

***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 10³ at 30 dpi to approximately 10⁸ 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 (10⁵ CN g⁻¹). Blotchy mottle symptoms were observed in trees with 10⁷ 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

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.*

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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 eritreae*) 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 conducted 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 30°C 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 conducted 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 50°C and 10 min at 95°C followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). 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® epMotion™ 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 $10 \text{ ng}\mu\text{l}^{-1}$ (2.1×10^9 CN) to $0.01 \text{ fg}\mu\text{l}^{-1}$ (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 [$y = -2.6131x + \ln(16S \text{ rDNA copy number}) + 40.352$, $R^2 = 0.9909$]. 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; $y = 0.0255x + 3.331$, $r^2 = 0.92$ (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 ($\approx 10^3$ 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 gene 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 $\approx 10^5$ CN g⁻¹ of tissue, and reached the maximum concentration of $\approx 10^7$ 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 $8.8 \text{ fg} \mu\text{l}^{-1}$ of plasmid) to 3.0×10^9 CN ($8 \text{ ng} \mu\text{l}^{-1}$), which was quite similar to the minimal value of the CLas concentration amplified by the TaqMan Real-Time PCR system (Wang et al. 2006), $4.39 \text{ fg} \mu\text{l}^{-1}$ ($1.30 \times 10^3 \text{ CN} \mu\text{l}^{-1}$). 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%) (McClellan 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 conducted 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) (McClellan 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.

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