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ORIGINAL ARTICLE

Latency and incubation of '*Candidatus* Liberibacter asiaticus' in citrus after vector inoculation

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Abstract

Huanglongbing (HLB) is a bacterial disease of citrus, transmitted by the Asian citrus psyllid *Diaphorina citri*. In Brazil, the disease is managed by insecticide spray targeting vector control and removal of trees when they first show HLB symptoms that aims to reduce '*Candidatus* Liberibacter asiaticus' (CLas) inoculum within the orchard. Albeit symptoms of HLB in field trees appear only months or years after the initial infection, the shortest incubation period (time between pathogen infection and symptom expression) reported for CLas in citrus was about 4 months after inoculation in studies in which this pathogen was graft-inoculated. In this work, an incubation period of 80 days (2.5 months) was recorded for citrus seedlings and 5 months for citrus nursery plants after inoculation with the psyllid vector itself. Psyllids were capable of acquire CLas from the infected seedlings and nursery plants 2.5 months after inoculation. These results show that the latency of CLas *in planta* is shorter than the incubation. Assessment of the presence of liberibacter in psyllids could be used as a proxy for the presence of HLB bacterium in presymptomatic stage of the plants.

Keywords Acquisition · Citrus huanglongbing · *Citrus sinensis* (L.) Osbeck · Fastidious bacteria · Inoculation · Presymptomatic infection

Introduction

Citrus grove inspections, in which inspectors walk or ride on platforms for examination of citrus trees looking for Huanglongbing (HLB) symptomatic branches, are one of the mandatory practices to identify and further eradicate HLB-infected citrus plants (Belasque et al. 2010; Bassanezi et al. 2013a, b). Removal of diseased plants typically reduces the source of inoculum and prevents HLB spread. This can be achieved when the infected plants are manifesting disease symptoms that are detectable by the human eye. The earlier the diseased plant is identified, the earlier it can be removed. However, infected plants do not always show symptoms and become a source of inoculum at the same time. Latency and incubation period, two major epidemiological components of plant diseases dynamics, are not necessarily simultaneous. Latency period is defined as the time between the pathogen inoculation in the host and that when the infected plant becomes a source of inoculum. Incubation period corresponds to the time between pathogen inoculation and the onset of symptom expression (Gottwald 2010).

HLB is currently the main citrus disease worldwide. The main pathogen associated with HLB in Brazil is the Gram-negative alpha-proteobacteria '*Candidatus* Liberibacter asiaticus' (CLas), transmitted in a persistent-propagative manner by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Gottwald 2010; Ammar et al. 2011; Canale et al. 2017). HLB-infected citrus plants typically develop blotchy-mottled leaves, and in a more advanced infection condition, the veins become thick and necrotic and the leaves exhibit a downward curvature (Bové 2006; Folimonova and Achor 2010). Incubation can be estimated by visual assessment of the plants inoculated with CLas and the record of the first evident HLB-symptom manifestation. Most of the literature reports HLB incubation period observed in grafted-

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inoculated plants with HLB-infected scions. Grafted-inoculated citrus trees manifest the first HLB symptoms (*e.g.* blotchy mottle) at 5 to 6 months after grafting, and CLas is typically detected by qPCR 2 to 3 months after the insertion of the scion (Folimonova and Achor 2010; Colleta-Filho et al. 2010; Lopes et al. 2009). However, information on the onset of HLB symptoms and PCR detection of the pathogen in plants inoculated by the vector *D. citri* is scarce. Inoue et al. (2009) observed blotchy mottle symptoms and positive qPCR detection of CLas in seedlings of *Citrus junos* 3 months after inoculation by *D. citri* adults that acquired the pathogen as nymphs. Hung et al. (2001) also detected CLas by conventional PCR in Chinese box-orange (*Severina buxifolia* (Poir.) Ten.) and in sweet orange (*Citrus sinensis* (L.) Osbeck) at three to 4 months after inoculation by *D. citri* and HLB symptoms on these two plant species were evident only 12 months after insect inoculation.

In spite of the epidemiological importance, estimates of latency and incubation periods for psyllid-transmitted CLas in HLB pathosystem are scarce. For vector-borne plant pathogens such as CLas, latency can only be estimated through transmission experiments with the psyllid vector, by determining the time after inoculation at which the pathogen can be acquired in infected hosts and subsequently transmitted to healthy hosts (Rimbaud et al. 2015). This work provides a detailed assessment of the latency of CLas and incubation of HLB in sweet orange seedlings and in nursery plants. The latency period was determined through a series of acquisition trials of CLas by *D. citri* at monthly intervals following inoculation of the plants by this psyllid vector, and the incubation period was evaluated through visual assessment of HLB-symptoms in the inoculated plants.

Material and methods

Citrus plants, *D. citri* rearing and experimental plastic cages

Healthy ACP were reared on orange jasmine plants (*Murraya paniculata* (L.) Jack) inside screened cages in a climate-controlled room, as described elsewhere (Canale et al. 2017). HLB-symptomatic sweet orange nursery trees (*C. sinensis* cv. Pera on *C. limonia* Osbeck rootstock) with 2 years after graft-inoculation with CLas, maintained in 5-L plastic bags (30 cm height × 10 cm diameter), were used as 'primary source plants' for acquisition of the bacterium by *D. citri* and subsequent inoculation of the plants that were used as sources of inoculum (hereafter termed 'source plants') in the experiments. In a first experiment, plants of sweet orange cv. Pera grown from seeds (hereafter referred as seedlings) in 2-L plastic bags were used either as 'source plants' for bacterial acquisition (when previously inoculated with CLas by *D. citri*) or as healthy 'test plants' (*ca.* 10 cm tall) for assaying infectivity of *D. citri* individuals that were previously fed on the 'source plants'; the healthy seedlings were also used to maintain the insects during the latency period (LP) of the bacterium in the psyllid vector. In a second experiment, 1.5-year old sweet orange nursery plants (*C. sinensis* cv. Pera grafted on *C. limonia*) grown in the 5 L pots were used as 'source-plants' (previously inoculated with CLas by *D. citri*), whereas the healthy seedlings of sweet orange cv. Pera were used as 'test plants' for assaying psyllid infectivity.

All 'source-plants' and 'test-plants' were pruned 3 weeks prior to psyllid exposure. The induction of young flushes by pruning provide higher bacterial titers and a more suitable tissue for insect feeding, either if the objective is pathogen acquisition or inoculation (Folimonova and Archor 2010; Ammar et al. 2013). Psyllid nymphs were used for acquisition in all experiments due to the higher efficiency of CLas transmission after acquisition by nymphs (Pelz-Stelinski et al. 2010; Canale et al. 2017). Cages made of clear plastic cup with removed bottom, sealed with a *voil* fabric and supported by wood sticks on the plants, were used for insect confinement during acquisition and inoculation.

All plants were grown in commercial potting mix for citrus (Tropstrato V8 Citrus®, Vida Verde, Mogi Mirim, SP) and kept in a vector-proof screenhouse, where they were fertilized *via* irrigation water as described by Esteves et al. (2019). After inoculation by psyllids, all 'test plants' were fortnightly sprayed with insecticide (dimethoate).

Experiment 1 - latency and incubation in citrus seedlings

Twenty healthy sweet orange seedlings were exposed to an inoculation access period (IAP) of 72 h by *D. citri* adults (10 individuals per plant), which were previously confined as third-instar nymphs on young shoots of the 'primary source plants' for a 10-day acquisition access period (AAP). The inoculated sweet orange seedlings were referred as 'source-plants', as described before (Fig. 1). Ten non-inoculated healthy seedlings were used as negative controls. Seedlings were fortnightly inspected for initial HLB symptoms, characterized by the downward curvature of leaves showing early corticated aspect, mild thickening, vein yellowing and subtle mottling. As soon as the first symptoms were visually detected, all the seedlings were tested by qPCR for CLas detection. Additionally, 10 healthy third instars of *D. citri* were confined on young shoots of each seedling for a 10-day AAP at monthly intervals following the 72-h IAP. Insects were subsequently tested for CLas infectivity by qPCR (Fig. 1).

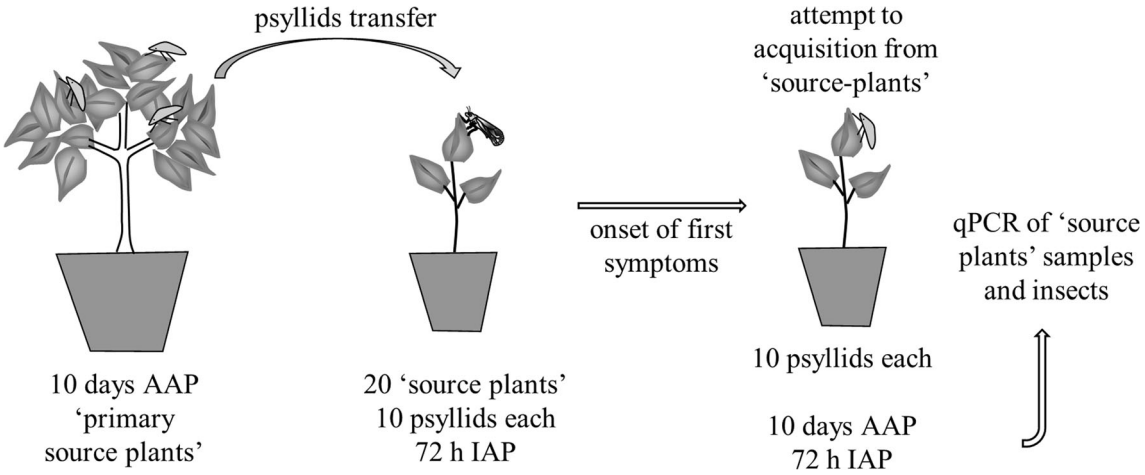

Fig. 1 Third instar nymphs of *Diaphorina citri* were exposed to 'primary source plants' infected with '*Candidatus* Liberibacter asiaticus' (CLas) for a 10-day acquisition access period (AAP). The psyllids were then transferred to 20 healthy citrus seedlings for a 72-h inoculation access period (IAP), using 10 insects per plant. These inoculated seedlings were regarded as 'source plants' and tested for CLas acquisition by psyllids after the onset of the first Huanglongbing symptoms, by allowing third instar nymphs a 10-day AAP on each plant. After the AAP, the 'source plants' and the psyllids were tested for infection by CLas by qPCR

Experiment 2 - latency and incubation in citrus nursery plants

Twenty 1.5-year old healthy sweet orange nursery plants were exposed individually to a group of 80 psyllids for an IAP of 7 days. These insects were previously confined as third-instar nymphs on young shoots of the 'primary source-plants' for a 96-h AAP, followed by 7 days on healthy sweet orange seedlings for completion of the latent period (LP) of CLas in the insects. The inoculated nursery plants were then referred as 'source-plants' (Fig. 2). Ten non-inoculated healthy nursery plants were used as negative control. Both groups of inoculated and non-

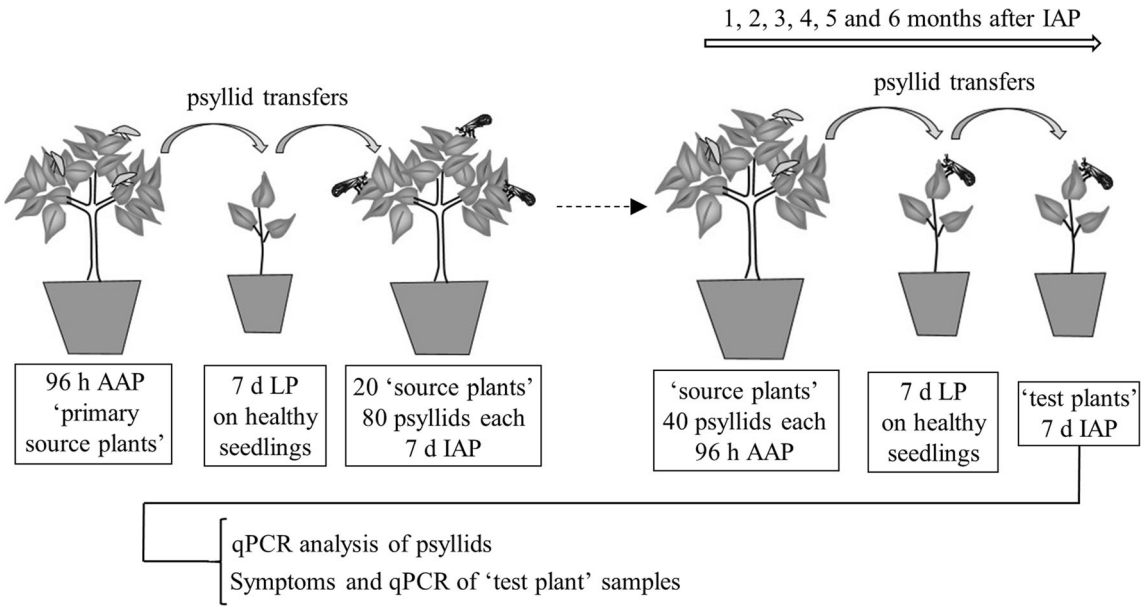

Fig. 2 Third-instar nymphs of *Diaphorina citri* were confined on 'primary source plants' infected with '*Candidatus* Liberibacter asiaticus' (CLas) for a 96-h acquisition access period (AAP), followed by 7 days on healthy citrus seedlings for completion of the bacterial latent period (LP) in the vector. The insects were then confined on 20 healthy citrus nursery trees (80 insects/plant) for an inoculation access period (IAP) of 7 days. These vector-inoculated nursery trees, now regarded as 'source plants', were submitted to monthly acquisition trials, each one using 40 fourth instars of *D. citri* per 'source plant' and 96-h AAP. The psyllids were then kept on healthy citrus seedlings during 7 days for completion of the bacterial LP and subsequently transferred to other healthy seedlings (indicators of CLas transmission and thus referred as 'test plants') for a 7-day IAP, using ten insects per 'test plant'. The psyllids were assayed for the presence of CLas using qPCR right after the IAP, and the 'test plants' were analyzed by qPCR and inspected for HLB symptoms 10 months later

inoculated 'source plants' were submitted monthly to (i) acquisition and transmission trials by *D. citri*, and (ii) HLB-symptom inspection and tissue sample collection (3–5 leaves/sample) for DNA extraction and analysis of CLas infection by qPCR (Fig. 2). The acquisitions trials were proceeded at a monthly interval, up to 6 months, after the nursery 'source plants' were inoculated, by confining 40 healthy 4th instar nymphs of *D. citri* on young shoots for a 96-h AAP. Those insects were then confined on healthy citrus seedlings during 7 days for completion of CLas latency in the vector, and subsequently transferred to healthy seedlings ('test plants') for the transmission trials, using ten psyllids per test plant and an IAP of 7 days. Right after IAP, groups of three psyllids from each 'source plant' were sampled for DNA extraction and qPCR for CLas detection in order to determine the acquisition rate, measured by the proportion of tested groups that were infected by CLas. The inoculated 'test plants' were analyzed by qPCR for CLas infection in order to determine the transmission rate, measured by the proportion of inoculated test plants that became infected.

DNA extraction from plants and insects and qPCR

Three to 5 citrus leaves of the 'source plants' and 'test plants' were collected and submitted to total DNA extraction following a modified cetyltrimethylammonium bromide (CTAB) method (Coletta-Filho et al. 2014; Murray and Thompson 1980). DNA extraction of psyllids was carried through maceration of three insects per sample in 1.5 mL microtubes, according the methodology also described by Coletta-Filho et al. (2014). CLas detection was performed by qPCR using the primers Las-I-F and Las-I-R and a FAM-labeled probe Las-P (Integrated DNA Technologies - IDT) designed based on Ts elongation factor gene, whose sequences are described by Lin et al. (2010). The reaction mix (13 μL) contained 0.8 μM of each forward and reverse primer, 0.2 μM of FAM probe, 6.5 μL of TaqMan Fast Universal PCR Master Mix (2X) (Life Technologies). Amplification cycles were ran in a 96-well ABI PRISM 7500 Fast Sequence Detection System, equipped with software version 2.0.5 for data acquisition and analyses. Each DNA sample was loaded in two wells in the reaction plate (two replicates). Positive control was represented by DNA samples from plant or insect previously tested positive for CLas. DNA extracts from healthy citrus leaf and psyllid were used as negative control. qPCR reactions were considered positive for CLas if mean cycle threshold (Ct) of replicates was ≤35 (Canale et al. 2017).

Results

Incubation and latency in citrus seedlings

Initial HLB symptoms, such as the downward curvature of leaves vein and a subtle mottling, was first observed at 80 days after IAP in four out of the 20 inoculated citrus seedlings ('source-plants'). Those plants were also qPCR-positive for CLas (Table 1). Therefore, the transmission rate was 20%. None of the other inoculated and non-inoculated (negative control) seedlings showed symptoms or CLas infection verified by qPCR until 6 months after the IAP. The four symptomatic 'source-plant' were tested as CLas sources at 80 days after IAP by confining 10 healthy third instar nymphs for a 10-day AAP. Psyllids successfully acquired CLas from the two source plants with the highest bacterial titers [Ct ≈ 20] (Table 1), showing that the latency of liberibacter in those plants was completed.

Incubation and latency in citrus nursery plants

Four citrus nursery 'source plants', out of a total of 20, were qPCR positive for the presence of CLas in at least one of the evaluated month after IAP (Ct cycles exhibited in Table 2). The first positive event was observed, in the 'source plant' no. 7, 8 weeks after IAP (Table 2). This plant was also positive for the upcoming 12 and 16 weeks after IAP, and negative then in the last two evaluations. 'Source plant' no. 18 resulted in positive for CLas twice, which were at 16 and 20 weeks after IAP. The majority of liberibacter detection was observed at 16 weeks after IAP (Table 2). Initial HLB-symptoms were observed in the 'source plants' no. 4, 7 and 18. Plant no. 4 showed typical HLB symptom 5 months after IAP. Phenotypical reactions were considered suspected as HLB symptoms in 'source plant' no. 7 and 18 from 3rd month after IAP, and it was certified over the time that they clearly were initial HLB symptoms.

All of the four liberibacter qPCR-positive 'source plants' consisted as source of inoculum of CLas, verified by the detection of the bacteria in the psyllid vector exposed on them for feeding (Table 2). The first psyllid samples in which

Table 1 Assessment of latency of '*Ca. L. asiaticus*' (CLas) in sweet orange through acquisition by *Diaphorina citri* from vector-inoculated seedlings ('source plants') at 80 days after inoculation

'Source plant'[x]	qPCR Ct values[y]	Acquisiton rate by psyllids[z]
1	20.64	9/10
2	30.50	0/10
3	34.35	0/10
4	20.54	10/10

[x] Four out of 20 inoculated 'source plants' tested qPCR-positive for CLas

[y] Mean cycle threshold (Ct) values of two replicates obtained by qPCR for the presence of CLas in the 'source plants'. Plants were considered infected if mean Ct was ≤35

[z] Infective insects/total

Table 2 Assessment of latency and incubation periods of '*Ca. L. asiaticus*' (CLas) in sweet orange through acquisition and transmission trials by *Diaphorina citri* from vector-inoculated nursery trees ('source plants') at monthly periods after inoculation

	Months after vector-inoculation of the 'source plants'																	
'Source plant'[w]	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	Ct values of qPCR in source plants[x]						Acquisition rate by psyllids[y]						Transmission rate to test plants[z]					
1	–	–	–	–	–	–	0/5	0/4	0/5	0/5	0/5	0/5	0/2	0/2	0/5	0/5	0/5	0/5
2	–	–	–	–	–	–	0/5	0/4	0/5	n.t.	n.t.	n.t.	0/2	0/2	0/6	0/6	0/1	0/2
3	–	–	–	–	–	–	0/5	0/4	0/5	0/5	0/5	0/5	0/2	0/2	0/5	0/6	0/3	0/4
4	–	–	–	–	**23.6***	–*	0/5	0/5	n.t.	n.t.	**4/4**	0/4	0/2	0/1	0/0	**3/5**	0/4	**1/4**
5	–	–	–	–	–	–	0/5	0/4	0/5	n.t.	0/5	n.t.	0/2	0/1	0/5	0/5	0/5	0/5
6	–	–	–	–	–	–	0/5	0/4	0/5	0/4	0/5	0/3	0/2	0/2	0/5	0/3	0/4	0/6
7	–	**28.7**	**23.7***	**31.5***	**-***	-*	0/5	**4/4**	**5/5**	n.t.	**2/2**	**1/1**	n.t.	**1/2**	**2/5**	0/3	**2/3**	**2/2**
8	–	–	–	–	–	–	0/5	0/4	**1/4**	n.t.	**4/5**	0/3	0/2	0/1	**2/5**	**3/5**	**1/5**	0/3
9	–	–	–	–	–	–	0/5	0/4	**3/5**	0/5	**3/3**	**2/3**	0/2	0/2	**1/4**	**1/3**	0/3	0/3
10	–	–	–	–	–	–	0/5	0/4	0/4	0/5	0/5	n.t.	0/2	0/1	0/5	0/5	0/4	0/4
11	–	–	–	–	–	–	0/5	n.t.	0/5	0/5	0/5	n.t.	0/2	0/2	0/5	0/3	0/4	0/3
12	–	–	–	–	–	–	0/5	0/5	0/4	0/5	0/4	0/3	0/2	0/1	0/2	0/3	0/3	0/3
13	–	–	–	–	–	–	0/5	n.t.	0/5	n.t.	0/4	n.t.	0/2	0/2	0/5	0/2	0/4	0/3
14	–	–	–	**31.7**	–	–	0/5	0/3	**1/5**	**1/1**	0/3	n.t.	0/2	0/1	**1/5**	0/3	0/2	0/3
15	–	–	–	–	–	–	0/5	0/5	**1/5**	0/5	n.t.	0/5	0/2	0/2	**2/6**	0/4	**1/2**	0/4
16	–	–	–	–	–	–	0/5	0/5	**1/5**	0/5	0/5	n.t.	0/2	0/2	**2/5**	**3/6**	0/3	0/3
17	–	–	–	–	–	–	0/5	0/5	0/5	0/2	0/1	0/4	n.t.	0/1	0/5	0/3	0/4	0/4
18	–	–	–*	**22.0***	**22.9***	–*	0/5	5/5	**5/5**	**5/5**	0/1	**1/1**	0/2	0/2	**4/5**	**4/4**	**1/3**	**1/3**
19	–	–	–	–	–	–	0/5	0/5	**2/5**	**4/5**	0/1	n.t.	0/2	**1/2**	**2/4**	**4/5**	0/3	0/3
20	–	–	–	–	–	–	0/5	0/5	0/5	**1/5**	0/5	n.t.	0/2	0/2	**1/6**	0/5	0/3	0/4

[w] 'Source plants' were exposed to a 7-day inoculation access period (IAP) by 80 *D. citri* individuals, which were previously submitted as third-instar nymphs for an acquisition access period (AAP) of 96 h on 'primary source-plants' infected by CLas, followed by 7 days on healthy citrus seedlings for completion of the latent period (LP) of CLas in the insects (Fig. 2)

[x] Mean cycle threshold (Ct) values of two replicates obtained by qPCR for the presence of CLas in the 'source plants'. Plants were considered infected if mean Ct was ≤35; presence of HLB symptoms was indicated by an asterisk. Symbol (−) indicates "undetermined" results in qPCR analysis

[y] Number of samples of three *D. citri* individuals that were qPCR-positive for CLas over the total number of samples tested. In each acquisition trial, 40 healthy nymphs (4th instar) of *D. citri* were confined per 'source plant' for a 96-h AAP; the psyllids were then kept on healthy citrus seedlings during 7 days for completion of the bacterial LP in the insect, followed by a 7-day IAP on healthy citrus seedlings ('test plants'), before they were tested by qPCR (Fig. 2). n.t. = not tested

[z] Number of citrus seedlings ('test plants') that were qPCR-positive for CLas over the total number of seedlings inoculated. These plants also developed HLB symptoms. Ten insects per plant inoculated the test plants during the 7-day IAP. n.t. = not tested

Results in bold have the purpose of only to highlight qPCR positive results and transmission events

liberibacter was detected were from those insects that fed from plant no. 7 at 8 weeks after IAP. Apparently, higher amounts of psyllids acquired liberibacter from 'source plants' which presented lower Ct values (higher bacterial titer).

Liberibacter was also detected in *D. citri* which fed from 'source plants' that were qPCR negative for CLas and symptomless for HLB at any evaluation month ('source plant' number 8, 9, 15, 16, 19 and 20, Table 2). Also, some indicator 'test plants' were qPCR positive even if liberibacter was not detected from the correspondent 'source plant'. The indicator 'test plants' were qPCR positive and symptomatic for HLB only if they were submitted to psyllids that could acquire liberibacter from the 'source plant', even if the 'source plant' tested negative for CLas.

Discussion

Most of the literature reports HLB incubation period observed in grafted-inoculated plants with HLB-infected scions. Latency, for vector-borne plant pathogens such as CLas, can only be estimated through transmission experiments with the psyllid vector. In this work, we studied the incubation and latency of CLas in seedlings and in nursery plants inoculated by its psyllid vector, *D. citri*. This was in attempt to determine these two epidemiological components simulating the natural condition of liberibacter transmission by its vector, rather than using the experimental grafting. Insect-inoculated citrus seedlings showed HLB-like symptoms 80 days (2.5 months) after inoculation and

psyllids could acquire CLas from those plants on this date. HLB symptoms were observed in citrus nursery plants 5 months after transmission of liberibacter by the insect vector. However, CLas was detected by molecular test as early as 2 months after inoculation, together with acquisition by the psyllid.

The incubation observed in seedlings in this work is in accordance with the already reported blotchy mottle symptoms and qPCR-positive for CLas in seedlings 3 months after inoculation by psyllids (Inoue et al. 2009). Incubation period of liberibacter in nursery plants was 5 months, similar to those results obtained in inoculation of plants using liberibacter-infected buds by grafting (Folimonova and Achor 2010; Coletta-Filho et al. 2010). The titer of liberibacter in the budwoods used for grafting was not reported and the same experimental plant could be inoculated several times (Folimonova and Achor 2010; Coletta-Filho et al. 2010), suggesting that the amount of bacterial cells inserted the receptor plants is large. Colleta-Filho et al. (2010) obtained 100% of transmission rate using inoculation by grafting whereas Canale et al. (2017) reported a liberibacter transmission rate by psyllids of 20% at most. Ammar et al. (2016) estimated that the CLas titer threshold for *D. citri* to be capable of transmit the pathogen is 10^6 copies of CLas DNA per psyllid. Perhaps a portion of this amount prevenient from the salivary gland is actually introduced in the plant phloem. Colleta-Filho et al. (2010) reported HLB symptom in plants grafted-inoculated when they reached almost 5×10^7 copies number per gram of plant tissue. It is apparent then, even if the amount of bacterium transmitted by the vector is inferior than that transmitted using an infected budwood, the progress of liberibacter colonization and establishment of HLB might succeed rapidly after inoculation by insect.

It has been difficult to consistently detect CLas in the nursery 'source plants', even if they were knowingly infected, due to liberibacter acquisitions by the insect vector. Albeit liberibacter distributes systemically in the plant and it is capable of infect a large sort of tissues that compose leaves, roots and fruit, the distribution is irregular and very dependent on the season time and on the effect of temperature on the population of liberibacter in the plant host (Li et al. 2009; Colleta-Filho et al. 2010; Lopes et al. 2013). Therefore, environmental factors could affect the amount of detectable bacteria from 'source plants', even if the plants used in this work were kept in a greenhouse. Besides detection of CLas in the nursery 'source plants' was based on sampling of some leaves for DNA extraction from plant midribs. In this case, even the choice of tissue sampling during collection of some leaves, especially the younger ones, where it is known that liberibacter develops best (Folimonova and Achor 2010) could interfere in the forthcoming monthly PCR analysis by hamper or interrupt the establishment of bacterial colonization within the plant, because of the removal of the source of bacterial cells that could multiply and eventually spread within the plant. Nevertheless, liberibacter acquisition was possible from those 'source plants' possibility due to the already reported ability of the nymphs (Inoue et al. 2009). Nymphs can be useful indicators of a liberibacter source plant in field, because they can acquire the bacterium when the plant is still in a presymptomatic phase (Manjunath et al. 2008).

Our study showed that psyllids were capable of acquire CLas from 'source plants' 2.5 months after insect-inoculation if those plants were seedling when inoculated. Also, psyllids could acquire liberibacter from nursery 'source plants' 2 months after these plants were inoculated. These results show that the latency of CLas *in planta* may be shorter than the incubation, *i.e.* the onset of evident symptom of HLB. It is observed that symptoms of HLB are not evident in field orchard trees for months or years after infection (Gottwald 2010; Shen et al. 2013). In addition, our results suggest that presymptomatic trees can serve as a source of inoculum of CLas for psyllids. Lee et al. (2015) presented an experimental evidence that young citrus flush become a source of inoculum within 15 days after receiving an inoculum of CLas. The authors provided a microsimulation that, considering the spread of HLB in an asymptomatic scenario, a 75% reduction in the psyllid population, that is a result of control strategy carried out during all flush periods, can delay the appearance of symptomatic trees by 1 year or more.

The current recommended strategies to manage HLB are the elimination of HLB-symptomatic trees and the use of chemicals to decrease vector population, both aimed to reduce source of inoculum of liberibacter, and must be applied in an extensive area. The removal of trees is based on visual inspections carried out in orchards in Brazil, and these measures are only effective if adopted at the regional level to maintain the disease in a satisfactory or unthreatening incidence (Bassanezi et al. 2013a, b, Bergamin Filho et al. 2016). However, a latent period shorter than the incubation period could be a caveat for the management strategy of HLB. A low-cost sampling and assaying tool is needed to detect asymptomatic HLB trees and prevent HLB dissemination even in a presymptomatic condition. Techniques based on spectroscopy, imaging profile of plant volatile organic compounds, for instance, which do not involve molecular assays are under study for early HLB disease detection (Valdés et al. 2016). Meanwhile, it is important to keep up the training of inspectors for visual diagnose for early diseased plant removal. Psyllid surveillance and molecular detection of liberibacter in the vector is a strategy to be aware of the promptness of the vector as a carrier of the bacterium even prior the onset of symptoms.

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Authors' contributions MCC, project initiation, performance of Experiment 1, data analysis and writing of manuscript; KMAK, maintenance of plants and insects, performance of Experiment 2 and data analysis; JRSL, project management (experimental working) and writing of manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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