accumulation in Las-negative plants.

Because Las was not detected in fully girdled plants in the branch opposite to the one that was inoculated (Table 1), we decided to investigate the extent of the impact of girdling on Las movement. To assess Las movement, we used one fully girdled and another nongirdled plant of exp. L-1, as illustrated in Figure 3. Las was detected in bark samples close to the Las-positive budstick of both plants, but at lower frequencies (10/12) and titers (avg. 2.7 ± 0.43 log Las cell g^{-1}) in fully girdled than in nongirdled plants (12/12, avg. 6.2 ± 0.06 log). In the inoculated branches of the nongirdled plant, Las moved greater distances, was uniformly distributed in both directions and sides, and reached higher titers with similar values at the inoculated and the opposite side (5.7 to 6.4 log) of the branch. In the branch of the fully girdled plant, Las was detected in all bark samples located at the same side of the inoculum, with higher values in the upward direction (4.2, 4.4, 4.7 log) than in the downward direction (2.3, 2.6, 2.2 log). On the side opposite to the Las-positive budstick of the fully girdled plant, Las was detected only up to 2 cm away from the inoculum in both upward and downward directions, with average titer of 2.9 ± 0.14 log Las (Fig. 3).

Vertical movement of Las. Vertical movement of Las from the middle portion of the stem was assessed in two experiments (V1 and V2), in plants that remained intact and in plants that were shoot- or root-pruned in order to stimulate production of new tissues. Young shoots with newly formed nondifferentiated leaves were present in both pruned and nonpruned plants at the time of the first evaluation, 30 days after inoculation (dai). Similarly, new white root tips, contrasting with the brownish older roots, were found on pruned and nonpruned roots (Supplementary Fig. S2). In general, greater numbers of newly formed tissues (leaves or roots) were detected on pruned plants. New and mature roots and leaves located at various distances above or below the inoculum, and stem bark located 10 cm above, were sampled periodically to estimate the percentage of Las-positive sites and bacterium titer. The way Las moved varied but followed a similar pattern in both experiments (Supplementary Fig. S3). Thus, the data were combined with the averages shown in Figure 4.

The time when Las was first detected in the sampled sites varied between treatments. In nonpruned plants, Las was first detected in stems and new leaves, and mature leaves 30 dai. The percentage of Las-positive sites and Las titers (log cell g^{-1} tissue [log]) in the tissue were of 90% and 4.0 ± 0.23 log, 70% and 3.8 ± 0.35 log, and 10% and 1.1 log, respectively (Fig. 4A and B). In root-pruned plants, stem was the only tissue infected after 30 days. In the roots of root-pruned plants, Las was found 45 dai, the same number of days as in nonpruned plants, but at higher incidence and titer (60%, 2.8 ± 0.23 log). At this time, Las was found in new and mature leaves of root-pruned plants, with percentage of Las-positive sites and Las titers of 40% and 3.0 ± 0.28 log, and 10% and 1.1 log, respectively (Fig. 4C and D).

Las movement in top-pruned plants was less pronounced than that of root-pruned plants. Although present at lower levels than in nonpruned plants, the pathogen reached the stem and new shoots of top-pruned plants on the same day after inoculation as it did in nonpruned plants, but at lower titers. However, in contrast to root-pruned plants, movement of the bacterium to roots took longer in top-pruned plants in which it was first detected 60 dai in 50% of plants and at 2.9 ± 0.20 log (Fig. 4E and F).

Pruning of plants also influenced the pattern of infection, with the impact of root removal greater than shoot removal on the incidence and titer in the Las-infected sites. From the first occasion that Las

Fig. 3. Schematic representation of the distribution and titers of '*Candidatus Liberibacter asiaticus*' in log cells g^{-1} bark tissue, 6 months after inoculation, in 12 bark sampling positions each of a graft-inoculated branch of fully girdled (dark columns) or nongirdled (gray columns) 1.5-year-old 'Pera' sweet orange potted plants in experiments L-1 and L-2. Girdling involved removing a 1 cm bark ring from the full perimeter of the plant main stem, 30 cm above soil level and 40 cm below the budstick used as inoculum. Sampling positions were between 0 and 1, 1 and 2, and 2 and 3 cm distant downward or upward from the edge of the budstick used as inoculum, at the same or opposite side in relation to that of the inoculum.

was detected in nonpruned and top-pruned plants to the final evaluation date, the incidence of Las-positive sites and titer values of the pathogen increased in most tissues. In contrast, incidence of Las-positive sites and titers of the pathogen in top-pruned plants increased in the new roots and stem tissues and decreased in the new and mature leaves in root-pruned plants (Fig. 4A to F). Statistical analysis of the overall Las titer data also showed higher impact of root pruning than top pruning on plant colonization. A significantly lower average value was attained from root-pruned than nonpruned plants ($n = 32$, $F = 23.24$, $P < 0.0001$), but no differences between top-pruned and nonpruned plants ($n = 32$, $F = 0.66$, $P = 0.4179$).

To better assess the impact of the new tissue growth on the direction of Las movement, logistic regression analysis of probability was applied to the overall data on the incidence of Las infected sites. The outcome is an estimate of the probability of detecting Las in each sampled site, in root-pruned or top-pruned plants in relation to nonpruned plants (Table 2). For the combined data of exp. V-1 and exp. V-2, the probabilities of finding Las in the new leaves, mature leaves, stem bark, and roots of the root-pruned plants were, respectively, 0.08, 0.44, 0.22, and 3.72 fold of those in the same sites of nonpruned plants, whereas the probability of detecting Las in the same sites in top-pruned plants were 0.68, 1.29, 0.76, and 1.47 fold, respectively, of those in nonpruned plants.

Las migration toward newly developed shoots of top-pruned plants was evaluated in more detail in plants of treatment D of experiment V1 and V2. At the inoculation date and every 15 days, a different group of inoculated plants was pruned. All 15-day-old shoots that had developed at or near the pruned site were sampled and analyzed by qPCR. Las was already present in the new shoots of 60% of the inoculated plants, at a mean titer of $\log 2.98 \pm 0.17$ cells g^{-1} of tissue, 15 days after inoculation. Thirty days later, all plants contained Las, with the titer reaching a maximum value of $\log 6.79$ cells g^{-1} of tissue. The monomolecular model best described titer increase overtime (Table 3, Fig. 5).

Discussion

This study was carried out to determine whether the basal portion of a potted citrus plant is an important route for Las to move from an infected site on one side of the canopy to an uninfected site on the opposite side of the same canopy. In the absence of a Las-immune rootstock, which would restrict pathogen multiplication in the lower stem and roots, girdling of the main stem was an artificial procedure used to physically separate phloem between the upper and lower portions of the plant.

The pathogen was restricted to the inoculated branches of plants that were fully girdled and inoculated with Las above the girdle for

Table 2. Odds ratio of '*Candidatus Liberibacter asiaticus*' detection over time, in different parts of root- or top-pruned 3-year-old plants of stem graft-inoculated 'Valencia' sweet orange scions on 'Volkamer' lemon rootstocks, in experiments V-1 and V-2, as compared with the nonpruned plants^a

Variable	Roots	Stem bark	Mature leaves	New leaves
Experiment V-1				
Top-pruned ^b	0.45	0.27	1.02	0.50
Root-pruned ^b	0.68	0.10	0.19	0.06
Experiment V-2				
Top-pruned	6.38	1.64	1.63	1.00
Root-pruned	39.1	0.37	0.78	0.11
Experiment V-1+2				
Top-pruned	1.47	0.76	1.29	0.68
Root-pruned	3.72	0.22	0.44	0.08

^a An odds ratio of 1.0 indicates that the chance of detecting the bacterium in the sampled tissue is the same as that in nonpruned plants.

^b Statistics of logistic regression: Pruning position ($\chi^2 = 140.66$, $DF = 2$, $P < 0.0001$), sampled tissues (new leaves, mature leaves, stem, and roots) ($\chi^2 = 106.06$, $DF = 3$, $P < 0.0001$).

Fig. 4. Average data from two experiments on the percentage of infected (A, C, E) and titers of '*Candidatus Liberibacter asiaticus*' (log cells g^{-1} tissue) (B, D, F) in distinct sampled sites (as indicated in A) of pruned and nonpruned graft-inoculated 3-year-old potted plants of 'Valencia' sweet orange grafted on 'Volkamer' lemon. Samplings were destructive and collected from five plants per treatment and evaluation date, undertaken every 15 days from 30 to 75 days after inoculation. A and B, nonpruned plants, C and D, root-pruned, and E and F, top-pruned plants.

6 months. It was never detected in the opposite branch or in the stem, shoots, or roots below girdle, an indication that the basal portion of a plant is an obligatory pathway for the bacterium to colonize an entire plant. However, bark removal led to accumulation of starch in the leaves, with the first signs already detected 1 month after bark removal. It is already known that, as a consequence of a blockage of phloem sap flow, girdling induces starch buildup in all plant tissues (Gonzalez et al. 2012). The apparent nonmovement of the bacterium within the above-girdled portion of the plants may, therefore, have resulted from an impact of bark removal on sap flow and starch accumulation due to phloem discontinuity (Wallerstein et al. 1978). Also, as indicated by Gonzalez et al. (2012), the starch granules that accumulate in girdled citrus plants are chemically distinct from those resulting from Las infection, a probable consequence of changes in sap composition. Therefore, we cannot rule out the possibility that the girdling procedure used in this work may also have had an indirect influence on bacterium multiplication through changes in sap constituents. The negative influence of sap obstruction on Las movement was further evidenced when the presence and titer of the bacterium were assessed at increasing distances from the inoculated budsticks of fully girdled and nongirdled plants. Las moved shorter distances and reached lower titers in branches of fully girdled plants.

With the objective of mitigating starch buildup in plant tissues, in a second experiment, the stem bark was only partially removed. This procedure did not inhibit Las from invading all sampled parts and because the inoculum was positioned at relatively long distances from the girdling site (ca. 40 cm), it is possible that not all vascular tissues that became connected with the infected budstick passed through the girdling site. Therefore, the partial girdling procedure may not have effectively blocked the stem phloem vessels that joined with those of the budstick used as source of inoculum.

In this work, Las was never able to cross the fully girdled site, in either upward or downward directions. This corroborates the information that Las colonizes exclusively phloem tissues (Jagoueix et al. 1994; Kumar et al. 2014), contrary to the hypothesis that the xylem vessels also play a direct role in the movement of Las infectious signal (Etcheberria et al. 2015, 2016).

In this study, we also investigated whether the new roots and shoots that developed on root- or top-pruned plants would influence on the vertical movement of Las. The chance of detecting Las in the roots of root-pruned plants was higher than in roots of nonpruned plants. Conversely, the chances of detecting Las in new shoots of top-pruned plants were lower than in new shoots of nonpruned plants. Thus, the influence of new root growths as a sink for Las seems to be stronger than the influence of new shoot growth, as is known to occur for carbohydrates (Blanke 2009; Fischer et al. 2012; Taiz et al. 2017). At the time Las was first detected in the roots, no symptoms were present on the leaves, similar to what was observed by Johnson et al. (2014). This rapid movement of the pathogen to the roots explains why skeletonization (severe pruning leaving only basal portions of major branches) and thermotherapy have not

been effective in curing citrus trees affected by HLB (Doud et al. 2017; Lopes et al. 2007, 2013).

Even though the chance of detecting Las in new shoots of top-pruned plants was lower than nonpruned plants, movement of Las to the shoots was nevertheless rapid and it reached high titer values (Fig. 5). The bacterium would have been present in these tissues earlier than 15 days after pruning, when the new shoots are at the early stage of development and, consequently, highly favorable to ACP feeding and reproduction (Cifuentes-Arenas et al. 2018). Therefore, the rapid movement of Las to new shoots explains the rapid increase in HLB incidence in orchards in Brazil where trees are topped and laterally pruned without adequate suppression of *D. citri* populations (S. Lopes, unpublished results). These procedures, which became a common practice to limit tree size and facilitate fruit harvest, stimulate intensive flushing, and thus contribute to spread of Las within orchards and regions. Opposite to roots and new leaves, Las movement from the inoculum site to the mature leaves that, at the time of inoculation, were already present on the stem, took longer than to other sampled tissues, especially in the root-pruned plants. Since in mature leaves, phloem sap influx is much less frequent than sap efflux (Ding et al. 1988), the longer time period of Las to move toward mature leaves provides further evidence that Las movement follows the sap flow, in a way proportional to the source: sink balance.

In this work, we describe our efforts to understand the Las pathway to move from an initial Las infection site on one side of potted plant canopies to uninfected sites on the opposite side of potted plant canopies. Although Las pathways of infection remain unresolved, an inability of the bacterium to move through the xylem, and a positive influence of new tissue growth on the predominant movement of the pathogen within citrus plants were clearly demonstrated.

Fig. 5. Monomolecular model that describes the increase in '*Candidatus Liberibacter asiaticus*' titers in newly formed 15-day-old shoots on the upper branches of stem graft-inoculated 3-year-old 'Valencia' sweet orange plants grafted on 'Volkamer' lemon. The model was adjusted to the combined data of two experiments (continuous line). Vertical dotted and continuous bars indicate standard errors of the mean for experiments V-1 or V-2, respectively. For detailed statistics for each experiment and for the combined data, see Table 3.

Table 3. Parameters and statistics of the monomolecular model describes increase over time of '*Candidatus Liberibacter asiaticus*' titers in young shoots developed on the upper branches of stem graft-inoculated 3-year-old 'Valencia' sweet orange plants grafted on 'Volkamer' lemon, in experiments V-1 and V-2

	Model statistics			Parameters of the model								
	F-value	P>F	R ² ^a	k _u ^b	SE ^c	Prob> t	δ ^d	SE	Prob> t	β ^e	SE	Prob> t
Exp. V-1	109.29	<0.0001	0.798	5.7806	0.5186	<0.0001	0.2191	1.4157	0.8786	0.0635	0.0224	0.0103
Exp. V-2	215.51	<0.0001	0.893	5.4928	0.3337	<0.0001	0.0318	0.9733	0.9743	0.0662	0.0168	0.0009
Exps. V-1+2	299.40	<0.0001	0.838	5.6389	0.2982	<0.0001	0.1251	0.8408	0.8824	0.0648	0.0139	<0.0001

^a Coefficient of determination.

^b Upper asymptote.

^c Standard error of the parameter.

^d Delay parameter.

^e Rate of increase.

Acknowledgments

We thank Dr. L. W. Timmer, Professor Emeritus, University of Florida, and Dr. G. A. C. Beattie, Adjunct Professor, Western Sydney University, and the anonymous reviewers for the valuable editing suggestions.

Literature Cited

- Achor, D., Welker, S., Ben-Mahmoud, S., Wang, C., Folimonova, S. Y., Dutt, M., Gowda, S., and Levy, A. 2020. Dynamics of *Candidatus* Liberibacter asiaticus movement and sieve-pore plugging in citrus sink cells. *Plant Physiol.* 182: 882-891.
- Albrecht, U., and Bowman, K. D. 2012. Tolerance of trifoliate citrus rootstock hybrids to *Candidatus* Liberibacter asiaticus. *Sci. Hortic. (Amsterdam)* 147: 71-80.
- Albrecht, U., Hall, D. G., and Bowman, K. D. 2014. Transmission efficiency of *Candidatus* Liberibacter asiaticus and progression of huanglongbing disease in graft- and psyllid-inoculated citrus. *HortScience* 49:367-377.
- Andrade, M. O., Pang, Z., Achor, D. S., Wang, H., Yao, T., Singer, B. H., and Wang, N. 2019. The flagella of '*Candidatus* Liberibacter asiaticus' and its movement in planta. *Mol. Plant Pathol.* 21:109-123.
- Blanke, M. M. 2009. Regulatory mechanisms in source sink relationships in plants – a review. *Acta Hortic.* 835:13-20.
- Boava, L. P., Sagawa, C. H. D., Cristofani-Yaly, M., and Machado, M. A. 2015. Incidence of '*Candidatus* Liberibacter asiaticus'-infected plants among citrandarins as rootstock and scion under field conditions. *Phytopathology* 105:518-524.
- Bonani, J. P., Fereres, A., Garzo, E., Miranda, M. P., Appezzato-Da-Gloria, B., and Lopes, J. R. S. 2010. Characterization of electrical penetration graphs of the Asian citrus psyllid, *Diaphorina citri*, in sweet orange seedlings. *Entomol. Exp. Appl.* 134:35-49.
- Bové, J. M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88:7-37.
- Bové, J. M. 2014. Huanglongbing or yellow shoot, a disease of Gondwanan origin: Will it destroy citrus worldwide? *Phytoparasitica* 42:579-583.
- Bové, J. M., Calavan, E. C., Capoor, S. P., Cortez, R. E., and Schwarz, R. E. 1974. Influence of temperature on symptoms of California stubborn, South Africa greening, India citrus decline, and Philippines leaf mottling diseases. 6th Conf. IOCV. 91:102.
- Canale, M. C., Tomaseto, A. F., Haddad, M. de L., Della Coletta-Filho, H., and Lopes, J. R. S. 2017. Latency and persistence of '*Candidatus* Liberibacter asiaticus' in its psyllid vector, *Diaphorina citri* (Hemiptera: Liviidae). *Phytopathology* 107:264-272.
- Capoor, S., Rao, D., and Viswanath, S. 1967. *Diaphorina citri* Kuway., a vector of the greening disease of citrus in India. *Indian J. Agric. Sci.* 37:572-579.
- Castle, B., Grosser, J., Bowman, K., and Stover, E. 2015. An HLB-tolerant citrus rootstock: What exactly does that mean? *Citrus Ind.* 96:16-19.
- Christensen, N. M., Nicolaisen, M., Hansen, M., and Schulz, A. 2004. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Mol. Plant-Microbe Interact.* 17:1175-1184.
- Cifuentes-Arenas, J. C., Beattie, G. A. C., Peña, L., and Lopes, S. A. 2019. *Murraya paniculata* and *Swinglea glutinosa* as short-term transient hosts of '*Candidatus* Liberibacter asiaticus' and implications for the spread of huanglongbing. *Phytopathology* 109:2064-2073.
- Cifuentes-Arenas, J. C., de Goes, A., de Miranda, M. P., Beattie, G. A. C., and Lopes, S. A. 2018. Citrus flush shoot ontogeny modulates biotic potential of *Diaphorina citri*. *PLoS One* 13:e0190563.
- Cimò, G., Lo Bianco, R., Gonzalez, P., Bandaranayake, W., Etxeberria, E., and Syvertsen, J. P. 2013. Carbohydrate and nutritional responses to stem girdling and drought stress with respect to understanding symptoms of Huanglongbing in citrus. *HortScience* 48:920-928.
- Coletta-Filho, H. D., Carlos, E. F., Alves, K. C. S., Pereira, M. A. R., Boscariol-Camargo, R. L., Souza, A. A., and Machado, M. M. 2010. In planta multiplication and graft transmission of '*Candidatus* Liberibacter asiaticus' revealed by real-time PCR. *Eur. J. Plant Pathol.* 126:53-60.
- da Graça, J. V. 1991. Citrus greening disease. *Annu. Rev. Phytopathol.* 29: 109-136.
- da Graça, J. V., Douhan, G. W., Halbert, S. E., Keremane, M. L., Lee, R. F., Vidalakis, G., and Zhao, H. 2016. Huanglongbing: An overview of a complex pathosystem ravaging the world's citrus. *J. Integr. Plant Biol.* 58:373-387.
- Dawson, W. O., Garnsey, S. M., Tatineni, S., Folimonova, S. Y., Harper, S. J., and Gowda, S. 2013. Citrus tristeza virus-host interactions. *Front. Microbiol.* 4:1-10.
- De Schepper, V., De Swaef, T., Bauweraerts, I., and Steppe, K. 2013. Phloem transport: a review of mechanisms and controls. *J. Exp. Bot.* 64:4839-4850.
- Ding, B., Parthasarathy, M. V., Niklas, K., and Turgeon, R. 1988. A morphometric analysis of the phloem-unloading pathway in developing tobacco leaves. *Planta* 176:307-318.
- Doud, M. M., Wang, Y., Hoffman, M. T., Latza, C. L., Luo, W., Armstrong, C. M., Gottwald, T. R., Dai, L., Luo, F., and Duan, Y. 2017. Solar thermotherapy reduces the titer of *Candidatus* Liberibacter asiaticus and enhances canopy growth by altering gene expression profiles in HLB-affected citrus plants. *Hortic. Res.* 4:17054.
- Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., and Gottwald, T. 2009. Complete genome sequence of citrus Huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. *Mol. Plant-Microbe Interact.* 22:1011-1020.
- Etxeberria, E., Gonzalez, P., and Brodersen, C. 2015. Evidence for alternative pathways of CLAS movement in citrus trees. *Proc. Fla. State Hortic. Soc.* 128:73-76.
- Etxeberria, E., Gonzalez, P., and Brodersen, C. 2016. Movement of HLB genetic signal within citrus trees: more questions than answers. *Proc. Fla. State Hortic. Soc.* 129:83-86.
- Fischer, G., Almanza-Merchán, P. J., and Ramírez, F. 2012. Source-sink relationships in fruit species: A review. *Rev. Colomb. Cienc. Hortic.* 6:238-253.
- Folimonova, S. Y., Folimonov, A. S., Tatineni, S., and Dawson, W. O. 2008. Citrus tristeza virus: survival at the edge of the movement continuum. *J. Virol.* 82:6546-6556.
- Garnier, M., and Bové, J. M. 1983. Transmission of the organism associated with citrus greening disease from sweet orange to periwinkle by dodder. *Phytopathology* 73:1358-1363.
- Gasparoto, M. C. G., Coletta-Filho, H. D., Bassanezi, R. B., Lopes, S. A., Lourenço, S. A., and Amorim, L. 2012. Influence of temperature on infection and establishment of '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus' in citrus plants. *Plant Pathol.* 61:658-664.
- Goldschmidt, E. E. 1999. Carbohydrate supply as a critical factor for citrus fruit development and productivity. *Hortic. Sci. (Prague)* 34:1020-1024.
- Gonzalez, P., Reyes-De-Corcuera, J., and Etxeberria, E. 2012. Characterization of leaf starch from HLB-affected and unaffected-girdled citrus trees. *Physiol. Mol. Plant Pathol.* 79:71-78.
- Gottwald, T. R. 2010. Current epidemiological understanding on citrus huanglongbing. *Annu. Rev. Phytopathol.* 48:119-139.
- Hartung, J. S., Paul, C., Achor, D., and Brilansky, R. H. 2010. Colonization of dodder, *Cuscuta indecora*, by '*Candidatus* Liberibacter asiaticus' and '*Ca. L. americanus*'. *Phytopathology* 100:756-762.
- Hilf, M. E., and Lewis, R. S. 2016. Transmission and propagation of '*Candidatus* Liberibacter asiaticus' by grafting with individual citrus leaves. *Phytopathology* 106:452-458.
- Hilf, M. E., Sims, K. R., Folimonova, S. Y., and Achor, D. S. 2013. Visualization of '*Candidatus* Liberibacter asiaticus' cells in the vascular bundle of citrus seed coats with fluorescence in situ hybridization and transmission electron microscopy. *Phytopathology* 103:545-554.
- Inoue, H., Ohnishi, J., Ito, T., Tomimura, K., Miyata, S., Iwanami, T., and Ashihara, W. 2009. Enhanced proliferation and efficient transmission of *Candidatus* Liberibacter asiaticus by adult *Diaphorina citri* after acquisition feeding in the nymphal stage. *Ann. Appl. Biol.* 155:29-36.
- Jagoueix, S., Bove, J. M., and Garnier, M. 1994. The floem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the proteobacteria. *Int. J. Syst. Bacteriol.* 44:379-386.
- Jiang, H., Wei, W., Saiki, T., Kawakita, H., Watanabe, K., and Sato, M. 2004. Distribution patterns of mulberry dwarf phytoplasma in reproductive organs, winter buds, and roots of mulberry trees. *J. Gen. Plant Pathol.* 70: 168-173.
- Johnson, E. G., Wu, J., Bright, D. B., and Graham, J. H. 2014. Association of '*Candidatus* Liberibacter asiaticus' root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. *Plant Pathol.* 63:290-298.
- Kim, J.-S., Sagaram, U. S., Burns, J. K., Li, J.-L., and Wang, N. 2009. Response of sweet orange (*Citrus sinensis*) to '*Candidatus* Liberibacter asiaticus' infection: microscopy and microarray analyses. *Phytopathology* 99:50-57.
- Kumar, K., Brodersen, C., Narciso, C., and Etxeberria, E. 2014. Enzyme digestion of HLB-infected tissues: A better approach to study phloem anatomy. *Proc. Fla. State Hortic. Soc.* 127:33-36.
- Li, W., Hartung, J. S., and Levy, L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *J. Microbiol. Methods* 66:104-115.
- Li, W., Levy, L., and Hartung, J. S. 2009. Quantitative distribution of '*Candidatus* Liberibacter asiaticus' in citrus plants with citrus huanglongbing. *Phytopathology* 99:139-144.
- Lopes, S., Frare, G., and Bertolini, E. 2009a. Liberibacters associated with citrus huanglongbing in Brazil: '*Candidatus* Liberibacter asiaticus' is heat tolerant, '*Ca. L. americanus*' is heat sensitive. *Plant Dis.* 93:257-262.
- Lopes, S. A., Bertolini, E., Frare, G. F., Martins, E. C., Wulff, N. A., Teixeira, D. C., Fernandes, N. G., and Cambra, M. 2009b. Graft transmission efficiencies and multiplication of '*Candidatus* Liberibacter americanus' and '*Ca. Liberibacter asiaticus*' in citrus plants. *Phytopathology* 99: 301-306.
- Lopes, S. A., Frare, G. F., Yamamoto, P. T., Ayres, A. J., and Barbosa, J. C. 2007. Ineffectiveness of pruning to control citrus huanglongbing caused by *Candidatus* Liberibacter americanus. *Eur. J. Plant Pathol.* 119:463-468.
- Lopes, S. A., Luiz, F. Q. B. F., Martins, E. C., Fassini, C. G., Sousa, M. C., Barbosa, J. C., and Beattie, G. A. C. 2013. '*Candidatus* Liberibacter asiaticus' titers in citrus and acquisition rates by *Diaphorina citri* are decreased by higher temperature. *Plant Dis.* 97:1563-1570.

- Madden, L. V., Hughes, G., and van den Bosch, F. 2007. The Study of Plant Disease Epidemics. APS Press, St Paul, MN.
- McClean, A. P. D., and Oberholzer, P. C. J. 1965. Citrus psylla, a vector of the greening disease of sweet orange. S. Afr. J. Agric. Sci. 8:297-298.
- Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4326.
- Oparka, K. J., and Cruz, S. S. 2000. The great shape: phloem transport and unloading of macromolecules. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:323-347.
- Patrick, J. W. 1997. Phloem unloading: sieve element unloading and post-sieve element transport. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 191-222.
- Pelz-Stelinski, K. S., Bransky, R. H., Ebert, T. A., and Rogers, M. E. 2010. Transmission parameters for *Candidatus* Liberibacter asiaticus by Asian citrus psyllid (Hemiptera: Psyllidae). J. Econ. Entomol. 103:1531-1541.
- Stover, E., McCollum, G. T., Driggers, R., Lee, R., Shatters, R., Jr., Duan, Y., Ritenour, M., Chaparro, J. X., and Hall, D. G. 2015. Resistance and tolerance to Huanglongbing in Citrus. Acta Hort. 1065:899-903.
- Taiz, L., Zeiger, E., Moller, I., and Murphy, A. 2017. Fisiologia e desenvolvimento vegetal. Sixth Edition. Artmed, Porto Alegre, Brazil.
- Takushi, T., Toyozato, T., Kawano, S., Taba, S., Taba, K., Ooshiro, A., Numazawa, M., and Tokeshi, M. 2007. Starch method for simple, rapid diagnosis of citrus huanglongbing using iodine to detect high accumulation of starch in the citrus leaves. Jpn. J. Phytopathol. 73:3-8.
- Tatineni, S., Sagaram, U. S., Gowda, S., Robertson, C. J., Dawson, W. O., Iwanami, T., and Wang, N. 2008. In planta distribution of "*Candidatus* Liberibacter asiaticus" as revealed by polymerase chain reaction (PCR) and real-time PCR. Phytopathology 98:592-599.
- Teixeira, D. D. C., Danet, J. L., Eveillard, S., Martins, E. C., Jesus Junior, W. C., Yamamoto, P. T., Lopes, S. A., Bassanezi, R. B., Ayres, A. J., Saillard, C., and Bové, J. M. 2005. Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the '*Candidatus* Liberibacter species associated with the disease. Mol. Cell. Probes 19:173-179.
- Wallerstein, I., Goren, R., and Monselise, S. P. 1978. Rapid and slow translocation of 14 c-sucrose and 14 c-assimilates in Citrus and Phaseolus with special reference to ringing effect. J. Hortic. Sci. 53:203-208.
- Yamamoto, P. T., Felipe, M. R., Garbim, L. F., Coelho, J. H. C., Ximenes, N. L., and Martins, E. C. 2006. *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae): Vector of the bacterium *Candidatus* Liberibacter americanus. Page 96 in: Proc. Huanglongbing Green. Int. Workshop, Ribeirão Preto, SP, Brazil.