Eur J Plant Pathol (2010) 126:53–60
DOI 10.1007/s10658-009-9523-2

In planta multiplication and graft transmission of '*Candidatus* Liberibacter asiaticus' revealed by Real-Time PCR

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Received: 12 December 2008 / Accepted: 4 August 2009 / Published online: 3 September 2009
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Abstract The bacterium *Candidatus* Liberibacter asiaticus (CLas) is associated with huanglongbing (HLB) in citrus in many countries. Despite the fact that many characteristics of the disease are known, the rate of multiplication of the bacterium within an infected tree is still poorly understood. To study this feature, we used the quantitative Real-Time polymerase chain reaction (Q-PCR) assay to follow and to quantify the multiplication of CLas in grafted infected young sweet orange plants. The rate of infection by grafting reached 100% at 120 days post-inoculation (dpi) showing that grafting could easily transmit CLas. A well-adjusted linear regression equation describing the bacterial growth *in planta* was obtained independently with measurements taken using repeated sampling in the same plant or different plants through the analysed period. The bacterial population, measured as copy number (CN) of the 16S rDNA target gene g^{-1} of tissue, increased 10,000 times from 10^3 at 30 dpi to approximately 10^8 CN at 240 dpi indicating that CLas multiplication was fastest in young citrus plants. We observed a direct relationship between the concentration of pathogen and the expression of symptoms. Yellowed leaves or shoots, are commonly the first observed symptom of HLB, and were present in trees with a low amount of bacteria (10^5 CN g^{-1}). Blotchy mottle symptoms were observed in trees with 10^7 CN g^{-1} of bacteria after 180 dpi. Buds taken from infected, but non-symptomatic branches were grafted on Rangpur lime and resulted in transmission rates ranging from 10 to 60%.

Keywords Huanglongbing · Greening · Citrus · Bacteria · *In vivo* quantification

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Introduction

Huanglongbing (HLB) is a devastating disease that affects all citrus varieties (Halbert and Manjunath 2004), causing significant economic losses (Gottwald et al. 2007). The disease is caused by a Gram-negative and phloem-inhabiting bacterium named *Candidatus* Liberibacter spp. (Garnier et al. 1984; Jagoueix et al. 1994), that was only recently cultivated (Sechler et al. 2009). Three different species of Liberibacter are associated with HLB: the *Ca*. L. africanus is present mainly on the African continent (Planet et al. 1995), the more widely spread *Ca*. L.

asiaticus (CLas), which is present on the Asian and American continents (Jagoueix et al. 1996; Coletta-Filho et al. 2005), and the recently reported *Ca*. L. americanus (Teixeira et al. 2005), which is associated to HLB disease plants in Brazil. All these HLB-associated species of Liberibacter are transmitted from infected to healthy plants by citrus psyllids (*Diaphorina citri* or *Trioza eritraea*) as well as by propagation of infected citrus tissues (Bové 2006). Therefore, the management of diseased orchards is achieved by the elimination of affected trees as soon as possible and control of the vector population to reduce the source of inoculum, as well as the use of healthy nursery trees for new plantings. The presence of a flying and widespread vector makes HLB difficult and expensive to control. In addition, the lag time between transmission of the pathogen and symptom expression is quite variable but usually more than three to four months after grafting (Lopes and Frare 2008), or eight months after psyllid feeding (Hung et al. 2001). The technique of transmitting HLB from diseased to healthy plants using infected tissues has been known for a long time. It has been shown that experimental conditions, such as temperature, the condition of the plant tissue used for grafting, and the species of Liberibacter have significant influences on disease transmission (McClean 1970; Schwarz 1970; Lopes and Frare 2008). Nonetheless, the temporal multiplication of Liberibacter within the host is poorly understood as well as its passage to the progeny by grafting buds taken from infected but non-symptomatic citrus branches.

Several Real-Time quantitative PCR (Q-PCR) assays have been designed to estimate the Liberibacter titers within the plant. Li et al. (2006) reported a Q-PCR assay capable of detecting and quantifying the three species of Liberibacter (africanus, asiaticus, and americanus). Wang et al. (2006) reported a Q-PCR assay detecting CLas and a method for estimating the copy number of a cloned Liberibacter gene as an indirect measure of bacterial populations in plants. We have also previously developed a Q-PCR assay based on the TaqMan system and the 16S rDNA genomic sequences of CLas as a template (Carlos et al. 2006).

Even though the transmission of CLas by budwood has been known for a long time, few attempts were made to estimate the bacteria growing within the plant after infection by grafting. The present work was undertaken to study the increase in number of CLas over time using the Q-PCR methodology in sweet orange plants (*Citrus sinensis*) inoculated by grafting, and to verify if the bacterium could be propagated to new trees by using budwood taken from infected but non-diseased citrus branches. Two experiments were conducted independently at distinct times (Spring and Autumn) and using different strategies (measuring bacterial growth in the same plant or in different plants within the studied time) to examine CLas growth in plants. A third experiment was conduced to test bacterial transmission to new trees using budwood infected with different amounts of bacteria. We used our published Q-PCR protocol (Carlos et al. 2006) for CLas detection and an adaptation of the Wang et al. (2006) method for population estimation.

Materials and methods

Source of inoculum and plant material

All infected budwood used to inoculate the experimental plants was taken from one 2 year-old sweet orange plants with typical symptoms of HLB maintained in the greenhouse as the only source of CLas for the experiments. The experimental plants used in the graft transmission experiments described below were healthy 8 month-old Valencia sweet orange (*C. sinensis*) plants on Rangpur lime (*C. limonia*) rootstock. For the CLas infection the plants were inoculated almost 5 cm above the grafting line, with two infected pieces of budwood (2 to 3 cm long), grafted onto the opposite site of the mainly trunk region.

All the experimental plants were grown in 4 l plastic bags containing Plantmax Citrus substrate (Eucatex) and maintained under greenhouse conditions with temperatures not exceeding 30ºC during all experiments. The plants were automatically irrigated every day and fertilised when necessary.

Graft transmission

The multiplication of CLas in the plants was estimated at 30, 60, 90, 120, 180, and 240 days post-inoculation (dpi) by using two strategically different experiments. In the first experiment, named ‘Autumn experiment’, the bacterial growth was estimated in the same plant over the period of

sampling, from April to December, by inoculating five trees. In the second experiment, named 'Spring experiment', the bacterial growth was estimated in different plants over the period of sampling, from September to May. For this experiment 30 trees were grafted at the same time and randomly separated into six batches (five trees/batch). Each batch was analysed once throughout the experiment, i.e., the batch analysed 30 days post-infection was not analysed again. The same applied for batches analysed between 60 and 240 days post-infection. However, in the latter experiment the bacterial growth was estimated during the period in different plants, contrary to the Spring experiment.

Graft perpetuation

To estimate if the population of bacteria present in the plants used as source buds was correlated with the transmission to the young nursery trees we conduced a third experiment where 10 pieces of budwood were taken from the infected trees in each of four non-symptomatic batches (batches 1 to 4) in the Spring experiment at the time of each regular sampling, and propagated on Rangpur lime seedlings. The presence of the pathogen in the propagated progeny was analysed once when the young nursery trees were approximately three to four months old.

Sampling and DNA extraction

Four leaves were collected from the canopy (two from the base and two from the top), pooled, and used for DNA extraction. The total DNA was extracted from 250 mg (fresh weight) of midribs and petioles according to the CTAB method of Murray and Thompson (1980). DNA was eluted in 100 µl of elution buffer (0.1 vol of Tris-EDTA, 20 µg µl^{-1} of RNAse), and the concentration and quality were analysed with gel electrophoresis using the known molecular weight standard of Lambda DNA (Invitrogen Corporation, Carlsbad, CA). All DNA were standardised to a concentration of around 10 ng µl^{-1}.

Q-PCR assay

The primers and probes used for the quantitative Real-Time PCR assay were designed by Carlos et al. (2006) using the Primer Express software (version 2.0; Applied Biosystems, Foster City, CA, U.S.A.) and were synthesised by the same company. The primer sequences (AS84F, 5'-TCACCGG CAGTCCCTATAAAAGT-3' and As180R, 5'- GGGTTAAGTCCCGCAACGA-3') and the probe (As111T, 5'-ACATCTAGGTAAAAACC-3') were based on the 16S rDNA sequence of CLas (GenBank AY919311). The optimised Q-PCR assay required 0.8 µM of each of the forward and reverse primers and 0.2 µM of the probe for CLas, 1X TaqMan fast universal Master Mix (Applied Biosystems), 4 µl of standardised DNA template (10 ng µl^{-1}), 1 µl of Eukaryotic 18S rRNA kit (Applied Biosystems) as an internal control for normalisation of the amount of total DNA in each reaction and autoclaved Milli-Q water to a final volume of 20 µl. The standard amplification protocol was 2 min at 50ºC and 10 min at 95ºC followed by 40 cycles of amplification (15 s at 95ºC and 1 min at 60ºC). The amplification, data acquisition, and data analysis were done with the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems) using the sequence detection software (version 1.4). Each run comprised of three replicates for the tested DNA, the HLB-negative and positive controls, and the non-template control (NTC). All templates were automatically dispensed by the Eppendorf ® ep*Motion* ™ 5070 workstation (Eppendorf, Ontario, Canada).

Standard curve

Total DNA from a HLB-affected sweet orange plant was used to amplify the CLas target with the primer pair OI1 and OI2c (Hocquellet et al. 1999). The amplicon (≈ 1200 bp) was inserted in a pGEM-T recombinant plasmid of 4.2 Kbp (Promega Corporation, Madison, Wisconsin, USA) and cloned into competent *E. coli* strain DH5α. The plasmid containing the insert was purified following the alkaline lysis method and subsequently sequenced to ensure that the amplicon content was indeed CLas 16S rDNA. The amount of DNA obtained from the lysis was estimated using electrophoresis, with known concentrations of standard fragments of the vector pGEM® 3Zf (+) (supplied by Applied Biosystems) as a control. The quantification of CLas based on copy number (CN) of the target gene was done as described by Wang et al. (2006). The plasmid solution was

serially diluted ten-fold from 10 ngμl^{-1} (2.1×10^9 CN) to 0.01 fgμl^{-1} (7×10^1 CN), and the Q-PCR assay was run by adding at each dilution point 100 ng of total DNA from healthy citrus plants. These standard dilutions were used to establish the calibration curve by plotting the Ct (y axis) against the $\log_{10}$ of the CN of the 16S rRNA gene (x axis).

Estimation of CLas concentration in plants

The quantification of the copy number (CN) of the CLas-16S rRNA gene (not bacterial number) present in the samples was estimated using the regression equation shown. To estimate the CN of the CLas 16S rDNA target gene g^{-1} of tissue (CN g^{-1} tissue) the estimated CN was multiplied by 100. This factor compensates for the use of 0.25 g in the extraction, and 25% of the extract obtained. Although recently Kim and Wang (2009) determined three sets of ribosomal genes in CLas our data were not corrected against the bacterial concentration obtained for the already published papers (Li et al. 2006, 2007, 2008; Manjunath et al. 2008; Tatineni et al. 2008), which assumed one copy of 16S rRNA gene in the CLas genome.

Results

The TaqMan based Q-PCR system

The quantification of CLas in the target samples was performed as an indirect measure of CN of the 16S rDNA insert. A linear relationship was obtained between the inverse magnitude of the Ct values and the amount of 16S rDNA copy input, resulting in a regression equation of [$y=-2.6131x+\ln$ (16S rDNA copy number)+40.352, $R^2=0.9909$]. Under the experimental conditions tested, the quantification of 16S rDNA CN of CLas was shown to be linear from almost 10^2 to 10^9 CN per amplification tube. The reproducibility of the standard curve was tested by running two independent assays using three dilution points. The coefficient of variation (CV) ranged from 0.40 to 1.28% within the triplicate reaction assays and from 0.17 to 1.53% between the two assays, thus confirming that the Q-PCR based on TaqMan methodology for CLas is highly reproducible.

Infection by grafting and CLas multiplication

The multiplication and the concentration of CLas in the artificially inoculated plants were estimated in two independent experiments (Autumn and Spring experiments). A high efficiency of CLas transmission by grafting was observed. After 30 dpi, two out of five plants from both experiments were positive for the bacteria and at 120 days, all the inoculated plants were infected (Table 1). The *in vivo* multiplication of CLas was estimated using only the samples from infected plants of both experiments. Three measurements were carried out for each DNA sample. A low coefficient of variation was obtained for each replicate of the samples from both experiments (maximum value of 0.24%), indicating high reproducibility of the experimental results. On the other hand, values obtained for different samples (using different plants for biological replication) yielded more variable Ct values (ranging 1.49 to 9.08%; data not shown). For both experiments the bacterial population (y) increased over the analysed period (x) as described by the linear regression equations; $y=0.0255\ x+3.331$, $r^2=0.92$ (Autumn experiment) and $y=0.0218x+3.1085$, $r^2=0.86$ (Spring experiment), therefore suggesting a temporal accumulation of CLas in citrus plants, with the maximum concentration of CLas for both experiment peaking at 240 dpi, with no difference in bacterial concentration between them.

The average of Ct values and the corresponding population of CLas are shown in Table 2. During the first 90 days relatively constant values ($\approx10^3$ to 10^4 CN of 16S rDNA amplicon g^{-1} of tissue) were

Table 1 Efficiency of transmission of *Ca*. Liberibacter asiaticus by grafting determined by quantitative Real-Time PCR

Experiment[a]	Days post-inoculation – DPI					
	30	60	90	120	180	240
Autumn	2/5[b]	3/5	4/5	5/5	5/5	5/5
Spring	2/5	2/5	2/5	5/5	5/5	5/5

[a] Autumn: five plants inoculated and the same plants analysed during the whole experiment. Spring: thirty plants inoculated and a set of five plants analysed on each of six dates of sampling

[b] Number of infected plants over the number of plants inoculated

Table 2 DNA amplification by quantitative Real-Time PCR and copy number of *Ca*. Liberibacter asiaticus 16S rDNA target gene from infected sweet orange plants[a]

Days post-inoculation	Autumn experiment [b]			Spring experiment		
	Ct, CLas	Ct, plant	16S rDNA CN g^{-1c}	Ct, CLas	Ct, plant	16S rDNA CN g^{-1}
30	29.21 a	14.35 a	1.8×10^3	28.53 ab	14.59 a	3.3×10^3
60	27.74 ab	14.56 a	6.7×10^3	29.94 a	14.39 a	1.0×10^3
90	26.94 b	14.11 a	1.3×10^4	28.48 ab	14.46 a	3.5×10^3
120	22.36 c	14.59 a	6.05×10^5	24.34 b	14.52 a	1.3×10^5
180	17.65 d	14.56 a	4.8×10^7	23.48 b	14.48 a	4.8×10^5
240	17.32 d	14.50 a	7.0×10^7	16.44 c	14.45 a	9.4×10^7

[a] Values of Ct, CLas were only from infected plants. Threshold cycles (Ct) for *Ca*. L. asiaticus and internal plant control were obtained using primers and probes for the CLas 16S rDNA target gene and for the 18S internal control from Applied Biosystems, respectively

[b] Letters on the right correspond to statistical analysis of intermeasures of Ct values. Means followed by the same letter in vertical position are not significantly different ($\alpha=0.05$)

[c] Copy number of *Ca*. Liberibacter asiaticus 16S rDNA target g^{-1} of tissue. These values were estimated using the equation from the standard curve and the values were corrected to g of tissues (see Materials and methods)

observed for the samples from both experiments, indicating that the CLas concentration remained almost constant during that time. At 120 dpi, the concentration of the pathogen increased to $\approx10^5$ CN g^{-1} of tissue, and reached the maximum concentration of $\approx10^7$ CN g^{-1} of tissue at 180 (Autumn experiment) or 240 (Spring experiment) dpi. The typical asymmetrical chlorosis (blotchy mottle), a symptom of HLB leaves, was first observed at 180 days on experimental plants of the Autumn experiment and at 240 days on the Spring experimental plants. It is interesting that once blotchy mottle symptoms had started, no significant change in the bacterial concentration was observed for samples analysed at 180 and 240 days for the Autumn experiment.

The internal control 18S rDNA used for normalisation of the amount of total DNA in the reaction yielded Ct values quite similar for all samples (average of 14.57), with no significant difference among the samples. That was strong evidence that a similar amount of DNA was loaded in each reaction (Table 2).

CLas propagation

A third experiment was conducted to verify if CLas could be transmitted to the progeny by the use of budwood taken from non-symptomatic but CLas-infected tissues. Budwood taken from the infected plants from the Spring experiment described above was grafted on Rangpur lime seedlings using the commercial method of budding. The budwood taken from plants infected with the lowest CLas population ($\approx 10^3$ CN mg^{-1} of tissue, i.e. plants from batches 1, 2, and 3) transmitted the bacteria to the progeny at efficiencies ranging from 10 to 30%. At 120 dpi, infection reached 60% for progeny obtained from budwood taken from plants infected with about 10^5 CN of CLas 16S rDNA g^{-1} of tissue (batch 4). Although the Ct values were quite variable among the four progenies and among trees within each progeny (Table 3), an increase in CLas concentration was observed in the progenies from budwood taken from trees infected with the highest concentration of CLas (batch 4, 10^5 CN).

Discussion

Knowledge on how fast CLas multiplication occurs in infected trees, and if bacteria can be transmitted to new citrus trees by buds taken from infected but non-symptomatic branches is important for the management of HLB. This information can be useful to legally endorse the well-accepted management of HLB, which is based in Brazil on the required use of healthy budwood to produce new citrus trees and the importance of decreasing the source of inoculum as much as possible by removing HLB-infected trees and controlling the psyllids. The present study was

Table 3 *Ca*. Liberibacter asiaticus propagation on young plants (progeny) grown from infected buds as determined by Real-Time quantitative PCR

Source of buds (plants from Spring experiment)	CLas propagation in the progeny						
	No. infected plants[b]	Ct value per plant					
Batch 1–30 dpi[a]	1/10	34.48					
Batch 2–60 dpi	1/10	21.30					
Batch 3–90 dpi	3/10	32.32	26.84	24.56			
Batch 4–120 dpi	6/10	34.27	34.05	28.90	26.69	25.36	22.72

[a] Ten buds were randomly taken from infected plants at the specified period (days post-inoculation, dpi) and propagated on Rangpur lime seedlings. The estimated concentration of Liberibacter in those plants can be seen in Table 2 (Spring experiment)

[b] Number of infected young plants in the 10 plants propagated by grafting obtained by propagating the buds from the infected plant

conducted to estimate the multiplication of CLas in artificially infected material as well as the spread of bacteria with propagation by budwood.

We quantified the predicted target from samples in a serial dilution series and we found a strong linear relationship between the log of the 16S rDNA copy number and the Ct value with highly reproducible results. The linearity of the quantification process ranged from 3.0×10^3 CN of the 16S rDNA insert (corresponding to 8.8 fgμl^{-1} of plasmid) to 3.0×10^9 CN (8 ngμl^{-1}), which was quite similar to the minimal value of the CLas concentration amplified by the TaqMan Real-Time PCR system (Wang et al. 2006), 4.39 fgμl^{-1} (1.30×10^3 CN μl^{-1}). For a correct quantification of the infection level among the trees it is necessary to normalise the input of total DNA into reactions. In the present paper, Ct values from 18S rDNA, also used by Christensen et al. (2004), showed a normalised input of total DNA in reactions.

One hundred percent of the trees were infected by CLas at 120 dpi as well some plants that were infected early in both Autumn and Spring experiments, an observation that contradicts the rates of infection obtained for *Ca*. L. africanus (50 to 65%) (McClean 1970; van Vuuren 1993) and for *Ca*. L. americanus (from 10 to 65%) (Lopes and Frare 2008; Lopes et al. 2009). It has been proposed that the time of the year when the experiments are conducted and the plant tissue used as the bacterial source can have a great influence on the efficiency of transmission (Schwarz 1970). Here, the Autumn and Spring experiments were conduced in a greenhouse with a controlled maximum temperature of 30°C and the same source of infected budwood was used for both

Fig. 1 Foliar symptoms of young Valencia sweet orange trees infected by *Candidatus* Liberibacter asiaticus (CLas). **a** The first leaf yellowing observed in plants infected with about 10^5 copy number (CN) of CLas 16S rDNA g^{-1} of tissue. **b** Blotching mottle in young shoots, first observed in plants infected with about 10^7 CN of 16S rDNA g^{-1} of tissue

experiments. However, the large difference in the rate of infection among these species of Liberibacter can be attributed to the easier transmission of the asiaticus species which can be a consequence of its systemic distribution *in planta* (Tatineni et al. 2008) and higher concentration in the infected tissues when compared to *Ca*. L. americanus (Lopes et al. 2009). Similar data for the infection of CLas by grafting were obtained by Stover et al. (2008), 90% efficiency with PCR-positive plants at 40 days after inoculation.

Although the growth phase of CLas cannot be determined *in planta* since bacteria in logarithmic growth phase can harbour multiple copies of their genome, the bacterial colony growth *in planta* for the Autumn and Spring experiments was a linear regression suggesting a temporal accumulation of bacteria in citrus tissue. However, the strategy of analysis of the bacterial growth using non-repetitive sampling in the same plant through time (Spring experiment) was considered adequate. This strategy could also be used for experiments where destructive sampling is necessary. During the first ninety days post-inoculation, no HLB-associated symptoms were observed in the infected trees with the highest values of Ct, corresponding to a population of almost 10^3 CN g^{-1} of tissue. The early symptoms of HLB are the appearance of yellow shoots in the infected trees (Bové 2006). These symptoms were first observed at 120 days post-inoculation in both experiments, with Ct values ranging from 22 to 24 and a population of about 10^5 CN mg^{-1} of tissue (Fig. 1a). The typical blotching mottle symptoms in the leaves (Bové 2006) were first observed at the time when CLas reached 10^7 CN g^{-1} of tissue at 180 to 240 dpi for the Autumn and Spring experiments, respectively (Fig. 1b). Similar Ct values (from 16 to 17) and a CLas concentration (10^7 CN g^{-1} of tissue) were observed by Li et al. (2007 and 2008) and Tatineni et al. (2008) in HLB symptomatic samples that had been naturally or artificially inoculated. Therefore, our estimate of the CLas population in symptomatic leaves of sweet orange plants inoculated by grafting seems consistent with published values.

A direct relationship between the concentration of bacteria present in the mother trees and CLas propagation through the progeny was observed with a high rate of newly infected citrus trees. Previous work has shown that both *Ca*. L. americanus and *Ca*. L. africanus were poorly transmitted by the progeny when using buds taken from symptomatic branches (2 and 4% respectively) (McClean 1970; Lopes and Frare 2008). In this study, we showed that CLas can be easily transmitted to new citrus tissues by grafting infected but asymptomatic buds.

In conclusion, using both the TaqMan-based Q-PCR system and the proposed strategy for estimating the concentration of the pathogen have provided us with a reasonable estimation of CLas in infected trees. Based on this work and using young plants we verified a strong multiplication of the Asian pathogen of above 10,000 times in a period of 210 days, with a linear relationship between the time required for symptom development and the pathogen concentration. A slower rate of bacterial growth is commonly observed in older plants in field conditions in Brazil (data not shown), but that is obviously not enough to prevent the spread of the disease. Therefore, we strongly reinforce the necessity of removing HLB-infected trees from orchards as early as possible as a rational management of HLB in infected areas. In addition, we showed that CLas can be easily propagated by buds taken from non-symptomatic but infected branches; this supports the ongoing programme in Brazil of using bud sources maintained under screen-house conditions and repeatedly tested for Liberibacter species.

Acknowledgements This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) project number 2005/00718-2. H.D. Coletta-Filho, M.A. Machado and A. A. de Souza are supported by the CNPq agency.

References

Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology, 88*, 7–37.

Carlos, E. F., Coletta-Filho, H. D., Targon, M. L. N. & Machado, M. A. (2006). Quantitative real-time PCR based on TAQMAN probes for the molecular detection of *Candidatus* Liberibacter asiaticus and *Ca*. L. americanus. In *Huanglongbing-Greening International Workshop* (p. 81), Ribeirão Preto, SP, Brazil, July 16–20, 2006 Proceedings.

Christensen, N. M., Nicolaisen, M., Hansen, M., & Schulz, A. (2004). Distribution of Phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions, 17*, 1175–1184.

Coletta-Filho, H. D., Takita, M. A., Targon, M. L. P. N., & Machado, M. A. (2005). Analysis of the 16S rDNA sequences from citrus-huanglongbing bacteria reveal a different "*Ca*. Liberibacter" strain associated to the citrus disease in Sao Paulo, Brazil. *Plant Disease, 89*, 848–852.

Garnier, M., Daniel, N. & Bové, J. M. (1984). The greening organism is a Gram-negative bacterium. (Paper presented at the *9th Conference International Organization Citrus Virologists*, University of California, Riverside, CA).

Gottwald, T. R., da Graça, J. V., & Bassanezi, R. B. (2007). Citrus Huanglongbing: the pathogen and its impact. *Plant Health Progress,* . doi:10.1094/PHP-2007-0906-01-RV.

Halbert, S. E., & Manjunath, K. L. (2004). Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease in citrus: a literature review and assessment of risk in Florida. *Florida Entomologist, 87*, 330–354.

Hocquellet, A., Toorawa, P., Bové, J. M., & Garnier, M. (1999). Detection and identification of the two *Candidatus* Liberobacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the beta operon. *Molecular and Cellular Probes, 13*, 373–379.

Hung, T. H., Wu, M. L., & Su, H. J. (2001). Identification of Chinese box orange (*Severinia buxifolia*) as an alternative host of the bacterium causing citrus Huanglongbing. *European Journal of Plant Pathology, 107*, 183–189.

Jagoueix, S., Bové, J. M., & Garnier, M. (1994). The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. *International Journal of Systematic Bacteriology, 44*, 379–386.

Jagoueix, S., Bové, J. M., & Garnier, M. (1996). PCR detection of the two '*Candidatus*' liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes, 10*, 43–50.

Kim, J., & Wang, N. (2009). Characterization of copy number of 16S rDNA and 16S rRNA of the *Candidatus* Liberibacter asiaticus and the implication in detection *in planta* using quantitative PCR. *BMC Research Notes, 2*, 37. doi:10.1186/1756-0500-2-37.

Li, W., Hartung, J. S., & Levy, L. (2006). Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *Journal of Microbiological Methods, 66*, 104–105.

Li, W., Hartung, J. S., & Levy, L. (2007). Evaluation of DNA amplification methods for improved detection of "*Candidatus* Liberibacter species" associated with citrus huanglongbing. *Plant Disease, 91*, 51–58.

Li, W., Li, D., Twieg, E., Hartung, J. S., & Levy, L. (2008). Optimized quantification of unculturable *Candidatus* Liberibacter spp. in host plants using real-time PCR. *Plant Disease, 92*, 854–861.

Lopes, S. A., Bertolini, G., Frare, G. F., Martins, E. C., Wulff, N. A., Teixeira, D. C., et al. (2009). Graft transmission efficiencies and multiplication of '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus' in citrus plants. *Phytopathology, 99*, 301–306.

Lopes, S. A., & Frare, G. F. (2008). Graft transmission and cultivar reaction of citrus to '*Candidatus* Liberibacter americanus'. *Plant Disease, 92*, 21–24.

Manjunath, K. L., Halbert, S. E., Ramadugu, C., Webb, S., & Lee, R. F. (2008). Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. *Phytopathology, 98*, 387–396.

McClean, A. P. D. (1970). Greening disease of sweet orange: its transmission in propagative parts and distribution in partially disease trees. *Phytophylactica, 2*, 263–268.

Murray, M., & Thompson, W. F. (1980). Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Research, 8*, 4321–4325.

Planet, P., Jagoueix, S., Bové, J. M., & Garnier, M. (1995). Detection and characterization of the African citrus greening liberibacter by amplification, cloning and sequencing of the rplKAJL-rpoBC operon. *Current Microbiology, 30*, 137–141.

Schwarz, R. E. (1970). Seasonal graft-transmissibility and quantification of gentisoyl glucoside marker of citrus greening in the bark of infected trees. *Phytophylactica, 2*, 115–120.

Sechler, A., Schuenzel, E. L., Cooke, P., Donnua, S., Thaveechai, N., Postnikova, E., et al. (2009). Cultivation of '*Candidatus* Liberibacter asiaticus', '*Ca*. L. africanus', and '*Ca*. L. americanus' associated with huanglongbing. *Phytopathology, 99*, 480–486.

Stover, E., Bowman, K., McCollum, G. & Niedz, R. (2008). Developing transgenic solutions for HLB resistant citrus at the US Horticulture Research Laboratory. Paper presented at the *International Research Conference on Huanglongbing*, Proceedings, Orlando Fl.

Tatineni, S., Sagara, U. S., Gowda, S., Robertson, C. J., Dawson, W. O., Iwanami, T., et al. (2008). *In planta* distribution of '*Candidatus* Liberibacter asiaticus' as revealed by polymerase chain reaction (PCR) and real-time PCR. *Phytopathology, 98*, 592–599.

Teixeira, D. C., Danet, J. L., Eveillard, S., Martins, E. C., Jesus Junior, W. C., Yamamoto, P. T., et al. (2005). Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the Candidatus Liberibacter species associated with the disease. *Molecular and Cellular Probes, 19*, 173–179.

Van Vuuren, S. P. (1993). Variable transmission of African greening to sweet orange. Paper presented at the *12th Conference International Organization Citrus Virologists*, University of California, Riverside, CA.

Wang, Z., Yin, Y., Hu, H., Yuan, Q., Peng, G., & Xia, Y. (2006). Development and application of molecular-based diagnosis for '*Candidatus* Liberibacter asiaticus', the causal pathogen of citrus huanglongbing. *Plant Pathology, 55*, 630–638.