Prevalent transmission of *'Candidatus* Liberibacter asiaticus' over '*Ca.* Liberibacter americanus' in a long-term controlled environment

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ABSTRACT

In Brazil, citrus huanglongbing (HLB) is associated with '*Candidatus* Liberibacter americanus' (CLam) and '*Ca.* Liberibacter asiaticus' (CLas). However, there are few studies about HLB epidemiology when both Liberibacter spp. and its insect vector, the Asian citrus psyllid (ACP, *Diaphorina citri*), are present. The objective of this work was to compare the transmission of HLB by ACP when both CLam and CLas are present as primary inoculum. Two experiments were performed under screenhouse conditions from April 2008 to January 2012 (experiment 1) and from February 2011 to December 2015 (experiment 2). The experiments were carried out with sweet orange plants infected with

CLam or CLas as inoculum source surrounded by sweet orange healthy plants. One hundred Liberibacter-free adult psyllids were monthly confined to the source of inoculum plants for 7 days with subsequent free movement inside the screenhouse. Fortnightly, nymphs and adults of psyllids were monitored. Psyllid and leaf samples were collected periodically for Liberibacter detection by PCR or qPCR. CLas was detected more frequently than CLam in both psyllid and leaf samples. No mixed infections were detected in the psyllids. A clear prevalence of CLas over CLam was observed in both experiments. The final HLB incidences were 16.7 and 14.5% of Liberibacter-positive test plants, and CLas was detected in 92.3 and 93.1% of these infected plants. Mixed infection was observed only in 3.8% of infected test plants in experiment 1. These results endorse the shift in the prevalence of CLam to CLas observed in citrus orchards of São Paulo, Brazil.

Keywords*:* Asian citrus psyllid, *Citrus sinensis,* comparative epidemiology, greening.

INTRODUCTION

Citrus huanglongbing (HLB) is a serious disease that is responsible for tree decline, the decrease of citrus yield, and the increase of production costs in citrus-producing countries (Bassanezi et al. 2020; Graham et al. 2020). This disease is associated with species of phloem-limited α-proteobacteria in the genus '*Candidatus* Liberibacter' (Bové 2006; Teixeira et al. 2005). '*Ca.* L. americanus' (CLam) (Teixeira et al. 2005) and '*Ca.* L. asiaticus' (CLas) (Coletta-Filho et al. 2004) are both associated with HLB in Brazil. These bacterial species are naturally transmitted from diseased to healthy citrus plants by the Asian citrus psyllid (ACP, *Diaphorina citri*) (Capoor et al. 1967; Yamamoto et al. 2006). While variables of CLas transmission by ACP are well documented, there are few

information for CLam (Darolt 2021; Nascimento 2010). CLas acquisition and transmission occurs when psyllids feed on the infected host for at least one hour and the rates are positively correlated with confinement time on infected plants (Capoor et al. 1974; Bonani et al. 2008; Pelz-Stelinski et al. 2010). The rate of CLas acquisition by nymphs is higher than by adults (Ammar et al. 2020; Canale et al. 2017). In addition, the efficiency of transmission of CLas by adult *D. citri* is higher when the vector acquired the pathogen as nymph compared with adult (Inoue et al. 2009; Pelz-Stelinski et al. 2010; Ammar et al. 2020). After a latent period of 17 days, psyllids can transmit CLas to a new host for up to 35 days (Canale et al. 2017). Since citrus plants are the most abundant inoculum source of both Liberibacter spp. in orchards, studies focusing on the behavior of CLam and CLas and its interaction with *D. citri* and citrus plants are necessary.

In sweet orange plants (*Citrus sinensis* (L.) Osbeck), early disease symptoms include the presence of one or more yellow shoots on infected plants. Leaves may become smaller, thicker, and leathery with enlarged and corky midribs (Bové 2006) on some trees showing yellow shoots. The most characteristic symptom is asymmetrical yellow mottling of the leaves, called blotchy mottle (Bové 2006). Eventually, defoliation and dieback occur in advanced stages of bacterial infection. Fruit from symptomatic branches is small, lopsided, more acidic and bitter, and has a lower juice percentage and total soluble solid content than healthy fruit (Bassanezi et al. 2011). Usually, yield loss is proportionally larger than the symptomatic area in the tree canopy (Bassanezi et al. 2011). The higher the disease severity, the lower the plant yield, especially because of premature fruit drop on affected branches. Despite the similarity of symptoms induced by CLam and CLas, at the same optimal environmental conditions for symptoms expression (22 to 24ºC), plants infected

with CLam show more conspicuous symptoms than plants with CLas (Lopes et al. 2009a, 2009b, Lopes and Frare 2008).

Initial HLB symptoms are observed three months after bacterial inoculation by ACP, though blotchy mottle symptom is usually found between four and six months, up to one year following inoculation (Canale et al. 2019; Lin et al. 2017; Yamamoto et al. 2006). The time interval between ACP inoculation to visual symptoms (i.e., the incubation period) can be related to several factors, such as canopy variety, plant age, and season (Bassanezi et al. 2010; Bassanezi and Primiano 2021; Gottwald 2010; Lin et al. 2017). Symptoms are expressed earlier in some varieties, but no commercial sweet orange is resistant to HLB (Lin et al. 2017). Most of infection occurs during the period of new vegetative flushes, usually in spring and summer, while more HLB-symptomatic plants are observed in autumn and winter periods (Bassanezi et al. 2010; Bassanezi and Primiano 2021). Despite of these factors, infected plants become a source of inoculum before symptom expression, i.e., ACP can acquire bacteria in asymptomatic plants (Canale et al. 2019; Coletta-Filho et al. 2014; Lin et al. 2017). Therefore, molecular techniques are necessary in studies that aim to detect HLB-asymptomatic plants. Molecular techniques can also detect which bacteria is affecting the plant because both CLam and CLas are associated with the same HLB symptoms. Conventional polymerase chain reaction (PCR) (Hocquellet et al. 1999; Teixeira et al. 2005, 2008b), loop-mediated isothermal amplification (LAMP) (Okuda et al. 2005), and quantitative PCR (qPCR) assays (Li et al. 2006; Teixeira et al. 2008a) have been developed for the detection, differentiation and/or quantification of '*Ca.* Liberibacter spp.' PCR and qPCR are more widely used in the molecular detection of '*Ca.* Liberibacter spp.', host-pathogen-vector interactions, and epidemiological studies (Alves et al. 2021; Canale et al. 2019; Gasparoto et al. 2012; Li et al. 2009; Teixeira et al. 2008a). Many studies focus

only on one '*Ca.* Liberibacter spp.' due to the complex interaction of all three components (host-Liberibacter-ACP) or due to the absence of a second HLB bacterium in the country where the studies were performed.

From the first report of HLB in the 2004-2005 season until the 2007-2008 season, CLam was the most prevalent species associated with infected citrus plants in Brazilian orchards and in orange jasmine located in non-commercial orchards (Lopes et al. 2010; Teixeira et al. 2005). In 2004, CLam was detected in 214 symptomatic leaf samples (98.2%), CLas in four samples (around 1.8%), and a mixed infection with both '*Ca*. Liberibacter spp.' were detected in only two samples (Teixeira et al. 2005). After the 2008-2009 season, CLas became the most common bacterial species associated with HLB in infected citrus plants and orange jasmine in Brazil, reaching 99.9% to 100% of infected plants (Bassanezi et al. 2020; Lopes et al. 2009a, 2010). This change in the prevalence of the bacterial species generated several new hypotheses related to the competitive ability of these '*Ca*. Liberibacter spp.' The uneven spatial distribution of CLam in the field, as well as the lower bacterium titer of CLam in relation to CLas (Lopes et al. 2009a), the influence of '*Ca*. Liberibacter spp.' on acquisition by ACP (Darolt 2021), and the environmental influence on bacterial infection and colonization in citrus plants (Gasparoto et al. 2012; Lopes et al. 2009b); among other factors, could explain the predominance of CLas after the 2008-2009 season. CLam infection and colonization in citrus plants is limited under a daily temperature regime of 27ºC/32ºC, in which this bacteria species is hardly or not detected (Gasparoto et al. 2012; Lopes et al. 2009b). This range of temperatures is commonly observed during the spring and summer of the main citrus production areas in Brazil, but some questions still remain, for instance: (i) Does bacterial transmission occur simultaneously by ACP? (ii) Do both bacteria co-exist before HLB symptom appearance?

(iii) Is the progress of HLB incidence similar when plants with CLam and CLas are concomitantly used as sources of inoculum? Comparative epidemiological studies between CLam and CLas are necessary to understand the Liberibacter-citrus-ACP interaction. The objective of this study was to compare the transmission of CLam and CLas by ACP and the subsequent polyetic progress of HLB, using infected and symptomatic *Citrus sinensis* plants as a source of inoculum in a screenhouse.

MATERIALS AND METHODS

Experimental design

Two experiments were carried out in an insect-proof screenhouse located at the municipality of Araraquara, SP, Brazil, 21°48'25.8"S 48°09'53.8"W, at the Fund for Citrus Protection (Fundecitrus). This screenhouse was composed of two isolated compartments of 150 m^2 each. The highest and lowest temperatures and relative humidity were obtained daily by thermo-hygrometers placed in the center of each compartment in both experiments. During experiment 1, photosynthetically active radiation (PAR) was measured with a plant canopy analyzer (LAI-2000, LiCor®) in three periods of the day: morning, midday, and afternoon inside each compartment. Four PAR measurements were performed in each compartment, in which one measurement was performed outside of the compartment and three measurements were performed inside the compartment: two in the borders and one in the central area of the compartment. These PAR measurements were performed from February 2010 to June 2010.

Experiment 1 was carried out from April 2008 to January 2012. One hundred and fifty-six potted healthy sweet orange plants ‘Valencia’ (*Citrus sinensis* (L.) Osbeck) grafted

144 on 'Rangpur' lime (*Citrus limonia* Osbeck) were distributed around four inoculum source
145 plants in each compartment (Fig. 1A). Plants were distributed in ten rows (R1 to R10) with
146 16 plants each (P1 to P16). Experiment 2 was carried out from February 2011 to December
147 2015. One hundred test plants were distributed around four inoculum source plants in each
148 compartment (Fig. 1B). Plants were distributed in eight rows (R1 to R8) with 13 plants
149 each (P1 to P13). At the beginning of each experiment, the test plants were one year old
150 and the inoculum source plants were two years old. In both experiments, all plants were
151 grown in 12 l plastic pots containing sterilized soil and decomposed pine bark substrate
152 (Plantmax citrus, Eucatex, Paulínia, SP, Brazil). Potted plants were irrigated twice a week
153 and fertilized periodically with calcium nitrate (0.8 g/l), Peters Professional Tropical
154 Foliage NPK 24-8-16 (0.8 g/l) with Fe-EDTA (0.04 g/l), and Kristalon NPK 20-8-8 (0.8
155 g/l) (Gasparoto et al. 2012).

156 Four sweet orange plants ('Natal' *C. sinensis*) grafted on 'Rangpur' lime were used
157 as source of inoculum: two graft-inoculated with CLam and two with CLas in each
158 experiment (Fig. 1). Each inoculum source plant was obtained by grafting three 4-cm-long
159 budwoods onto the stem of a healthy citrus plant (Lopes and Frare 2008). Budwoods were
160 taken from selected 5-year-old 'Valencia' sweet orange trees naturally infected by CLam or
161 CLas and showing typical symptoms of HLB. Prior to grafting, CLam- or CLas-infected
162 'Valencia' trees that were used to obtain the budwoods were confirmed by conventional
163 PCR (Hocquellet et al. 1999; Teixeira et al. 2005). The Liberibacter's source plants were
164 exclusively obtained by grafting CLam or CLas infected bud on health citrus plants which
165 were kept under greenhouse. The graft transmission was confirmed 24 months after
166 grafting by PCR (Teixeira et al. 2008a). The source of inoculum plants remained in the
167 compartments until the latest ACP confinement.

168

169 **Psyllid confinement and assessment**

170 CLam and CLas transmission in the test plants occurred naturally by infected adult
171 ACPs. First, Liberibacter-free ACPs were reared on healthy orange jasmine (*Murraya*
172 *paniculata* (L.) Jack) plants maintained in insect-proof cages. The cages were maintained in
173 a room with a controlled temperature (25 ± 1°C), air humidity (70 ± 20%), and photoperiod
174 (dark/light of 10/14 h). Subsequently, 25 of these Liberibacter-free adult ACPs were
175 monthly confined for 7 days (acquisition access period-AAP) onto the entire plant of each
176 inoculum source plant, for a total of 100 caged insects per compartment. Test plants and
177 inoculum source plants were pruned 28 days before the first insect confinement to induce
178 new shoot emissions, which corresponded to the most favorable stage for insect oviposition
179 (Cifuentes-Arenas et al. 2018). After AAP, psyllids were released for free movement inside
180 the compartment.

181 Psyllid confinement and release after AAP on the inoculum source plants were
182 performed monthly until September 2009 (for 18 months) in experiment 1. Fortnightly, the
183 population of psyllids was monitored inside both compartments until December 2009 (21
184 months). The number of nymph and adult psyllids were counted in each evaluation on three
185 branches of 32 predetermined test plants systematically distributed in the compartment
186 (Fig. 1A). After this, all remaining insects were killed, and all test plants were transferred to
187 another insect-proof screenhouse. In experiment 2, psyllid confinements were monthly
188 performed from March 2011 until April 2013 (25 months). The ACP population was
189 counted on three branches of 16 predetermined plants (Fig. 1B). The position of the
190 predetermined test plants inside each compartment was taken into account to establish a
191 dispersal gradient of psyllids distribution for experiments 1 and 2.

192 During both experiments, the presence of other insects (mites, aphids, etc.) was
193 continually monitored inside the compartments. If pests were observed, methods that did
194 not affect the psyllid population inside the compartments were undertaken, e.g., manual
195 control of pests (crushing) and/or washing infested leaves with water.
196
197 **'*Ca*. Liberibacter spp.' detection in psyllids and plant material**
198 Adult psyllids were collected for '*Ca*. Liberibacter spp.' detection by conventional
199 PCR. One sample consisted of 3 psyllids (pooled sample), and a total of 3 to 22 pooled
200 samples were collected per timepoint in experiment 1. The number of pooled samples
201 varied as a function of the total number of psyllids present in each compartment. In
202 experiment 2, one sample consisted of one psyllid, and a total of 1 to 11 samples were
203 collected per timepoint. DNA from samples was extracted with the CTAB protocol
204 (Murray and Thompson 1980) and was ajusted with water to final volume of 30 µl. A 5 µl
205 DNA aliquot was assayed for the presence of CLam and/or CLas in conventional duplex
206 PCR. The detection of CLam and CLas was carried out as described in Teixeira et al.
207 (2008b).
208 Two types of sampling were performed in the test plants for '*Ca*. Liberibacter spp.'
209 detection: scheduled samples and samples following the appearance of HLB symptoms.
210 Scheduled sampling was calendar-based, regardless of the presence of HLB symptoms,
211 totaling 10 scheduled samples. In the scheduled samples, four leaves from four adjacent test
212 plants (one leaf of each plant) composed a pooled sample. DNA extraction and '*Ca*.
213 Liberibacter spp.' detection by quantitative PCR (qPCR) were performed in each pooled
214 sample (Teixeira et al. 2008a). In the case of a positive detection, samples were collected

from each of the same four plants belonging to the pooled sample, and '*Ca*. Liberibacter spp.' detection was carried out in each individual sample.

In the second sampling type, plants presenting typical or doubtful HLB visual symptoms were collected for molecular detection. All plants were inspected weekly for HLB-like symptoms, namely blotchy mottle leaves, enlarged, swollen, and corky midribs and lateral veins. If any of these symptoms were observed, samples were collected from each symptomatic plant, and '*Ca*. Liberibacter spp.' detection was performed by conventional PCR (Teixeira et al. 2008b). Conventional PCR and qPCR present similar specificity to detect the presence of '*Ca.* Liberibacter spp.' in symptomatic plants (Li et al. 2007).

'*Ca*. Liberibacter spp.' detection from leaf samples collected on scheduled dates in experiment 1 were evaluated by qPCR using SYBR Green I (Applied Biosystems, Foster City, CA, USA) as the detection method, with 500 ng of template DNA, using cycling parameters and primers from Teixeira et al. (2008a). Samples with Ct value below 35 were considered positive for individual detection of either CLam and CLas. Scheduled samples of experiment 2 were evaluated using hydrolyzing probes (TaqMan probe and Path ID master mix from Applied Biosystems/ThermoScientific) to detect CLam or CLas using 300 ng of template DNA (Li et al. 2006). Each sample was amplified in duplicate, and cycling parameters were used according to Li et al. (2006). The baseline and threshold were automatically assigned and manually inspected using analysis software (StepOnePlus, Applied Biosystems/ThermoScientific). Each amplification batch contained a negative control (healthy sweet orange), a positive control (CLam or CLas infected sweet orange), and a non-template control. The detection limit was set with a dilution series of positive

238 samples as well as with a plasmid dilution containing the target amplification of each
239 primer pair, and a Ct of 34 or less was considered positive for the detection of both species.
240
241 **Data analyses**
242 The amount of ACP nymphs (proportion considering the total amount in two
243 compartments) in test and inoculum source plants were compared in each experiment by
244 the non-parametric two-proportion z-test at a significance level of alpha = 0.05 (Zar 1999).
245 The same analysis was performed to compare the following variables: ACP adults in test or
246 inoculum source plants; bacterioliferous psyllids with CLam or CLas; and the final
247 incidence of test plants with CLam or CLas. Analyses were performed with the R function
248 'prop.test' using RStudio Version 1.2.5033 software. A dispersal gradient of nymph and
249 adult psyllids was established from the source of inoculum plants to the test plants with 1 m
250 radial distance. The power model $y = a\ x^b$, where y is the total number of nymph or adult
251 psyllids, x is the distance (m), a is the amount of insects at distance 1 and b is the gradient
252 slope, was fitted to the data by non-linear regression with software Statistica (Statsoft).
253 The average number of nymph and adult psyllids per inoculum source (CLam and
254 CLas) and test plant were plotted over time. Day 1 was considered the first day that psyllids
255 were confined to the inoculum source plants for the first time. The incidence of test plants
256 with psyllids was calculated as the proportion of assessed test plants with nymph or adult
257 psyllids per assessment date. The CLam and CLas incidence in each compartment were
258 calculated as the accumulated proportion of PCR-positive plants for CLam and/or CLas.
259 The sum of all Liberibacter-positive plants to the total test plants corresponded to HLB
260 incidence in each experiment. All disease incidences (CLam, CLas, and HLB incidence)
261 were plotted over time.

262

263 **RESULTS**

264 The proportion of nymph and adult psyllids were consistently higher on test plants (P
265 < 0.001) than on both inoculum source plants throughout both experiments, in which more
266 than 0.6 of nymph and adult psyllids were observed on test plants (Table 1). However, the
267 number of psyllids per plant was higher on inoculum source plants than on test plants (Figs.
268 2, 3, Supplementary Fig. S1). The steepness of dispersal gradient for nymphs and adults of
269 *D. citri* from experiment 1 was high and the slope of the curve ranged from -1.49 to -0.68
270 m^{-1} (Supplementary Fig. S1, A and C) confirming that a high concentration of psyllids was
271 established at plants close to the source of inoculum. Gradient dispersal model was not
272 significant for data from experiment 2, but an average of 11.9 nymphs/plant and 28.8
273 adults/plant were observed over the distances (Supplementary Fig. S1, B and D). Overall,
274 there was no clear evidence of psyllid preference for inoculum source plants with CLam or
275 CLas (Table 1). Although a homogeneous light intensity was observed throughout
276 compartments, some psyllids were found settling on the screen walls located in the
277 direction of the sunset. Photosynthetically Active Radiation (PAR) inside compartment 1
278 varied from 382.76 to 420.9 μmol/m²/s, and inside compartment 2, it varied from 367.3 to
279 419.4 μmol/m²/s. PAR measured outside of both compartments varied from 786.1 to 801.9
280 μmol/m²/s. The thermal amplitude during the period of the experiment was, on average,
281 19.6±0.5ºC, in which the average highest and lowest temperatures were 36.3 and 16.8ºC,
282 respectively (Supplementary Fig. S2). The mean relative humidity varied between 25.6 and
283 83.8% inside both compartments (Supplementary Fig. S2).

284 The psyllids' settling behavior within compartments varied over the experimental
285 assessments (Figs. 2 and 3). Some peaks of nymph and adult psyllids were observed on

CLam and CLas inoculum source plants during experiments 1 (Fig. 2) and 2 (Fig. 3). A more regular distribution in the number of nymphs and adults per test plant was observed over assessments in experiment 1 (Fig. 2C and F). However, the mean incidence of test plants with nymph and adult psyllids was 4 and 13%, respectively (Supplementary Fig. S3). Although the population of nymph and adult psyllids also presented a regular distribution over the test plants, an increase in the psyllid population settled on the test and source of inoculum plants was observed about 2 years after the first confinement in experiment 2 (Fig. 3C and F). In spite of this, the mean incidence of test plants with psyllids in experiment 2 was similar to experiment 1 ($P = 0.99$). In experiment 2 the mean incidences of test plants with nymph and adult psyllids were 5 and 15%, respectively (Supplementary Fig. S3).

The proportion of CLas-positive samples was higher than CLam-positive samples for both psyllid and test plants, in both experiments (Table 2). One hundread and 91.8% of bacterialiferous psyllids and 92.3 and 93.1% of infected test plants were CLas positive in experiments 1 and 2, respectively. No psyllid was positive for CLam in experiment 1. CLam was detected, but its incidence remained in 2% of psyllid samples in experiment 2. No mixed infection of CLam and CLas was detected in the psyllids. Although no psyllid was positive for CLam in experiment 1, on average, 1.3% of test plants were CLam-positive (Table 2).

The earliest detection of CLas and CLam occurred, on average, 7 and 22 months after the first psyllid confinement, respectively, in experiment 1 (Fig. 4). Mixed infections of CLam and CLas were detected in only two plants (1.3% of the test plants or 3.8% of infected test plants) and both bacteria remained simultaneously in the plants until the end of

experiment. HLB incidence continued to increase, with 16.7% of test plants positive for CLam and/or CLas at 46 months, by the end of experiment 1 (Fig. 4C).

The first detection of CLas in test plants occurred at 115 days (about 4 months) after the first psyllid confinement in experiment 2 (Fig. 5). Positive samples of CLas were found earlier than those of CLam, which occurred 36 months after the first psyllid confinement (Fig. 5). No mixed infections of CLam and CLas were detected in experiment 2. HLB incidence increased from 2 to 14.5% of test plants positive for CLam and/or CLas in experiment 2 (Fig. 5C).

DISCUSSION

This study showed a higher efficiency of ACP in spreading CLas than CLam in the concurrent presence of both inoculum sources. CLas acquisition and transmission by the insect vector and the subsequent HLB progress were higher than CLam in both experiments carried out over 4-5 years in a screenhouse. These results are consistent with current field HLB sampling in Brazil, in which CLas was detected in more than 99% of Liberibacter-positive samples (Bassanezi et al. 2020). CLam was the prevalent species in citrus orchards from 2005 to 2007, and a sharp increase of CLas was subsequently observed (Bassanezi et al. 2020; Lopes et al. 2009a).

Nymphs of *D. citri* settled on test plants, suggesting that psyllids dispersed from the inoculum source plants, settled on, and laid eggs on test plants in the screenhouse compartments. Probably, the increase of psyllid population per plant observed around the second year after the psyllid confinement is related to the growth of test plants. Older plants may have more branches, and consequently, more new shoot emissions, which corresponded to the most favorable stage for insect development (Cifuentes-Arenas et al.

2018). The insect distribution within compartments was not homogeneous, as the average number of psyllid nymphs and adults in each inoculum source plant was higher than that in each test plant. However, more than 60% of psyllid nymphs and adults were found on test plants in both experiments. In experiment 1, the total number of psyllid nymphs and adults was 10 times higher than that in experiment 2. In spite of this, the mean incidence of plants with adult psyllids was similar, with 13 and 15% of test plants in experiments 1 and 2, respectively. However, the incidence of Liberibacter-positive psyllids in experiment 2 was higher than in experiment 1 (26.9% *vs*. 15.5%, respectively). Nevertheless, the final incidence of HLB was similar, with 16.7 and 14.5% Liberibacter-positive plants in experiments 1 and 2, respectively. For many vector-borne diseases, pathogen spread is not proportional to the extent or duration of insect infestation of a crop (Broadbent 1957). Capoor et al. (1974) observed that 68% of plants exposed to one psyllid presented HLB symptoms, and 100% HLB incidence was observed when plants were exposed to groups of five or more psyllids. Epidemics of vector-borne diseases are also driven by the length of (i) time for the vector to acquire the pathogen from the diseased plant, i.e., acquisition access period, (ii) time for the vector to transmit the pathogen to a new healthy plant, i.e., latent period, and (iii) time that the vector remains infectious after acquiring the pathogen, i.e., persistence of transmission (Kruse et al. 2019). The relationship of *D. citri* with CLam or with CLas is persistent and propagative. For CLas-*D. citri* relationship, there is an acquisition and access period (AAP) of at least one hour, latent period of 17 days, and continued transmission of the bacteria up to 4-5 weeks (Ammar et al. 2016; Canale et al. 2017; Nascimento 2010). There is few information for CLam-*D. citri* relationship, and 30% of bacterialiferous adult psyllids were detected either with CLam and CLas after the same AAP (Darolt 2021; Nascimento 2010).

Acquisition and transmission of '*Ca.* Liberibacter spp.' by psyllids may be influenced by several factors, derived from the host, the psyllid and the bacterium to be transmitted (Bonani et al. 2008; Coletta-Filho et al. 2014; Galdeano et al. 2020; Lopes et al. 2009a). The detection of '*Ca*. Liberibacter spp.' in the hosts is influenced by the bacterium titer and the molecular method for bacterial detection (Lopes et al. 2009a; Teixeira et al. 2008a). Although different qPCR protocols were used in experiments 1 and 2, the detection limit of both techniques are sufficient to detect CLas and CLam in infected plants even before symptom appearance (Lopes et al. 2009a, 2009b). Sequential sampling scheme used in the current study, would provide enough chance of detection of a sample that had failed in a previous sampling due to low titer for detection. That would happen with any PCR technique. Although no CLam-positive adults were detected in experiment 1, CLam-positive plants were detected around 2 years after the first confinement, indicating either the presence of CLam in the psyllids beyond the detection limit or that psyllids that were not sampled transmitted CLam from source to test plants. It is important to highlight that only psyllid adults were analyzed for the presence of '*Ca*. Liberibacter spp.' and a low bacterial titer in the psyllid gut may not be detected using conventional PCR. The detection of CLam or CLas in psyllids by PCR instead of the more sensitive qPCR could have affected the percentage of positive psyllid samples in this report, but the detection efficiency would be similar to both Liberibacters, since the detection limit is a function of template copy number in the sample (Li et al. 2006; Teixeira et al. 2008a). Nymphal ACPs, mainly at 4^{th} and 5^{th} stages, are more efficient in acquiring CLas and CLam than adult ACP (Ammar et al. 2016; Nascimento 2010). Therefore, if psyllids acquired the bacteria as adults, the bacteria titer would reach lower levels than adult psyllids that acquired the bacteria at nymphal stages (Ammar et al. 2016). Another hypothesis for the absence of

CLam-positive psyllids is that *D. citri* may have acquired CLam and, subsequently acquired CLas. In a few situations of controlled consecutive acquisition experiments, where *D. citri* acquired CLam and subsequently CLas, only the second species was detected in the insect (Nascimento 2010), while when the opposite was carried out, CLam and CLas could both be detected in *D. citri* (Darolt 2021).

Phenotypical changes due to symptom severity in sweet orange plants caused by CLam may also have contributed to the lower acquisition efficiency of CLam by psyllids, consequently causing a lower transmission efficiency. CLam inoculum source plants presented few new shoots, strong symptoms of leaf yellowing in a branch, fast mottling progression that crossed the leaf lateral veins, and thicker and leathery leaves with corky veins (Supplementary Fig. S4). These severe symptoms were associated with CLam infection (Lopes et al. 2009a). This leathery and corky aspect of leaves from CLam inoculum source plants may make stylet penetration and ingestion activities by *D. citri* difficult (Bonani et al. 2008). High mortalities of psyllids confined to CLam inoculum source plants during an acquisition access period were previously observed (Nascimento 2010). Further experiments of the effect of CLam infection on leaf morphology and consequently on the feeding behavior of *D. citri* should be performed to investigate if the psyllid stylet is able to reach the phloem.

The titer of CLam and CLas in the inoculum source plants varied over the assessments, but this variation is inherent for both Liberibacter species' biology (Li et al. 2009; Lopes et al. 2009a; Teixeira et al. 2008a). In the present work, inoculum source plants were obtained by graft transmission and not by natural bacterial transmission from a psyllid, but were symptomatic at the beginning of the experiments, indicating systemic distribution of each bacterium over the canopy. Despite this, samples from the CLam

inoculum source always presented a higher average Ct (27.4 to 31.3), i.e., lower bacterial titer in plants than samples from the CLas inoculum source (Ct between 20.5 and 22.7) during experiment 1. There was no relationship between the bacterial titer in the phloem vessels and symptom severity. CLas inoculum source plants had higher bacterial titers but less severe visual HLB symptoms in citrus plants than CLam (Supplementary Fig. S4), similar to previous reports (Lopes et al. 2009a, 2009b; Lopes and Frare 2008). A higher bacterial titer of CLas in plants may have increased the probability of the psyllid acquiring and, consequently, transmitting CLas more efficiently than CLam (Lopes et al. 2009a; Lopes and Frare 2008). These results and observations indicate that CLas has a higher competitive ability than CLam in aspects related to both the insect vector and host colonization and could clarify the shift in predominance of '*Ca*. Liberibacter spp.' 3-4 years after the first detection of the disease in Brazil. HLB is a destructive disease of citrus associated with '*Ca.* Liberibacter spp.' and the absence of genetic control methods forces citrus growers to eradicate symptomatic trees and control the vector by insecticide sprays. Therefore, determining the present bacterial species within a country may enable new approaches for HLB management, such as developing strategies to disrupt pathogen transmission and expanding breeding programs (Alves et al. 2021; Curtolo et al. 2020) to obtain plants resistant to '*Ca*. Liberibacter spp.' infection and colonization.

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List of Table Captions:

TABLE 1. Proportion of nymphs and adults of *Diaphorina citri* observed on test plants and both inoculum source plants in experiments 1 and 2.

TABLE 2. Proportion of '*Candidatus* Liberibacter spp.' detected in adult psyllid *Diaphorina citri* and in plant (final disease incidence) by PCR and/or qPCR in experiments 1 and 2.

List of Figure Captions:

Fig. 1. Schematic representation of the screenhouse with two individual compartments (compartment 1 and compartment 2). Red circles represent symptomatic 'Natal' sweet orange source plants infected with '*Candidatus* Liberibacter americanus'. Yellow circles represent symptomatic 'Natal' sweet orange source plants infected with '*Ca.* Liberibacter asiaticus'. Green circles represent 'Valencia' sweet orange potted plants exposed to *Diaphorina citri* released after 7-day acquisition access periods on inoculum source plants. R represents the rows where 16 plants per row were distributed within the compartments in experiment 1, **A**, and 13 plants per row in experiment 2, **B**. Marked plants (✳) were assessed for psyllid population.

Fig. 2 Population fluctuation of the psyllid *Diaphorina citri* per plant in experiment 1. Nymphs, **A to C**, and adults, **D to F**, of psyllids were assessed in 'Natal' plants infected with '*Candidatus* Liberibacter americanus' (CLam, **A and D**, $n = 2$) or '*Ca.* Liberibacter asiaticus' (CLas, **B and E**, $n = 2$), and 'Valencia' healthy plants (test plants, C and F, $n = 32$). Bars represent the standard errors of the mean between compartments. Arrows indicate the confinement periods of *D. citri* on CLam and CLas inoculum source plants.

Fig. 3 Population fluctuation of the psyllid *Diaphorina citri* per plant in experiment 2. Nymphs, **A to C**, and adults, **D to F**, of psyllids were assessed in 'Natal' plants infected with '*Candidatus* Liberibacter americanus' (CLam, **A and D**, $n = 2$) or '*Ca.* Liberibacter asiaticus' (CLas, **B and E**, $n = 2$), and 'Valencia' healthy plants (test plants, C and F, $n = 32$). Bars represent the standard errors of the mean between compartments. Arrows indicate the confinement periods of *D. citri* on CLam and CLas inoculum source plants.

633

634 **Fig. 4** Progress of cumulative Liberibacter-positive trees incidence (proportion) in
635 experiment 1. Plants infected with **A**, '*Candidatus* Liberibacter americanus' (CLam), **B**,
636 '*Ca.* Liberibacter asiaticus' (CLas), and **C**, HLB incidence represents the sum of CLam-
637 and CLas-positive plants to the total assessed plants. Bars represent the standard errors of
638 the mean between compartments.

639

640 **Fig. 5** Progress of cumulative Liberibacter-positive trees incidence (proportion) in
641 experiment 2. Plants infected with **A**, '*Candidatus* Liberibacter americanus' (CLam), **B**,
642 '*Ca.* Liberibacter asiaticus' (CLas), and **C**, HLB incidence represents the sum of CLam-
643 and CLas-positive plants to the total assessed plants. Bars represent the standard errors of
644 the mean between compartments.

645

Table 1 Proportion of nymphs and adults of *Diaphorina citri* observed on test plants and both inoculum source plants in experiments 1 and 2.

Plants[a]	Experiment 1		Experiment 2	
	Nymphs[b]	Adults[b]	Nymphs[b]	Adults[b]
Test plants	0.60 a	0.77 a	0.65 a	0.64 a
Source of CLam	0.18 c	0.09 c	0.20 b	0.20 b
Source of CLas	0.22 b	0.13 b	0.15 b	0.17 b
P-value	< 0.001	< 0.001	< 0.001	< 0.001

[a] Plants with '*Candidatus* Liberibacter americanus' (CLam) or with '*Ca.* Liberibacter asiaticus' (CLas).

[b] Proportion of nymph or adult psyllids considering the total number of psyllids collected in both compartments. Values followed by different letters in the column are significantly different ($P < 0.05$) by a two-proportion z-test.

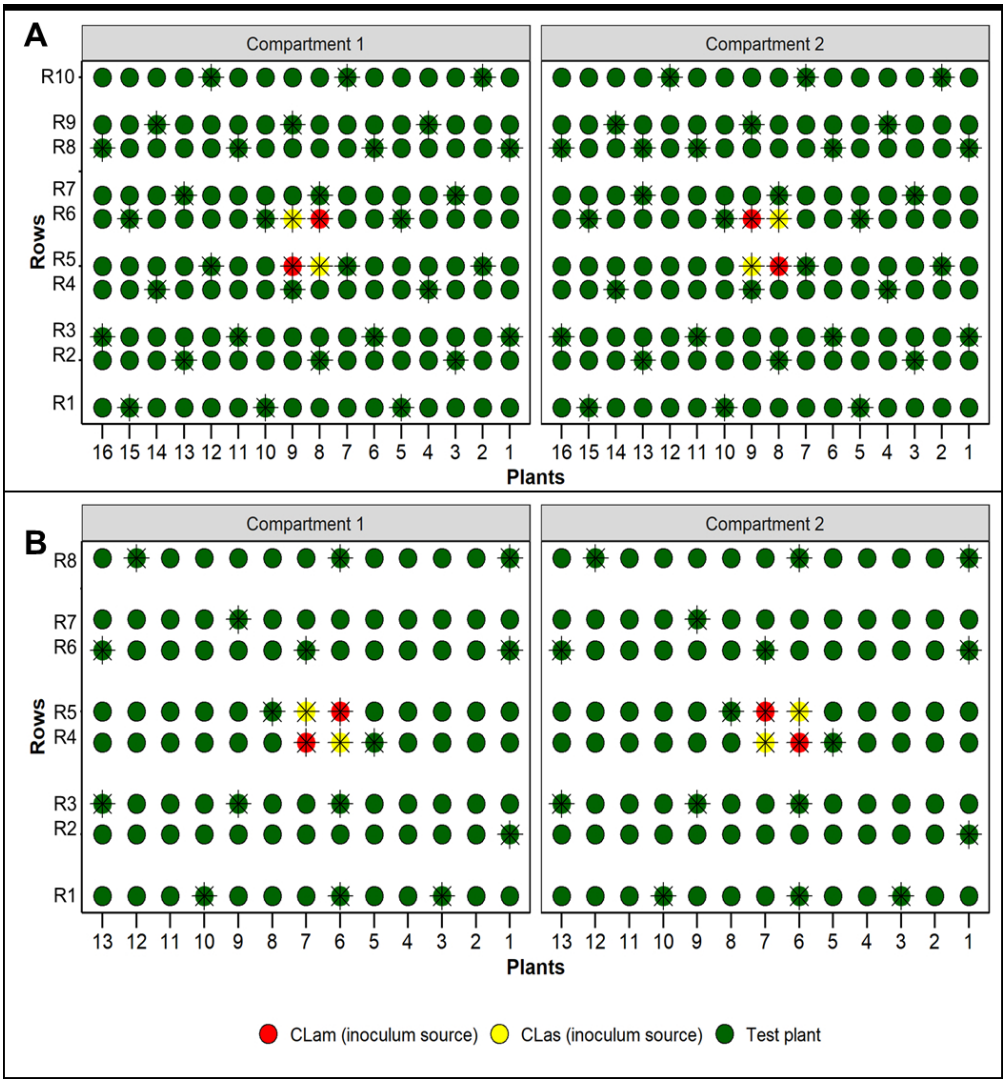
652 Table 2 Proportion of '*Candidatus* Liberibacter spp.' detected in adult psyllid *Diaphorina citri* and in plants (final disease incidence)
653 by PCR and/or qPCR in experiments 1 and 2.

Experiment	Liberibacter species[a]	Bacterialiferous psyllid (proportion)[b]	Final disease incidence (proportion)[c]
1	CLam	0.00 (0/97) b	0.01 (4/312) b
	CLas	0.15 (15/97) a	0.16 (50/312) a
	P-value	0.01	< 0.0001
2	CLam	0.02 (4/182) b	0.01 (2/200) b
	CLas	0.25 (45/182) a	0.14 (27/200) a
	P-value	< 0.0001	0.002

654 [a] CLam refers to '*Ca.* Liberibacter americanus' and CLas to '*Ca.* Liberibacter asiaticus'.

655 [b] Proportion of Liberibacter-positive psyllid considering the total number of psyllids collected in both compartments. Values followed
656 by different letters in the column are significantly different ($P < 0.05$) by a two-proportion z-test.

657 [c] Final cumulative incidence of plants from compartments 1 and 2 infected by CLam or CLas (proportion) Values followed by different
658 letters in the column are significantly different ($P < 0.05$) by a two-proportion z-test.



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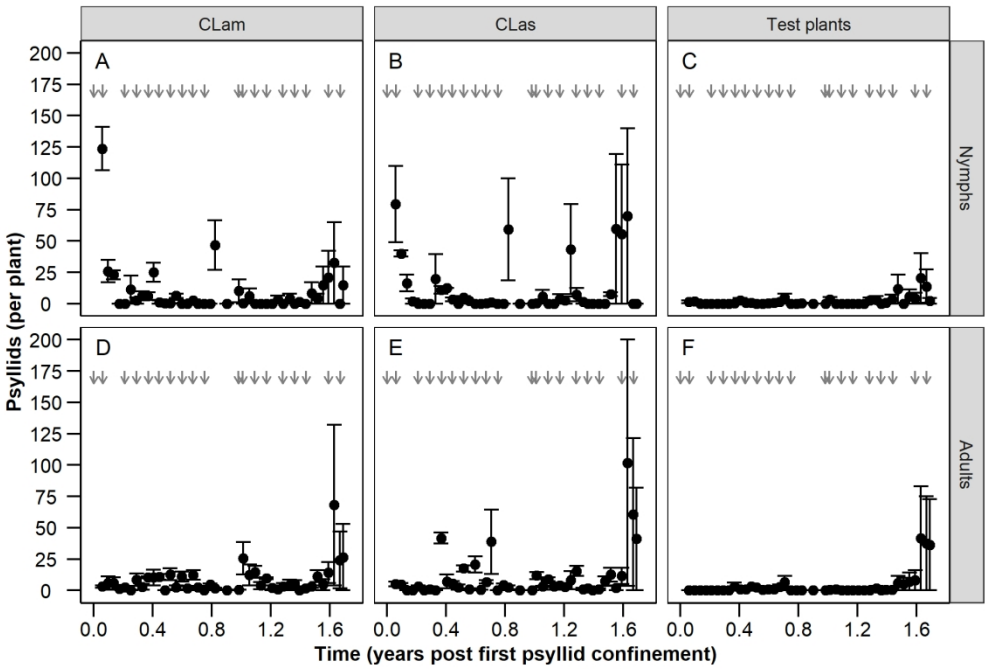

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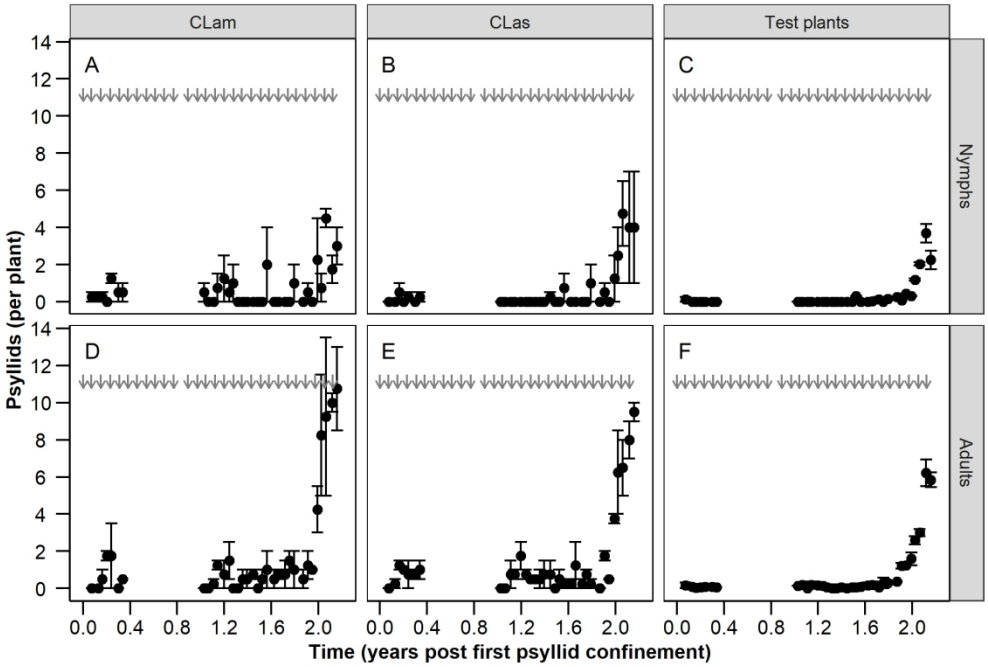

Fig. 3 Population fluctuation of the psyllid *Diaphorina citri* per plant in experiment 2. Nymphs, A to C, and adults, D to F, of psyllids were assessed in 'Natal' plants infected with 'Candidatus Liberibacter americanus' (CLam, A and D, n = 2) or '*Ca*. Liberibacter asiaticus' (CLas, B and E, n = 2), and 'Valencia' healthy plants (test plants, C and F, n = 32). Bars represent the standard errors of the mean between compartments. Arrows indicate the confinement periods of *D. citri* on CLam and CLas inoculum source plants.

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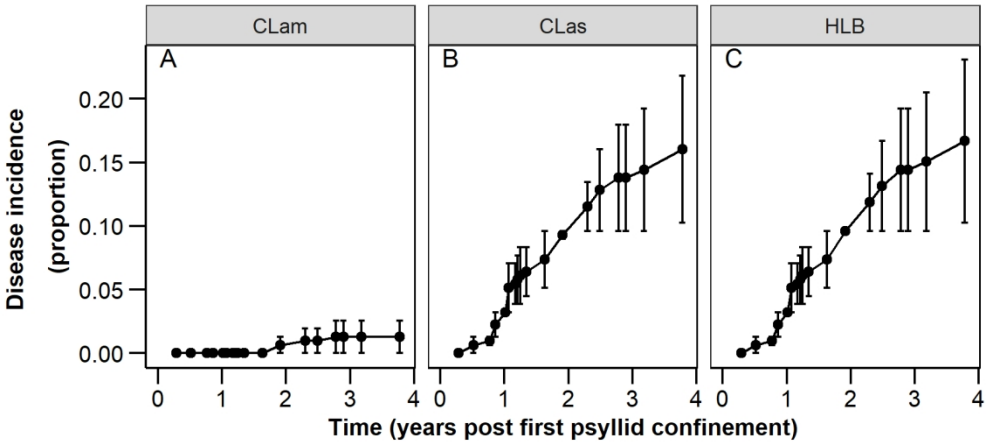

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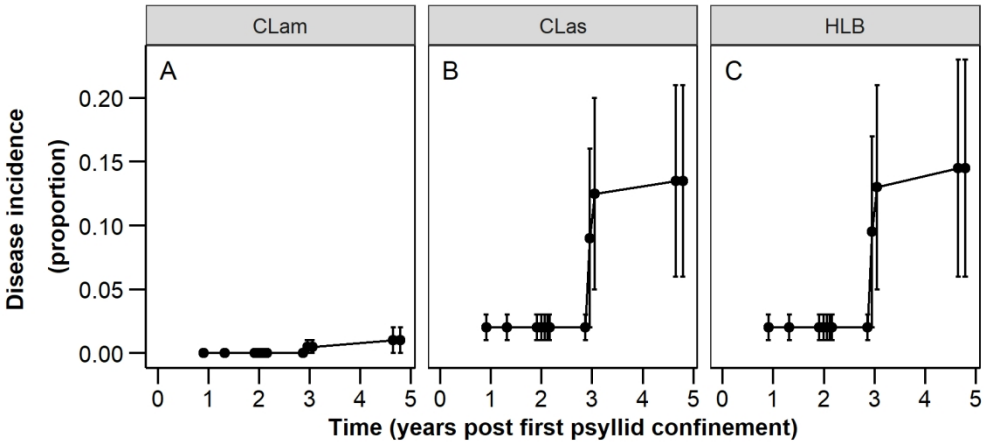

Fig. 5 Progress of cumulative Liberibacter-positive trees incidence (proportion) in experiment 2. Plants infected with A, '*Candidatus* Liberibacter americanus' (CLam) , B, 'Ca. Liberibacter asiaticus' (CLas), and C, HLB incidence represents the sum of CLam- and CLas-positive plants to the total assessed plants. Bars represent the standard errors of the mean between compartments.

152x76mm (300 x 300 DPI)

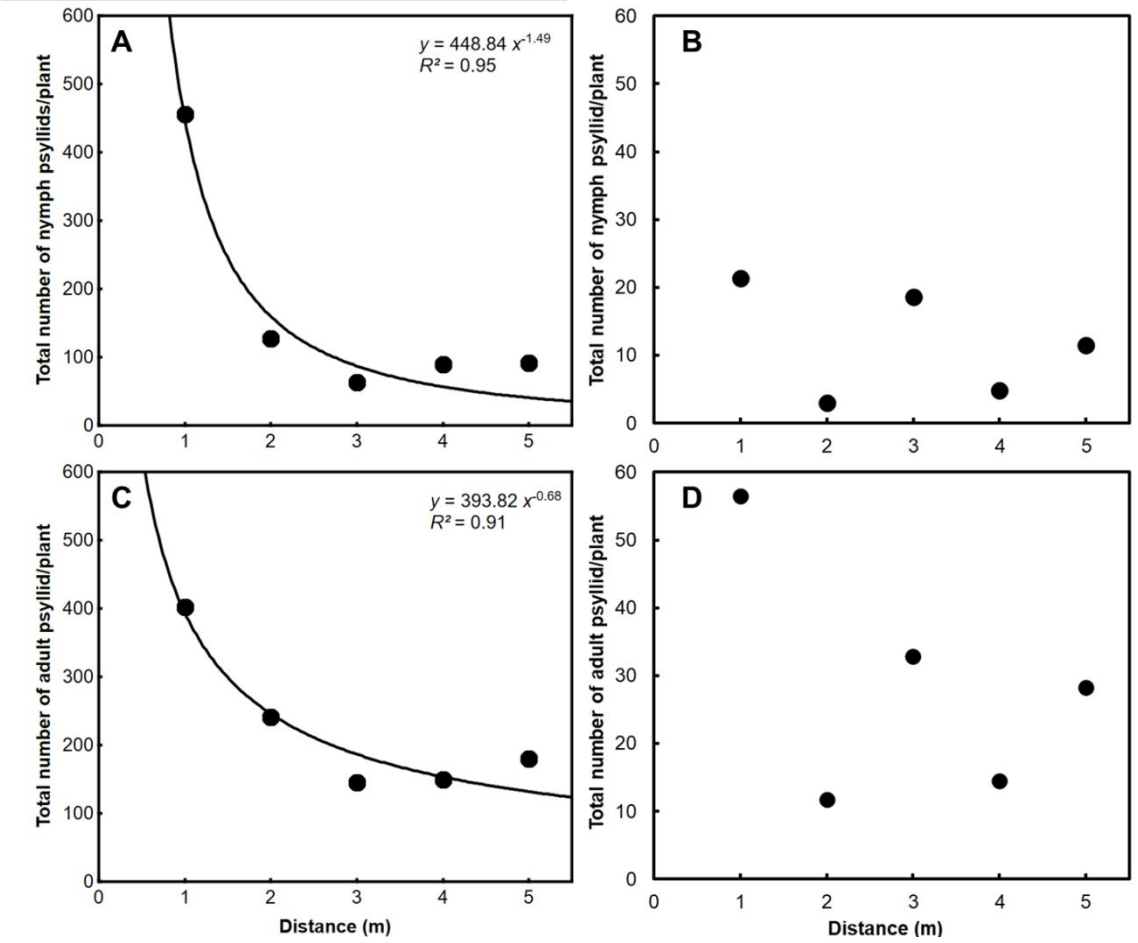

Fig. S1 – Total number of **A and B**, nymph and, **C and D**, adult psyllids per plant as a function of distance from inoculum source plants in experiment 1 (**A and C)** and experiment 2 (**B and D)**. Lines in **A and C** represent power model: $y = a\ x^b$, where y is the total number of nymph or adult psyllids, x is the distance (m) from the inoculum source plants, a is the amount of insects at distance 1 and b is the gradient slope. R^2 is the determination coefficient.

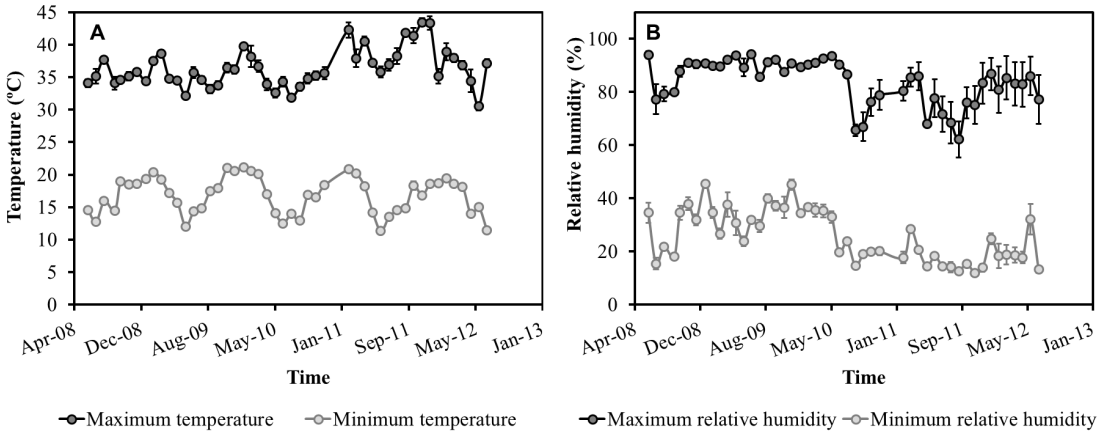

Fig. S2 – A, Highest (maximum) and lowest (minimum) temperatures (ºC) and, **B**, relative humidity inside compartments of the screenhouse. Mean values were based on daily measurements. Bars represent the standard errors of the mean between compartments.

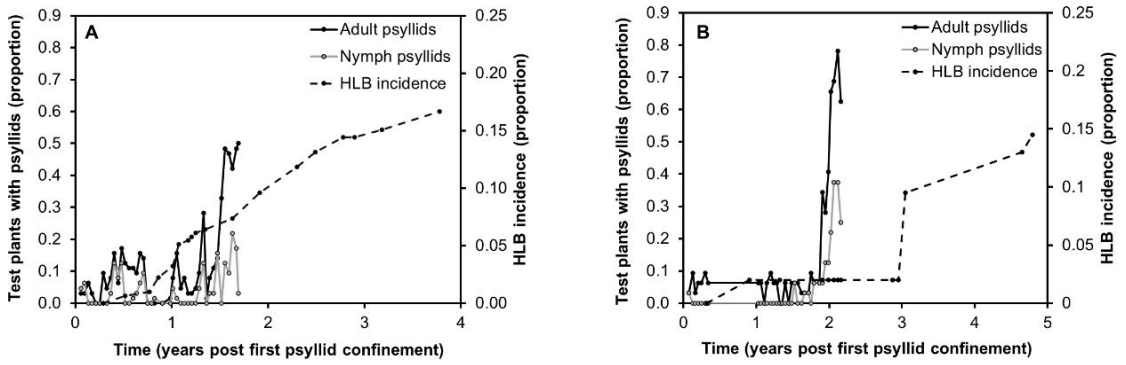

Fig. S3 Progress of incidence of test plants with nymph or adult psyllids (proportion) and of Huanglongbing (HLB) in **A,** experiment 1 and, **B,** experiment 2. A total of 64 and 32 test plants were assessed in experiments 1 and 2, respectively for psyllids population. HLB incidence represents the sum of '*Candidatus* Liberibacter americanus' and '*Ca.* Liberibacter asiaticus'-positive plants to the total assessed plants in experiments 1 (n = 312) and 2 (n = 200). The average incidences of plants with *Diaphorina citri* in experiments 1 and 2 were compared by a two-proportion z-test (P = 0.99).

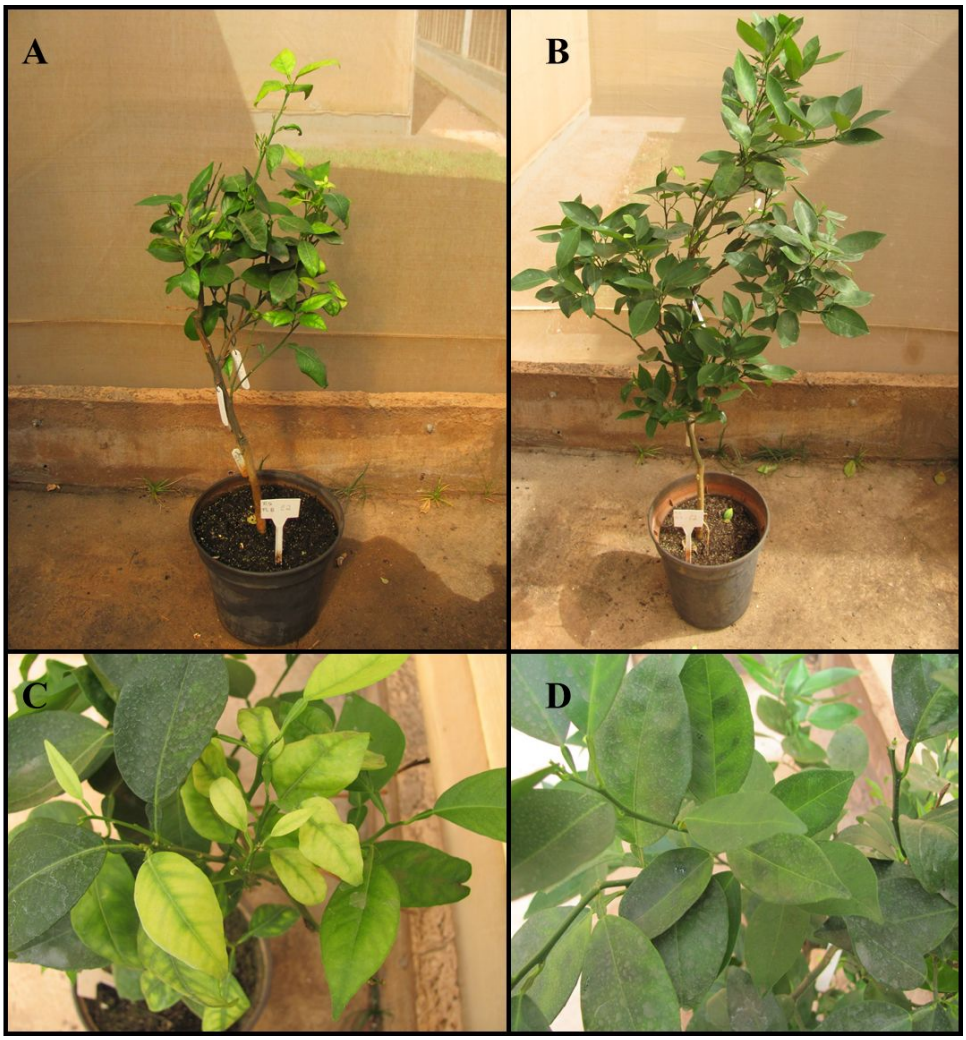

Fig. S4 A and C, Huanglongbing symptoms in 'Natal' sweet orange inoculum source plants of '*Candidatus* Liberibacter americanus' and, **B and D**, '*Ca.* Liberibacter asiaticus'. **C and D** are viewed from the top.