Liberibacters Associated with Citrus Huanglongbing in Brazil: '*Candidatus* Liberibacter asiaticus' Is Heat Tolerant, '*Ca. L. americanus*' Is Heat Sensitive

S. A. Lopes and G. F. Frare, Fundecitrus, Av. Adhemar de Barros, 201, Araraquara, SP, CEP 14807-040, Brazil; E. Bertolini and M. Cambra, Instituto Valenciano de Investigaciones Agrarias, Carretera Moncada Náquera, km 4.5, Moncada, Spain; N. G. Fernandes, A. J. Ayres, and D. R. Marin, Fundecitrus, Brazil; and J. M. Bové, University of Bordeaux 2 and INRA, 71, Av. E. Bourlaux, 33883 Villenave d'Ornon, France

ABSTRACT

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In São Paulo State, Brazil, '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus' are associated with huanglongbing (HLB). Affected municipalities occur mainly in the central and southern regions, where the annual number of hours above 30°C is two to five times lower than that in the extreme northern and western regions. The influence of temperature on sweet orange trees infected with '*Ca. L. asiaticus*' or '*Ca. L. americanus*' was studied in temperature-controlled growth chambers. Symptom progression on new shoots of naturally infected and experimentally graft-inoculated symptomatic sweet orange trees was assessed. Mottled leaves developed on all infected trees at 22 to 24°C, but not on any '*Ca. L. americanus*'-infected trees at 27 to 32°C. Quantitative, real time-PCR was used to determine the liberibacter titers in the trees. After 90 days, '*Ca. L. asiaticus*'-infected trees had high titers at 32 and 35°C, but not at 38°C, while '*Ca. L. americanus*'-infected trees had high titers at 24°C, but at 32°C the titers were very low or the liberibacters could not be detected. Thus, the multiplication of '*Ca. L. asiaticus*' is not yet affected at 35°C, while a temperature of 32°C is detrimental to '*Ca. L. americanus*'. Thus, '*Ca. L. americanus*' is less heat tolerant than '*Ca. L. asiaticus*'. The uneven distribution of these two liberibacters in São Paulo State might be in relation with these results.

In São Paulo State (SPS), Brazil, two Liberibacter species are associated with huanglongbing (HLB) disease of citrus: '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus' (2,5,17). Recently, a phytoplasma of group 16SrIX was also found to be associated with HLB symptoms in SPS (18). Today, HLB is probably the most serious and destructive disease of citrus in the world (2). Since 2004, more than three million sweet orange trees have succumbed to the disease in SPS, and the number of affected municipalities has increased from 39 in August 2004 to 182 in August 2008. HLB affects all commercial varieties of sweet orange, mandarin, and grapefruit trees, whatever their age and type of rootstock (2,6). However, variations in susceptibility exist among local citrus species and varieties (9). Both Liberibacter species also affect ornamental trees of orange jasmine (*Murraya paniculata*) in urban areas (11,12).

In SPS, the most probable site where the HLB outbreak occurred is the Araraquara area (7), located within the geographic center of the State, where the highest density of affected trees occurred. From there, the disease has moved longer distances toward the south than toward the north, west, or east (10). Unrecognized environmental factors could be the cause of this uneven disease distribution. Indeed, it has been demonstrated that '*Ca. L. asiaticus*' isolates from India and the Philippines and '*Candidatus* Liberibacter africanus' isolates from South Africa differ in their sensitivities to high temperatures (3,14). While sweet orange seedlings infected with '*Ca. L. africanus*' or '*Ca. L. asiaticus*' developed severe HLB symptoms within 30 weeks when maintained under "cool" phytotron conditions (22°C for 8 h in the dark and 24°C for 16 h in the light), only seedlings infected with '*Ca. L. asiaticus*' exhibited severe HLB symptoms when kept for 30 weeks under "warm" conditions (27°C for 8 h in the dark and 32°C for 16 h in the light); no symptoms were observed on the '*Ca. L. africanus*'-infected seedlings, even when they stayed for 72 weeks rather than 30 weeks under the warm conditions (3). These results explain why in Africa, Madagascar, Reunion Island, Mauritius Island, and Yemen, where '*Ca. L. africanus*' is the heat-

sensitive HLB agent, the disease is seen only in cool, elevated areas. The Cape Town region, at the southern tip of Africa, is the only area where '*Ca. L. africanus*'-associated HLB occurs at sea level: here, latitude compensates for altitude. The heat sensitivity of the African HLB psyllid vector, *Trioza erythrae*, is an additional reason why '*Ca. L. africanus*'-associated HLB occurs only in cool environments (4).

Here, we show that '*Ca. L. americanus*'-infected sweet orange trees, in contrast to '*Ca. L. asiaticus*'-infected trees, do not develop HLB blotchy mottle leaf symptoms and become '*Ca. L. americanus*'-negative when maintained under warm conditions. These results may partially explain the distribution of HLB in SPS and the increase of '*Ca. L. asiaticus*'-infected trees in comparison with '*Ca. L. americanus*'-infected trees, as observed over the last 2 years.

MATERIALS AND METHODS

Sweet orange trees. The influence of temperature was tested on naturally and experimentally infected sweet orange trees grafted on Rangpur lime rootstock. The young trees were maintained for 60 or 90 days within three growth chambers (CMP4030, Conviron, Winnipeg, Canada). All infected trees showed blotchy mottle leaf symptoms, characteristic of HLB, on at least one branch. Infection was confirmed by polymerase chain reaction (PCR) with primers specific for '*Ca. L. americanus*' and '*Ca. L. asiaticus*', as described previously (15). Field-grown, 1.5-year-old 'Hamlin' sweet orange trees were from a farm in Motuca, SPS, and became naturally infected with '*Ca. L. americanus*' in the field, presumably through the psyllid *Diaphorina citri*, shown to be a vector of '*Ca. L. americanus*' in SPS (19). The trees were pruned approximately 50 cm above the trunk branching point, removed from the soil, transplanted into 30-liter plastic containers filled with the field soil in which the trees were initially growing, and transferred to a cooled greenhouse kept below 32°C. Two months later, the trees were pruned again and transferred to the growth chambers. Experimentally infected trees were 1-year-old, container-grown 'Hamlin', 'Natal', 'Pera', and 'Valencia' sweet orange trees grafted on Rangpur lime rootstock and raised in a closed, in-

Corresponding author: S. A. Lopes
E-mail: slopes@fundecitrus.com.br

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sect-proof greenhouse. They were graft-inoculated at the scion trunk with 4-cm-long budwood sticks as described previously (9). The budwood sticks were collected on symptomatic branches of field trees infected with ‘*Ca. L. americanus*’ or ‘*Ca. L. asiaticus*’, as determined by PCR. Healthy field-grown and container-grown trees were also included in the experiment as negative controls.

Light and temperature conditions. Each 24-h day had 16 h of continuous light and 8 h of continuous dark. Table 1 summarizes the eight distinct daily temperature cycles used in the two growth chambers of experiments I and II, and in the three chambers of experiments III and IV. In Table 1, for each temperature cycle, the hours in the dark are indicated in bold style. For instance, the daily temperature cycle in growth chamber 1 of experiment I was as follows: after 5 h (in the dark) at 22°C, the temperature increased from 22 to 24°C within 3.5 h (darkness for the first 3 h), and remained at 24°C for 12 h, when it decreased back to 22°C in 3.5 h. Table 1 also lists the type of trees in the chambers of the various experiments. For example, chamber 1 in experiments I contained 10 ‘Hamlin’ sweet orange trees naturally infected with ‘*Ca. L. americanus*’ as well as two healthy ‘Hamlin’ trees. In experiment I, the sweet orange trees were naturally infected with ‘*Ca. L. americanus*’ in the field. In experiments II, III, and IV, infection with ‘*Ca. L. americanus*’ or ‘*Ca. L. asiaticus*’ was by graft inoculation in the greenhouse. Trees in experiment I were maintained inside the chamber for 60 days and then transferred to a cooled greenhouse with temperatures kept well below 32°C most of the time—environmental conditions that were favorable for HLB symptom expression, in particular for ‘*Ca. L. americanus*’-infected trees (9). In experiments II, III,

and IV, the trees remained in the chambers for 90 days.

Symptom expression and PCR. The sweet orange trees were examined for symptom expression and analyzed for liberibacter infection by SYBR green quantitative real-time PCR (qPCR) at time zero, when they were transferred to the chambers, and at the end of the experiments, namely at “60 days” for experiment I and “90 days” for experiments II, III, and IV. Plants of experiments III and IV were also evaluated after 60 days of incubation. For symptom expression, the presence of leaves with blotchy mottle and/or mineral deficiency-like patterns, known to be frequently associated with HLB (2,6,9), was recorded. For PCR analysis of naturally infected, field-grown trees, for each tree several samples were collected from two to four branches, one sample per branch. Each sample contained five mature leaves. For PCR analysis of the greenhouse-grown graft-inoculated trees, only one four-leaf sample was collected per tree, as these trees were smaller than the field-grown trees. The samples (0.5 g of fresh midribs) were processed for DNA extraction using the CTAB protocol (13) as described previously (15). Yield and quality of DNA were measured with a Lambda EZ 201 spectrophotometer (Perkin Elmer), and the concentration was adjusted to 10 ng/μl for qPCR assays. All DNA samples were kept at –20°C. qPCR assays were carried out with the newly developed *rpl*-primer pairs *f-rpl/LAs/r-rpl/LAs* and *f-rpl/JAm/r-rpl/JAm* for detection and quantification of ‘*Ca. L. asiaticus*’ and ‘*Ca. L. americanus*’, respectively (16). qPCR assays were performed in a StepOne Plus thermocycler (Applied Biosystems). The reaction mixture contained, in a volume of 25 μl, the following: 1xPower SYBR green master mix (Applied Biosystems), 0.15 μM of each primer, and 50 ng of total DNA. In order to

obtain high endpoint fluorescence and low cycle thresholds, the annealing temperature had to be optimized. Final amplification conditions consisted of an initial step at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 63°C for 1 min. To obtain the melting curve data, after PCR the temperature was increased by 0.5°C every 10 s from 69 to 95°C with a continuous measurement of fluorescence. Data acquisition and analysis were performed with the StepOne Plus 2.0 software (Applied Biosystems). Melting curve peaks were at 75.4°C for ‘*Ca. L. americanus*’ and 77.4°C for ‘*Ca. L. asiaticus*’.

Liberibacter DNA quantification. Amplification products of both Liberibacter species were obtained by conventional PCR with primers *f-rpl/LAs/r-rpl/LAs* for ‘*Ca. L. asiaticus*’, giving an amplicon of 161 bp, and *f-rpl/JAm/r-rpl/JAm* for ‘*Ca. L. americanus*’, giving an amplicon of 127 bp (16). With both primer pairs, after an initial denaturation step at 94°C for 3 min, PCR included 40 cycles of 95°C for 30 s, 67°C for 30 s, and 72°C for 30 s, followed by an elongation step at 72°C for 5 min. Reaction mixture in a final volume of 25 μl contained 500 ng of total DNA, 150 μM of each *rpl*-primers, 200 μM dNTP, 300 μM MgCl₂, and 1.25 U of *Taq* DNA polymerase (Invitrogen) in the reaction buffer supplied by the manufacturer. Amplified products were purified using the Wizard SV Gel kit and the PCR Clean-up System (Promega), inserted into pGem-T easy vector (Promega), and cloned in *E. coli* DH5α. Transformants were selected for ampicillin resistance in media also containing X-gal and IPTG to screen for inserts. The white (Gal[–]) colonies were grown in LB medium supplemented with 100 μg/ml ampicillin. Plasmid DNA was prepared with the Wizard Miniprep kit (Promega), linearized with the restriction enzyme *Eco*RI, confirmed by electropho-

Table 1. Daily temperatures and light cycles per experiment, and nature of sweet orange trees used in the growth chambers

Experiment	Chamber and daily temperature cycles ^z	Total trees tested
I	Chamber 1 - 22°C(5h), ↑(3h and 0.5h), 24°C(12h), ↓(3.5h)	5 ‘ <i>Candidatus</i> Liberibacter americanus’, naturally infected ‘Hamlin’; 1 asymptomatic
	Chamber 2 - 27°C(5h), ↑(3h and 0.5h), 32°C(12h), ↓(3.5h)	Same
II	Chamber 1 - 22°C(5h), ↑(3h and 0.5h), 24°C(12h), ↓(3.5h)	11 ‘ <i>Ca. L. americanus</i> ’, graft-infected (5 ‘Valencia’, 2 ‘Hamlin’, 2 ‘Natal’, 2 ‘Pera’); 20 ‘ <i>Ca. L. asiaticus</i> ’, graft-infected (5 ‘Valencia’, 6 ‘Hamlin’, 4 ‘Natal’, 5 ‘Pera’); 2 asymptomatic ‘Valencia’
	Chamber 2 - 27°C(5h), ↑(3h and 0.5h), 32°C(12h), ↓(3.5h)	10 ‘ <i>Ca. L. americanus</i> ’, graft-infected (4 ‘Valencia’, 2 ‘Hamlin’, 3 ‘Natal’, 1 ‘Pera’); 19 ‘ <i>Ca. L. asiaticus</i> ’, graft-infected (5 ‘Valencia’, 5 ‘Hamlin’, 4 ‘Natal’, 5 ‘Pera’); 2 asymptomatic ‘Valencia’
III	Chamber 1 - 24°C(8h and 7h), ↑(4h), 32°C(1h), ↓(4h)	6 ‘ <i>Ca. L. americanus</i> ’, graft-infected ‘Valencia’; 6 ‘ <i>Ca. L. asiaticus</i> ’, graft-infected ‘Valencia’; 2 asymptomatic ‘Valencia’
	Chamber 2 - 24°C(8h and 1h), ↑(3h), 32°C(9h), ↓(3h)	Same
	Chamber 3 - 24°C(3h), ↑(4.5h), 32°C(0.5h and 11.5h), ↓(4.5h)	Same
IV	Chamber 1 - 24°C(8h and 1h), 24°C(3h), ↑(3h), 32°C(6h), ↓(3h)	11 ‘ <i>Ca. L. americanus</i> ’, graft-infected (5 ‘Valencia’, 6 ‘Natal’); 12 ‘ <i>Ca. L. asiaticus</i> ’, graft-infected (6 ‘Valencia’, 6 ‘Natal’); 2 asymptomatic ‘Valencia’
	Chamber 2 - 24°C(8h and 1h), 24°C(3h), ↑(3h), 35°C(6h), ↓(3h)	Same
	Chamber 3 - 24°C(8h and 1h), 24°C(3h), ↑(3h), 38°C(6h), ↓(3h)	Same

^z Numbers of hours in the dark are in boldface.

resis in a 1.2% agarose gel, and sequenced using T7/Sp6 primers. The sequences were confirmed by BLAST search and alignment (1). The amount of plasmid DNA was estimated by UV densitometry. Conversion of μg of double-stranded (ds) DNA to pmol was performed considering the average molecular mass of a DNA base pair (660 Da) and the number of bases of the dsDNA fragment (Nb). The following formula was applied: $\text{pmol of dsDNA} = \mu\text{g of dsDNA} \times (10^6 \text{ pg/1 } \mu\text{g}) \times (1 \text{ pmol/660 pg}) \times (1/\text{Nb})$. The Avogadro constant (6.023×10^{23}) was used to estimate the number of plasmid DNA copies. Tenfold serial dilutions of the plasmid DNA copies were prepared from 5.0×10^{10} to 5.0×10^1 for 'Ca. L. americanus', and from 3.7×10^{10} to 3.7×10^1 for 'Ca. L. asiaticus', aliquoted, and stored at 4°C until use. Dilutions from 5.0×10^8 to 5.0×10^2 for 'Ca. L. americanus' or 3.7×10^8 to 3.7×10^2 for 'Ca. L. asiaticus' were employed to generate the standard curve. To determine the theoretical sensitivity and the reliability of the qPCR, three repetitions of the assay were performed, using the 10-fold serial dilutions of the previously prepared plasmid DNA

copies. The relative amount of liberibacter cells per gram of fresh leaf midrib was estimated taking into consideration the amount of liberibacter DNA obtained in 50 ng of total DNA used per reaction mix, and the total amount of DNA extracted from 500 mg of leaf tissue. The SAS system (SAS Institute, Cary, NC) was used for statistical analysis of the data. The Tukey's student range test was used for comparing means of qPCR cycle threshold (Ct) data, with a significance level of $P = 0.05$.

RESULTS

Twenty young sweet orange trees naturally field-infected with 'Ca. L. americanus', as well as 88 young sweet orange trees experimentally graft-inoculated with 'Ca. L. americanus' and another 89 graft-inoculated with 'Ca. L. asiaticus', were included in four experiments involving eight distinct daily temperature cycles (Table 1). The four sweet orange varieties used in the experiments ('Hamlin', 'Valencia', 'Natal', and 'Pera') were among the most important Brazilian sweet orange varieties. qPCR analysis was applied to

total DNA extracted from leaf samples collected at time zero, i.e., the time when the trees were transferred to the chambers. The analyses indicated that all infected trees carried only one species of Liberibacter, and all asymptomatic trees used as controls were PCR negative. At time zero, average Cycle thresholds (Cts) ranged from 22.99 to 28.97 for the field-grown trees naturally infected with 'Ca. L. americanus', from 20.35 to 27.00 for the trees graft-inoculated with 'Ca. L. americanus', and from 18.02 to 27.67 for those inoculated with 'Ca. L. asiaticus'.

Experiment I. In experiment I (Table 2), symptomatic, field-grown, sweet orange trees naturally infected with 'Ca. L. americanus', as well as PCR-negative, asymptomatic trees from the same field were used. After 60 days of incubation at 22 to 24°C, i.e., under "cool" conditions (Table 1, experiment I, chamber 1), the trees had produced new shoots. The size of the shoots averaged 40.4 cm for the 'Ca. L. americanus'-infected trees and 49.2 cm for the asymptomatic trees. On infected trees, but not on asymptomatic ones, the older leaves showed the blotchy mottle symptoms characteristic of HLB, and the younger leaves at the tip of the shoots exhibited symptoms of iron and zinc deficiencies (Table 2). The zinc deficiency patterns were characterized, in many cases, by small green islands on a yellow leaf-blade background (9), a sign of severe deficiency. The control tree maintained at 22 to 24°C also exhibited some deficiency-like symptoms, but these were much milder than those observed on the liberibacter-infected trees. 'Ca. L. americanus' was detected in most shoots from infected trees, and the Ct values varied from 25.96 and 27.69 (Table 2).

In the case of the trees maintained for 60 days at 27 to 32°C, namely under "warm" conditions (Table 1, experiment I, chamber

Table 2. Leaf symptoms of, and quantitative real-time polymerase chain reaction (qPCR) results from, healthy and 'Candidatus Liberibacter americanus'-affected 'Hamlin' trees exposed for 60 days to 22/24°C or 27/32°C

Plant no.	Daily temperature cycles					
	22 and 24°C (chamber 1)			27 and 32°C (chamber 2)		
	Symptoms ^x	PCR+ ^y	Ct ^z	Symptoms	PCR+	Ct
1	mt, df++	2/2	25.96	df+	1/4	29.69
2	mt, df+++	3/5	27.69	df+	0/4	—
3	mt, df+++	2/2	27.56	—	0/3	—
4	mt, df+	3/6	27.02	df+	0/3	—
5	mt, df++	2/3	26.52	df+	0/3	—
Control	df+	0/3	—	—	0/4	—

^x mt = leaf mottling, df = mineral deficiency, with df+ meaning slight general leaf chlorosis, df++ and df+++ respectively, initial and severe zinc deficiency, and — = no symptoms.

^y Number of new branches that tested positive by qPCR over total number of new branches.

^z Ct = Average qPCR cycle threshold values, with — meaning no fluorescence.

Table 3. Leaf symptoms of, and quantitative real-time polymerase chain reaction (qPCR) results from, healthy and 'Candidatus Liberibacter americanus'— or 'Ca. L. asiaticus'—affected trees of 'Valencia', 'Hamlin', 'Natal', and 'Pera' exposed for 90 days to 22/24°C or 27/32°C

Sweet orange variety	Daily temperature regimens							
	22 and 24°C (chamber 1)				27 and 32°C (chamber 2)			
	Total	Symptoms ^x	PCR+ ^y	Ct values ^z	Total	Symptoms	PCR+	Ct values
'Ca. L. americanus'								
'Valencia'	5	mt, df+++	5/5	25.21 a	4	df+	1/4	29.35
'Hamlin'	2	mt, df++	2/2	23.69 a	2	df+	0/2	—
'Natal'	2	mt, df++	2/2	23.50 a	3	—	0/3	—
'Pera'	2	mt, df++	2/2	24.90 a	1	df+	0/1	—
'Ca. L. asiaticus'								
'Valencia'	5	df++	5/5	19.38 b B	5	df+	5/5	21.64 A
'Hamlin'	6	df+	6/6	19.63 b A	5	—	5/5	20.38 A
'Natal'	4	df+	4/4	19.02 b B	4	—	4/4	21.27 A
'Pera'	5	df+	5/5	18.48 b B	5	df+	5/5	20.69 A
Control	2	—, —	0/2	—	2	—, —	0/2	—

^x mt = leaf mottling, df = mineral deficiency, with df+ meaning slight general leaf chlorosis, df++ and df+++ respectively, initial and severe zinc deficiency, and — = no symptoms.

^y Number of trees that tested positive by qPCR over total number of trees.

^z Ct = Average qPCR cycle threshold values, with — meaning no fluorescence. Means with different letters are significantly different ($P < 0.05$). Comparisons were done between Liberibacter species within each sweet orange variety and temperature regimen (small letters) and between chambers within 'Ca. L. asiaticus' (capital letters).

2), the new shoots were more vigorous. Their average size reached 48.6 cm for the '*Ca. L. americanus*'-infected trees and 49.6 cm for the asymptomatic control trees. However, in strong contrast to the '*Ca. L. americanus*'-infected trees maintained under "cool" conditions, similar trees kept under the "warm" 27 to 32°C conditions did not show the characteristic HLB leaf mottle, even if previously they had shown such symptoms (Table 2). Also, the general leaf chlorosis and mineral deficiency patterns were much milder than those observed under the cool conditions. '*Ca. L. americanus*' could be detected by qPCR (Ct: 29.69) only in one single asymptomatic branch of a previously symptomatic '*Ca. L. americanus*'-infected tree (Table 2). All other trees previously infected with '*Ca. L. americanus*', as well as the healthy control trees, were PCR negative. An additional experiment, identical to experiment I, was carried out and gave almost the same results (data not shown).

Experiment II. Experiment II (Table 3) was carried out under the two same daily temperature cycles as experiment I, but involved graft-inoculated sweet orange trees infected with '*Ca. L. americanus*' or '*Ca. L. asiaticus*', while in experiment I all trees were naturally infected with '*Ca. L. americanus*'. Also, in experiment I, only one sweet orange variety ('Hamlin') was used, while in experiment II, four of the major Brazilian varieties ('Hamlin', 'Valencia', 'Natal', 'Pera') were dealt with. The results for the '*Ca. L. americanus*'-infected trees of experiment II were very similar to those of experiment I. After 90 days at 22 to 24°C, the newly developed shoots exhibited the characteristic blotchy mottle and mineral deficiency leaf patterns, and were all PCR positive for '*Ca. L. americanus*', with an average Ct value of 24.56, ranging from 23.50 for 'Natal' to 25.21 for 'Valencia'. The trees maintained at 27 to 32°C lacked blotchy mottle leaf symptoms, their leaf yellowing was less intense, and '*Ca. L. americanus*' was de-

tected in only one of the four 'Valencia' trees tested, with a Ct value of 29.35, close to a negative PCR test. In the case of trees infected with '*Ca. L. asiaticus*', leaf blotchy mottle and mineral deficiency patterns were present on the trees in the "cool" (22/24°C) chamber, but these symptoms were not as strong as in the case of '*Ca. L. americanus*'-infected trees under the same conditions. In the "warm" (27/32°C) chamber, the symptoms were even less intense than at 22/24°C or even absent on certain plants. However, '*Ca. L. asiaticus*' was detected by PCR in all the trees, not only those from the "cool" chamber (average Ct value of 19.16, ranging from 18.48 for 'Pera' to 19.63 for 'Hamlin'), but also those from the "warm" chamber (average Ct value of 20.98, ranging from 20.38 for 'Hamlin' to 21.64 for 'Valencia'), under conditions (27/32°C) where '*Ca. L. americanus*'-infected trees were essentially PCR negative. The lowest Ct values (corresponding to the highest liberibacter titers) were those of the '*Ca. L. asiaticus*'-infected trees in the "cool" chamber. These values were significantly different from those of the '*Ca. L. americanus*'-infected trees maintained in the same chamber, as well as from those of the '*Ca. L. asiaticus*'-infected trees in the "warm" chamber, except for the 'Hamlin' variety (Table 3).

Experiment III. In experiment III, the 24-h daily temperature cycles differed by the length of time at which the trees were kept at 24 and 32°C (Table 1). In chamber 1, the trees were at 24°C for 15 h and at 32°C for 1 h, with a period of 4 h to go from 24 to 32°C, and from 32 to 24°C. In chamber 2, the time at 24°C as well as at 32°C was 9 h, with a period of 3 h to go from one temperature to the other. In the third chamber, the periods at 24 and 32°C were, respectively, 3 and 12 h, with two 4.5-h periods in between. Leaf samples were collected individually from each '*Ca. L. americanus*'- or '*Ca. L. asiaticus*'-inoculated 'Valencia' sweet orange tree as well as from the uninoculated control trees at three different times: when the trees were transferred to the chambers, and 60 and 90 days later.

Temperature affected similarly the development of infected trees and uninoculated control trees. The greater the number of hours at 32°C, the longer the newly developed shoots (results not shown). Pronounced blotchy mottle and strong yellowing were present only on leaves of '*Ca. L. americanus*'-affected trees maintained for 1 or 9 h per day at 32°C (chambers 1 and 2) but not on trees maintained for 12 h at 32°C (chamber 3). The leaves of '*Ca. L. asiaticus*'-affected trees exhibited less pronounced blotchy mottle and less intense yellowing than those observed on '*Ca. L. americanus*'-infected leaves. qPCR revealed that the highest liberibacter titers, or cells per gram of fresh leaf midrib, were

Fig. 1. Log of liberibacter titers (cells per gram of fresh leaf midrib as determined by quantitative real-time polymerase chain reaction [qPCR]) from '*Candidatus Liberibacter americanus*'- or '*Ca. L. asiaticus*'-infected sweet orange trees exposed for 60 and 90 days at 32°C for 1, 9, or 12 h, and at 24°C for 15, 9, or 3 h per day (see Table 1).

close to 10^6 for '*Ca. L. americanus*'-infected trees and 10^7 for '*Ca. L. asiaticus*'-infected ones (Fig. 1). The liberibacter titers in '*Ca. L. asiaticus*'-infected trees were only slightly affected by the number of daily hours at 32°C: 1, 9, or 12 h. In contrast, the liberibacter titers of '*Ca. L. americanus*'-infected trees decreased significantly from $\sim 10^6$ to $\sim 10^4$ cells per gram of leaf midrib when the trees were exposed during 90 days at 32°C for 9 h per day (Fig. 1). When the trees stayed even longer at 32°C, namely 12 h per day, during 60 or 90 days, the liberibacters became barely detectable (Fig. 1). However, when the daily exposure to 32°C was only for 1 h, the liberibacter titer did not decrease statistically during 60 or 90 days.

Experiment IV. In experiment 4, the three chambers were programmed for a fixed daily period of 12 h at 24°C (known from the above experiments to be favorable to both liberibacters) and an additional daily period of 6 h at 32, 35, and 38°C, respectively, in chambers 1, 2, and 3. Samples were collected and analyzed by qPCR 60 and 90 days after transferring the plants to the chambers. As shown in Figure 2, in the case of '*Ca. L. americanus*'-infected trees, the liberibacters could not be detected or had very low titers in the trees held at 35 and 38°C. Even the liberibacters in '*Ca. L. asiaticus*'-infected trees were affected in the 38°C chamber since their titers in these trees varied between $\sim 10^2$ and 10^4 , while in the two other chambers they were between 10^6 and 10^7 cells per gram of leaf midrib. In the experiment shown in Figure 2, where the infected trees were kept for 6 h at 32°C, the '*Ca. L. asiaticus*'-infected trees had liberibacter titers close to 10^7 , while the '*Ca. L. americanus*'-infected trees had very much lower titers, close to 10^3 or 10^4 cells per gram of leaf midrib, suggesting that 6 h a day at 32°C for 60 or 90 days was detrimental to '*Ca. L. americanus*', but not a 1-h daily period, as observed in experiment III (Fig. 1).

DISCUSSION

The results from this work clearly show that '*Ca. L. asiaticus*' is more heat tolerant than '*Ca. L. americanus*'. The titer of '*Ca. L. asiaticus*' in sweet orange trees, as revealed by qPCR, was not affected by a daily exposure of the trees at 32°C for 90 days, while under the same conditions, '*Ca. L. americanus*' could not be detected or was detected only at very low titers. Even a daily temperature of only 9 h at 32°C decreased the titer of '*Ca. L. americanus*', and the decrease was noticeable after 60 days. As shown in Figure 2 for 'Natal' as well as 'Valencia' sweet orange trees, the highest temperature tested at which '*Ca. L. asiaticus*' was not affected was 35°C, but at 38°C, a drastic reduction of '*Ca. L. asiaticus*' titers was observed. At 35 and 38°C, '*Ca. L. americanus*' was

present only at very low titers or could not be detected. While 35°C seemed to be close to the highest temperature at which '*Ca. L. asiaticus*' was not affected, a similar temperature for '*Ca. L. americanus*' has not yet been determined. In the experiments reported here, '*Ca. L. americanus*' was not affected by a temperature of 24°C, and the titers at time 0 and those after 60 or 90 days were similar, i.e., 5.45 versus 5.58 log of liberibacter cells per gram of fresh leaf midribs for the field-grown trees used in experiment I and 6.14 versus 5.99 log of liberibacter cells per gram of fresh leaf midribs for the greenhouse-grown trees in experiment II. Temperatures between 24 and 32°C have not been tested yet.

As a whole, these results suggest that multiplication of '*Ca. L. asiaticus*' in sweet orange trees is not affected by temperatures up to 35°C, but at 38°C growth of '*Ca. L. asiaticus*' is drastically reduced. Multiplication of '*Ca. L. americanus*' is

already severely affected at 32°C. Furthermore, after 60 days at 27/32°C, most '*Ca. L. americanus*'-infected trees of experiment I had no HLB symptoms and were PCR-negative, and they remained so for 2 years under cool greenhouse conditions, at which time the trees from the 22/24°C chamber died of HLB or had strong HLB symptoms, indicating that the 27/32°C treatment had eliminated '*Ca. L. americanus*', but not '*Ca. L. asiaticus*'. '*Ca. L. americanus*' is thus more heat sensitive than '*Ca. L. asiaticus*'.

It has been known since 1974 that the HLB-associated bacteria from India and the Philippines, known today to be isolates of '*Ca. L. asiaticus*', were more heat tolerant than those from South Africa, now known to be isolates of '*Ca. L. africanus*' (3). '*Ca. L. africanus*'-infected sweet orange trees developed strong blotchy mottle symptoms at a daily temperature cycle of 22°C for 8 h in the dark and 24°C for 16 h in the light ("cool" conditions), and the

Fig. 2. Log of liberibacter titers (cells per gram of fresh leaf midribs) from '*Candidatus Liberibacter americanus*'- or '*Ca. L. asiaticus*'-infected 'Natal' (Nat) and 'Valencia' (Val) sweet orange trees exposed for 60 or 90 days for 6 h a day at 32, 35, or 38°C, and for 9 h a day at 24°C (see Table 1).

HLB agent ('*Ca. L. africanus*') could be detected by electron microscopy. However, at 27°C for 8 h in the dark and 32°C for 16 h in the light ("warm" conditions), no HLB symptoms developed, and electron microscopy was negative. In the case of '*Ca. L. asiaticus*', HLB symptoms were obtained under both warm and cool conditions, and electron microscopy was positive. Therefore, '*Ca. L. asiaticus*' became known as "heat tolerant" and '*Ca. L. africanus*' as "heat sensitive", and the heat sensitivity of '*Ca. L. africanus*' was one of the two factors which explained why in Africa, Madagascar, Reunion Island, and other regions, African HLB occurred only in cool, elevated areas, not at sea level, except at the southern tip of Africa, in the Cape Town region, where latitude compensates for altitude. The second factor involved in the distribution of HLB in the above regions is the heat sensitivity of *Trioza erytreae*, the African psyllid vector of HLB, and even more so the simultaneous occurrence of high temperatures and low humidity, which produce lethal conditions for the African psyllid (4).

From the work reported here, it appears that '*Ca. L. americanus*', like '*Ca. L. africanus*', is a heat-sensitive liberibacter, while '*Ca. L. asiaticus*', not only in Asia but also in America, is a heat-tolerant liberibacter not affected by 35°C. Recently, a new Liberibacter species has been discovered in potato, tomato, and capsicum (Lia Liefteing, New Zealand, *personal communication*). It will be interesting to see the behavior of the new species toward temperature.

It has been observed in SPS that the characteristic blotchy mottle leaf symptoms are much better expressed in the fall and winter months, from March to September in the Southern Hemisphere, than in spring and summer. The same also seems to be true in Florida in the Northern Hemisphere, where the period favorable for HLB symptom expression is from September to March. In SPS, the fall and winter daily temperatures stay most of the time below 32°C, and are thus favorable for liberibacter multiplication and symptom expression. Indeed, it is well known that strong HLB fruit and leaf symptoms are associated with high liberibacter titers (16). In SPS, HLB control involves identification and elimination of symptomatic HLB-infected trees, and the fall/winter months are well suited for symptomatic tree identification.

When, in 2004, HLB was first reported in SPS, '*Ca. L. americanus*' was the major Liberibacter species, as more than 95% of symptomatic HLB trees tested were infected with it. In 2008, the situation has

reversed, and most symptomatic trees tested are found to be infected with '*Ca. L. asiaticus*'. This evolution might be related to the fact that the concentration of '*Ca. L. asiaticus*' in sweet orange trees is higher than that of '*Ca. L. americanus*' (this work; 8; S. A. Lopes, *unpublished*), a situation that might favor transmission of '*Ca. L. asiaticus*' rather than '*Ca. L. americanus*' by the Asian HLB psyllid vector.

Also, in SPS, an uneven distribution of HLB exists. From the Araraquara region, the initial focus of the disease, HLB has moved longer distances to the south than to the north/northwest, despite the widespread presence of the psyllid vector and the many citrus farms, which provide a spatial continuum of adequate psyllid hosts and sources of inoculum (10). The south is characterized by lower air temperatures than the north/northwest. Data available for 2004, when HLB broke out in Araraquara, show Araraquara, in the center of SPS, Tatuí, in the south, and Jales, in the northwest, to have TH30 values, i.e., total number of hours above 30°C per year, respectively, of 529, 253, and 1,103. Four years later, in 2008, HLB had increased in the Araraquara region, was now present in Tatuí, but not yet at Jales. The heat sensitivity of '*Ca. L. americanus*', affected by temperatures of 32°C and above, but also of '*Ca. L. asiaticus*', affected by temperatures of 38°C and above, might explain the absence of HLB in the hot northwest region of SPS.

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