Reaction of sweet orange cultivars expressing the *attacin* A gene to '*Candidatus* Liberibacter asiaticus' infection

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Abstract – The objective of this work was to evaluate the reaction of four sweet orange cultivars expressing the *attacin* A gene to '*Candidatus* Liberibacter asiaticus' (Las) infection, a bacterium associated to huanglongbing (HLB) disease. Transgenic sweet orange plants of Hamlin, Natal, Pêra, and Valência cultivars, as well as nontransgenic controls received inocula by grafting budwood sections of HLB-infected branches. Disease progression was evaluated through observations of leaf symptoms and by polymerase chain reaction (PCR) analysis, eight months after inoculation. A completely randomized design was used, with four experiments (one for each cultivar) performed simultaneously. Bacteria title was estimated by quantitative PCR (qPCR). HLB symptoms and Las titers were present in nontransgenic and transgenic plants expressing the *attacin* A gene of the four sweet orange cultivars, eight months after bacteria inoculation. Five transgenic lines (transformation events) of 'Pêra' sweet orange expressing the *attacin* A gene have significantly lower Las titers in comparison with nontransgenic plants of this cultivar.

Index terms: *Citrus sinensis*, antibacterial peptide, disease resistance, genetic transformation, huanglongbing.

Reação de cultivares de laranjeira doce que expressam o gene *atacina* A à infecção com '*Candidatus* Liberibacter asiaticus'

Resumo – O objetivo deste trabalho foi avaliar a reação de quatro cultivares de laranjeira doce que expressam o gene *atacina* A à infecção por '*Candidatus* Liberibacter asiaticus' (Las), bactéria associada à doença huanglongbing (HLB). Plantas transgênicas das cultivares Hamlin, Natal, Pêra e Valência, bem como controles não transgênicos, receberam inóculos, por enxertia, de seções de ramos infectados por HLB. A progressão da doença foi avaliada pela observação de sintomas foliares e por análise da reação em cadeia da polimerase (PCR), oito meses após a inoculação. Utilizou-se o delineamento inteiramente casualizado, com quatro experimentos (um para cada cultivar) realizados simultaneamente. A concentração bacteriana foi estimada por PCR quantitativo (qPCR). Sintomas de HLB e Las estavam presentes em plantas não transgênicas e em plantas transgênicas que expressaram o gene *atacina* A, nas quatro cultivares de laranjeira doce, oito meses após a inoculação. Cinco linhagens transgênicas (eventos de transformação) de laranjeira 'Pêra', que expressam o gene *atacina* A, apresentam concentrações de Las significativamente inferiores às das plantas não transgênicas desta cultivar.

Termos para indexação: *Citrus sinensis*, peptídeo antibacteriano, resistência a doenças, transformação genética, huanglongbing.

Introduction

Huanglongbing (HLB) is one of the most serious diseases affecting citrus cultivation. Although Koch's postulates have not yet been fulfilled, due to the constant association between symptoms and presence of '*Candidatus* Liberibacter spp.' in the phloem, these bacteria are believed to be the causal agent of the disease. Three following species are associated with HLB: '*Candidatus* Liberibacter africanus' (Laf), present mainly in the African continent; '*Candidatus* Liberibacter asiaticus' (Las), present in the Asian and American continents; and '*Candidatus* Liberibacter americanus' (Lam), reported only in Brazil (Bové, 2006; Teixeira et al., 2008). Of the three species, Las now predominates in Brazil (Lopes et al., 2009).

Despite reports of 'Liberibacter' cultivation in vitro (Sechler et al., 2009), only molecular techniques have been used for its detection in plants (Teixeira et al., 2008; Sechler et al., 2009). Las and Lam transmissions occur through the psyllid vector *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae) (Bové, 2006). Las transmission can also be achieved artificially by grafting, using branch segments or buds removed from infected plants (Lopes & Frare, 2008; Coletta-Filho et al., 2010).

Regardless of Liberibacter species, the management of HLB in commercial areas includes the planting of healthy nursery trees, the scouting and eradication of symptomatic trees, and the chemical control of insect vectors (Belasque Junior et al., 2009). Although these practices can reduce the spread of the disease if implemented jointly by different neighboring growers, they are costly, difficult for widespread adoption, and involve constant insecticide applications, which are harmful to the environment. Although genetic resistance is potentially the best management strategy, it has not been used against HLB due to a lack of resistant or immune genotypes among the commercial varieties (Gottwald et al., 2007). Nevertheless, genetic transformation may be a useful tool to confer disease resistance to susceptible cultivars. Moreover, this approach has been successfully applied to achieve disease resistance in other citrus-pathogen systems (Barbosa-Mendes et al., 2009; Borejsza-Wysocka et al., 2010; Cardoso et al., 2010; Mendes et al., 2010). Therefore, citrus genetic transformation can also be of great potential for HLB management.

The attacins belong to a class of peptides of approximately 20 kDa that are secreted in the hemolymph of *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), in response to bacterial infections. There are basic (A, B, C, and D) and acidic (E and F) forms of attacin, and both forms contain three hydrophobic portions in the amino-terminal region (Hultmark et al., 1983). Attacins generally act against Gram-negative bacteria by binding with lipopolysaccharides present in the outer membranes of these bacteria, altering both membrane structure and permeability, and by the direct inhibition of the synthesis of various membrane proteins, including OmpA, OmpC, and OmpF (Hultmark et al., 1983; Carlsson et al., 1991). The rapid and efficient action of antimicrobial peptides against pathogenic bacteria and their low toxicity to mammalian cells increase their potential use in medicine and agriculture (Wang et al., 2010).

Plants with genes that encode attacins have been proven to act against phytopathogenic bacteria. Apple trees containing the *attacin* E peptide exhibited resistance to *Erwinia amylovora* [(Burrill) Winslow et al.] (Borejsza-Wysocka et al., 2010); as well, the sweet orange cultivars Hamlin, Natal, Pêra, and Valência, which contain the *attacin* A gene, had increased resistance against *Xanthomonas citri* subsp. *citri* (Schaad et al.) and *Xylella fastidiosa* (Wells et al.) (Boscariol et al., 2006; Cardoso, 2008; Cardoso et al., 2010). These promising results obtained for sweet orange encourage this study.

The objective of this work was to evaluate the reaction of four sweet orange cultivars expressing the *attacin* A gene to '*Candidatus* Liberibacter asiaticus' (Las) infection, a bacteria associated to huanglongbing (HLB) disease.

Materials and Methods

The experiment was carried out from February 2008 through October 2009, in a screenhouse, at the Laboratório de Biotecnologia de Plantas Hortícolas, from Departamento de Produção Vegetal, at Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, São Paulo State, Brazil.

Transgenic 'Hamlin', 'Natal', 'Pêra', and 'Valência' sweet orange cultivars (*Citrus sinensis* [L.] Osbeck), which contain *attacin* A (*att*A) gene, were used in the experiments. The transgenic plants were obtained by transformation with *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The detailed description of these plants regarding gene construct has been described (Boscariol et al., 2006; Cardoso et al., 2010). Propagation of the four cultivars containing the *att*A gene and of the nontransgenic plants occurred by bud grafting onto 'Rangpur' lime rootstock plants (*Citrus limonia* Osbeck).

Las-infected buds were collected from branches of three-year-old plants of 'Valência' sweet orange grafted onto 'Rangpur' lime, grown in the field: plot 51B, at the Marchesan Farm, located in Matão, SP, Brazil. The presence of Las was confirmed by PCR. The branches were removed and transported to the greenhouse on the day of inoculation. Approximately 5 cm-long buds were grafted, in February 2009, onto the stems of the eight-month-old experimental plants (one bud per plant) according to protocols of Lopes & Frare (2008).

The buds were fixed to the stems with a plastic ribbon which was removed after 40 days.

Evaluations were done for sweet orange transgenic lines (12 of 'Hamlin' – H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, and H12; five of 'Natal' – N1, N4, N5, N8, and N9; six of 'Pêra' – P6, P7, P8, P10, P11, and P15; and 16 of 'Valência' – V1, V2, V3, V4, V7, V8, V10, V11, V12, V13, V15, V16, V17, V18, V19, and V20).

The experimental design was completely randomized. Four experiments (one for each cultivar) were performed simultaneously. Each experiment included a treatment with nontransgenic plants of the same cultivar. In addition, two transgenic plants from each treatment (transformation event) and two nontransgenic plants of the same cultivar did not receive inocula and were used as controls. Each transgenic plant (transgenic line or transgenic event) was considered a treatment with four replicates, except for H6 (3 replicates) and V11 (2 replicates).

The visual symptoms of HLB were assessed as early as four months after inoculation, and continued until eight months after inoculation (October 2009), when the experiment was concluded. Typical HLB and HLB-associated symptoms (Lopes & Frare, 2008; Lopes et al., 2009) were recorded. For a more precise comparison among plants with inocula, a photographic scale of the two main symptoms, namely leaf mottling (m) and manganese (Mn) deficiency, was prepared. Indices were recorded on a three-point scale, by which 1 was considered as mild; 2 as moderate; and 3 as severe (Figure 1). HLB-associated symptoms, such as

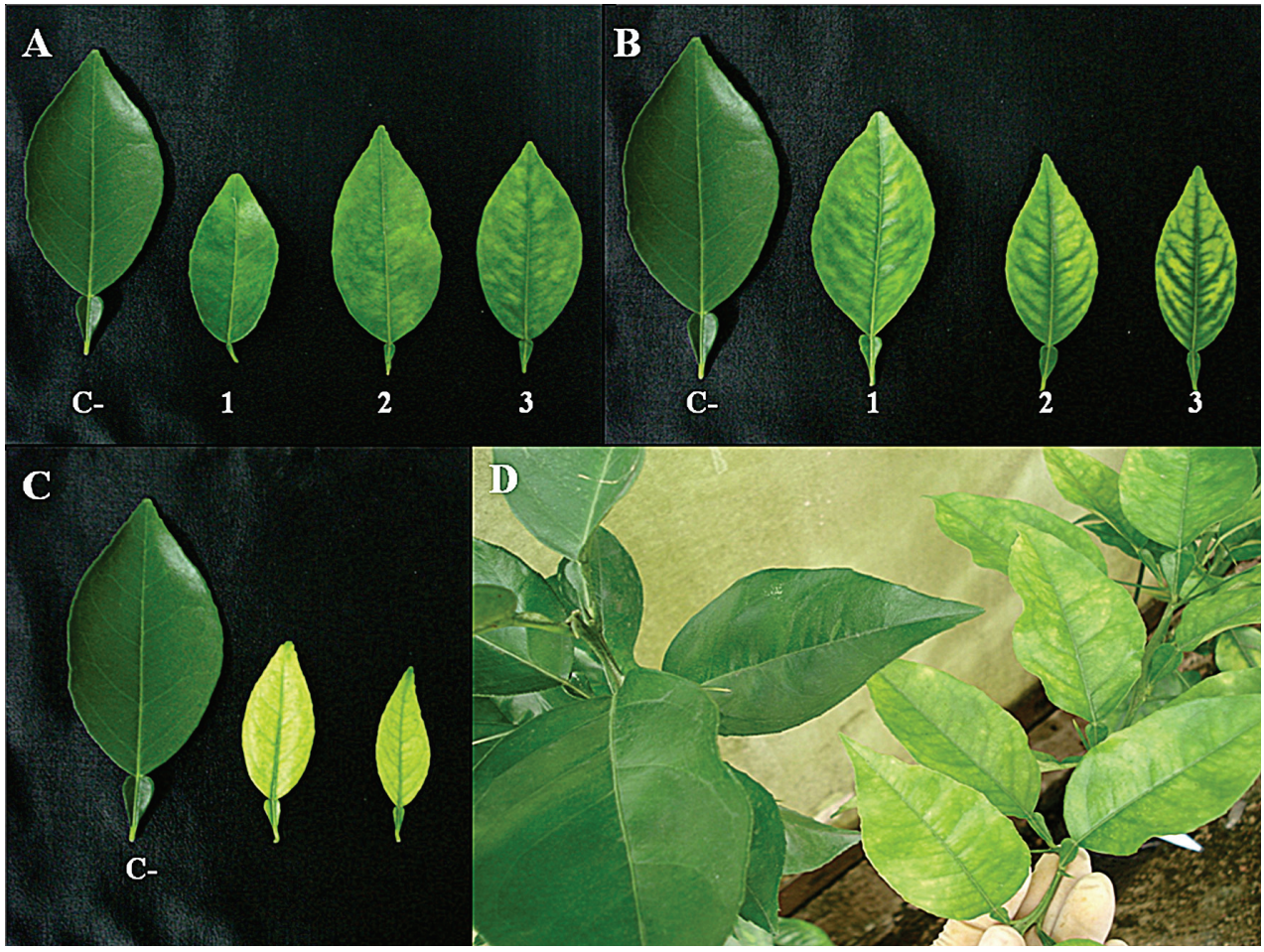

Figure 1. HLB and HLB-associated leaf symptoms observed in sweet orange plants eight months after inoculation with Las-infected buds. C-, control (without inoculum); A, mottling intensities 1, 2, and 3 (mild, moderate, and severe, respectively); B, intensity of manganese (Mn) deficiency 1, 2, and 3 (mild, moderate, and severe, respectively); C, chlorosis symptoms; D, comparison of HLB-infected (right) and healthy (left) plants.

chlorosis (Figure 1 C), iron (Fe) deficiency, and zinc (Zn) deficiency were also recorded.

The detection of Las was assessed by PCR, in samples composed by leaf petioles and midribs, from each plant collected eight months after inoculation. DNA extraction followed the method of Murray & Thompson (1980). Approximately 250 mg of a mixture of petioles and midribs of 3-5 symptomatic leaves (when present) were collected from the sub-apical region between the 2nd and 10th nodes. The samples were then cut into small pieces, macerated in liquid nitrogen with a mortar and pestle, and stored at -80°C until extraction.

For PCR analysis, the primers RPLA2: 5' – TAT AAA GGT TGA CCT TTC GAG TTT – 3' and RPLJ5: 5' – ACA AAA GCA GAA ATA GCA CGA ACA – 3' were used to amplify a Las fragment of 703 bp. The reactions were run in 25 µL of a solution containing 0.5 µmol L^{-1} of each primer, 200 µmol L^{-1} dNTP, 2.0 µmol L^{-1} $MgCl_2$, 2.5 µL of 10X buffer, 1.5 U of Taq DNA polymerase, 1 µL of DNA, and 17.2 µL of ultrapure water. Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc.) with the following cycle program: an initial cycle at 94°C for 30 s; 34 cycles with denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 1 min; and a final elongation cycle at 72°C for 1 min (Hocquellet et al., 1999). After amplification, 10 µL aliquots of each sample were analyzed by electrophoresis on a 1.0% agarose gel stained with ethidium bromide (0.03 µg mL^{-1}) in TAE buffer (1X) at 60 V voltage for at least one hour. Gel was then visualized and photographed using the EDAS 120 program (Kodak, Rochester, NY, USA).

Las populations (bacteria titers) in experimental plants were estimated by quantitative PCR (qPCR) eight months after inoculation. DNA samples used in the qPCR analysis were the same as those previously described for the PCR analysis. A spectrophotometer NanoDrop 1000, (Thermo Scientific, Wilmington, DE, USA) was used to quantify and verify DNA quality. DNA was then diluted to 100 ng μL^{-1} final concentration. The qPCR analysis was performed using a StepOne Plus thermocycler (Applied Biosystems, Carlsbad, CA, USA). The reaction was performed in a 25 µL volume containing 1.25 µL 2X SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.15 µmol L^{-1} of the primer f-*rpl*LAS: 5' – CGC CCG TTT CCG TTG – 3', 0.15 µmol L^{-1} of the primer *rpl*LAS: 5' – AGC CTC TTT AAG CCC TAA ATC AG – 3', 500 ng genomic DNA, and ultrapure water, to make up the remaining volume. For DNA amplification, the following program was used: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s followed by 63°C for 60 s, and 69 to 95°C for 20 s, to obtain the melting curve. Samples were analyzed in duplicate followed by calculations of means. Data were analyzed with StepOnePlus Real-Time PCR System software (Applied Biosystems, Carlsbad, CA, USA) (Teixeira et al., 2008). The cycle threshold (C_T) was used to identify the cycle at which the reaction reached the exponential phase threshold, which allowed for quantification based on the fluorescence of each sample.

To validate the detection limit of amplicon quantification, standard curves were obtained. The curves were derived from plasmids containing the target sequence. Following cloning, sequencing, and confirmation with Blast (basic local alignment search tool), plasmids containing amplicons were diluted to 10 ng μL^{-1} final concentration and were kept at 4°C until use. The standard curves were prepared by obtaining serial dilutions (ranging from 150,000 to 1.5 copies of the target amplicon) from the plasmid DNA solution, which had 10 ng μL^{-1} concentration (Teixeira et al., 2008). The results were calculated, and the values were expressed in Log_{10} (Liberibacter cells g^{-1} of leaf petiole and midribs).

Analysis of variance was used to test for differences in the Las concentrations, obtained by qPCR analysis, between plants. First, the Lilliefors and Levene's tests were used to check for normality and homogeneity of variances, respectively (Freud & Wilson, 2003). After verification, the F test at 5% of probability was applied for comparison of means. When the F test was significant, Dunnett's test at 5% of probability was applied to compare transgenic versus nontransgenic plants. Even after transformation, data from experiments with the 'Hamlin', 'Natal', and 'Valência' sweet orange cultivars did not meet assumptions of normality or homogeneity in variance. In those cases, the nonparametric test of Kruskal-Wallis at 5% of probability was used. However, no significant differences were confirmed. Thus, comparisons between transgenic and nontransgenic plants from 'Hamlin', 'Natal', and 'Valência' sweet orange cultivars

were performed using the deviations in the standard errors of the mean (±Ɛ). A t test at 5% of probability was applied to evaluate the differences in the Las concentrations among nontransgenic control plants from 'Hamlin', 'Natal', 'Pêra', and 'Valência' sweet orange cultivars.

Results and Discussion

Plant propagation of the transgenic lines and nontransgenic plants of 'Hamlin', 'Natal', 'Pêra', and 'Valência' sweet orange cultivars was successful. All plants containing the *att*A gene exhibited development and phenotypes similar to those observed for nontransgenic plants.

Eight months after inoculation, leaf symptoms related to HLB were recorded at different intensities in several plants. PCR analysis revealed that all plants with symptoms were positive for Las (Table 1). Moreover, two symptomless 'Hamlin' sweet orange plants (one H3 and one H4) were also positive for Las by PCR and qPCR analyses.

Detection of HLB symptoms or Las bacteria were not possible in few transgenic and nontransgenic plants, eight months after inoculation (Table 1). Further evaluations showed that Las inoculation failed in such plants, which were not further evaluated in this experiment. Nevertheless, the inoculation method used in this study can be considered highly efficient: 80% for 'Hamlin', 79% for 'Natal', 75% for 'Pêra', and 86% for 'Valência' sweet orange. Although current research on HLB evaluation suggests Las inoculation by the vector (*D. citri*) feeding, the method used in this experiment was considered the most appropriate at the time of the research was carried out (Lopes & Frare, 2008; Folimonova et al., 2009; Coletta-Filho et al., 2010).

After confirmation of Las presence by PCR, in all four sweet orange cultivars with HLB symptoms (transgenic and nontransgenic lines) (Table 1), bacteria population was accessed in all experimental plants by qPCR, eight months after inoculation. Transgenic and nontransgenic plants without inocula were used as controls. Results from qPCR analyses confirmed the infection rates found by PCR. These results indicate that the interval of eight months after inoculation seems to be an adequate period of time for Las incubation and distribution within the experimental plants. Similar research work also found that maximum concentration of Las can be reached by temporal accumulation of about 240 days after infection (Coletta Filho et al., 2010).

As data collected from 'Hamlin', 'Natal', and 'Valência' sweet oranges did not meet assumptions of normality or homogeneity in variance, comparisons between transgenic and nontransgenic plants were restricted for these cultivars. Nevertheless, bacterial titers and HLB symptoms were similar among transgenic and nontransgenic plants. However, Las titers were significantly higher in nontransgenic 'Pêra' sweet orange (C_T value = 23.19) as compared by t test to the nontransgenic plants from the other sweet orange cultivars, in which C_T values averaged as follows: 25.03 for 'Valência', 26.47 for 'Hamlin', and 31.14 for 'Natal'. The genetic background of each sweet orange cultivar may influence HLB resistance and Las distribution and titers within the plants, as HLB symptoms may vary among different citrus genotypes according to Folimonova et al. (2009).

In contrast, five evaluated transgenic lines of 'Pêra' sweet orange (P7, P8, P10, P11, and P15) had significantly lower Las titers, as estimated by qPCR; consequently, they had greater C_T results than nontransgenic plants (Figure 2). Moreover, Line P11 displayed a lower intensity of symptoms than nontransgenic plants (Table 1).

Attacin A, synthesized from the gene *att*A, is known to act against Gram-negative bacteria. *Attacin* A inhibits the synthesis of various outer membrane proteins and changes the structure and permeability of the outer membrane (Hultmark et al., 1983; Carlsson et al., 1991). We found that *attacin* A may have influenced the multiplication of Las bacteria responsible for citrus HLB in five transgenic lines of 'Pêra' sweet orange (P7, P8, P10, P11 and P15). However, apparently, no transgenic lines of 'Hamlin', 'Natal', and 'Valência' were found resistant to HLB, in comparison to the nontransgenic plants of the respective cultivars. The effect of the genetic background on disease resistance by transgenic approach, especially against *X. citri* subsp. *citri*, has been reported (Cardoso et al., 2010; Mendes et al., 2010).

Interestingly, the multiplication of other bacterial pathogens was also influenced in the same events used in the present study. Cardoso (2008) identified a reduced multiplication rate for *Xylella fastidiosa*,

Table 1. HLB and HLB-associated symptoms and detection of Las by PCR, in leaves of sweet orange cultivars 'Hamlin', 'Natal', 'Pêra', and 'Valência' with or without the *att*A gene. Plants were inoculated with infected buds, and measurements were taken eight months after inoculation.

Event	Number of inoculated plants	Symptoms[1]	Number of PCR-positive plants
		'Hamlin'	
Nontransgenic	4	3 (m3), 1 (m2), 3 (Mn3), 1 (Mn2)	4
H1	4	2 (-), 1 (m3), 1 (m2), 1 (Mn3), 1 (c)	2
H2	4	2 (-), 2 (m3)	2
H3	4	1 (-), 2 (m3), 1 (Mn3), 1 (Mn2)	4
H4	4	1 (-), 1 (m3), 1 (m2), 1 (Mn3), 1 (D Fe)	4
H5	4	1(-), 2 (m3), 1 (m2), 1 (Mn2), 1 (Mn1)	3
H6	3	1 (-), 2 (m2), 1 (Mn2)	2
H7	4	1 (m3), 1 (m2), 1 (m1), 1 (Mn3), 2 (Mn2), 1 (D Fe)	4
H8	4	1 (m2), 3 (m1), 1 (Mn3), 2 (Mn2), 1 (c)	4
H9	4	1 (-), 1 (m1), 2 (m2), 1 (Mn3)	3
H10	4	1 (-), 2 (m3), 1 (m2), 2 (Mn2)	3
H11	4	3 (m2), 1 (m1), 1 (Mn2), 2 (Mn1)	4
H12	4	2 (-), 2 (m1), 1 (Mn2)	2
Control without inoculum	26	26 (-)	0
		'Natal'	
Nontransgenic	4	1 (-), 2 (m3), 1 (m2), 1 (Mn3), 1 (Mn2)	3
N1	4	2 (m3), 1 (m2), 1 (m1), 1 (Mn3), 1 (Mn2)	4
N4	4	2 (-), 1 (m3), 1 (m2), 1 (Mn3), 1 (Mn2)	2
N5	4	4 (m3), 2 (Mn2)	4
N8	4	1 (-), 3 (m3), 1 (Mn3), 2 (Mn2)	3
N9	4	1 (-), 2 (Mn2), 1 (Mn1), 1 (Mn2), 1 (Mn1)	3
Control without inoculum	12	12 (-)	0
		'Pêra'	
Nontransgenic	4	1 (-), 1 (m3), 2 (m2), 1 (Mn3), 1 (D Fe), 1 (D Zn)	3
P6	4	1 (-), 2 (m3), 2 (Mn3), 1 (D Fe), 1 (c)	3
P7	4	2 (-), 2 (m3), 1 (Mn3), 1 (Mn2), 1 (D Zn)	2
P8	4	1 (-), 2 (m3), 1 (m2), 2 (Mn3), 1 (c)	3
P10	4	2 (m3), 2 (m2), 4 (Mn3)	4
P11	4	1 (-), 2 (m2), 1 (D), 1 (c)	2
P15	4	2 (m3), 1 (m1), 2 (Mn3), 1 (Mn2)	4
Control without inoculum	14	14 (-)	0
		'Valência'	
Nontransgenic	4	2 (m3), 2 (m1), 2 (Mn3), 2 (Mn2)	4
V1	4	1 (-), 2 (m1), 3 (Mn2)	3
V2	4	1 (-), 2 (m2), 1 (Mn3), 2 (Mn2), 1 (c)	3
V3	4	2 (m3), 1 (m2), 1 (m1), 1 (c)	4
V4	4	2 (-), 1 (m3), 2 (D Fe)	2
V7	4	1 (-), 2 (m3), 1 (m2), 1 (Mn2), 2 (Mn1)	4
V8	4	1 (-), 1 (m3), 2 (m2), 1 (Mn2)	3
V10	4	3 (m3), 3 (Mn3)	4
V11	2	2 (m3), 1 (Mn3)	2
V12	4	2 (m3), 2 (m2), 2 (Mn3), 1 (Mn2)	4
V13	4	2 (m3), 1 (m2), 1 (Mn3), 1 (Mn2), 1 (D), 1 (c)	3
V15	4	1 (-), 1 (m3), 1 (m2), 2 (Mn3), 1 (Mn2), 1 (c)	3
V16	4	1 (Mn3), 3 (c)	4
V17	4	4 (m3), 4 (Mn3), 2 (c)	4
V18	4	2 (-), 1 (m1), 1 (m2), 2 (Mn2)	2
V19	4	1 (m3), 2 (m2), 1 (m1), 1 (Mn3), 3 (Mn2), 1 (c)	4
V20	4	1 (m3), 3 (m2), 3 (Mn3), 1 (Mn2), 1 (c)	4
Control without inoculum[2]	33	33 (-)	0

[1]Number of plants with symptoms: m, leaf mottling; Mn, manganese deficiency (numbers 1, 2, and 3 following each symptom letter means mild, moderate, and severe, respectively); c, chlorosis; D, uncertain HLB symptoms; Fe, iron deficiency; Zn, zinc deficiency; (-), no symptom. [2]V11 with one plant without inoculum as control.

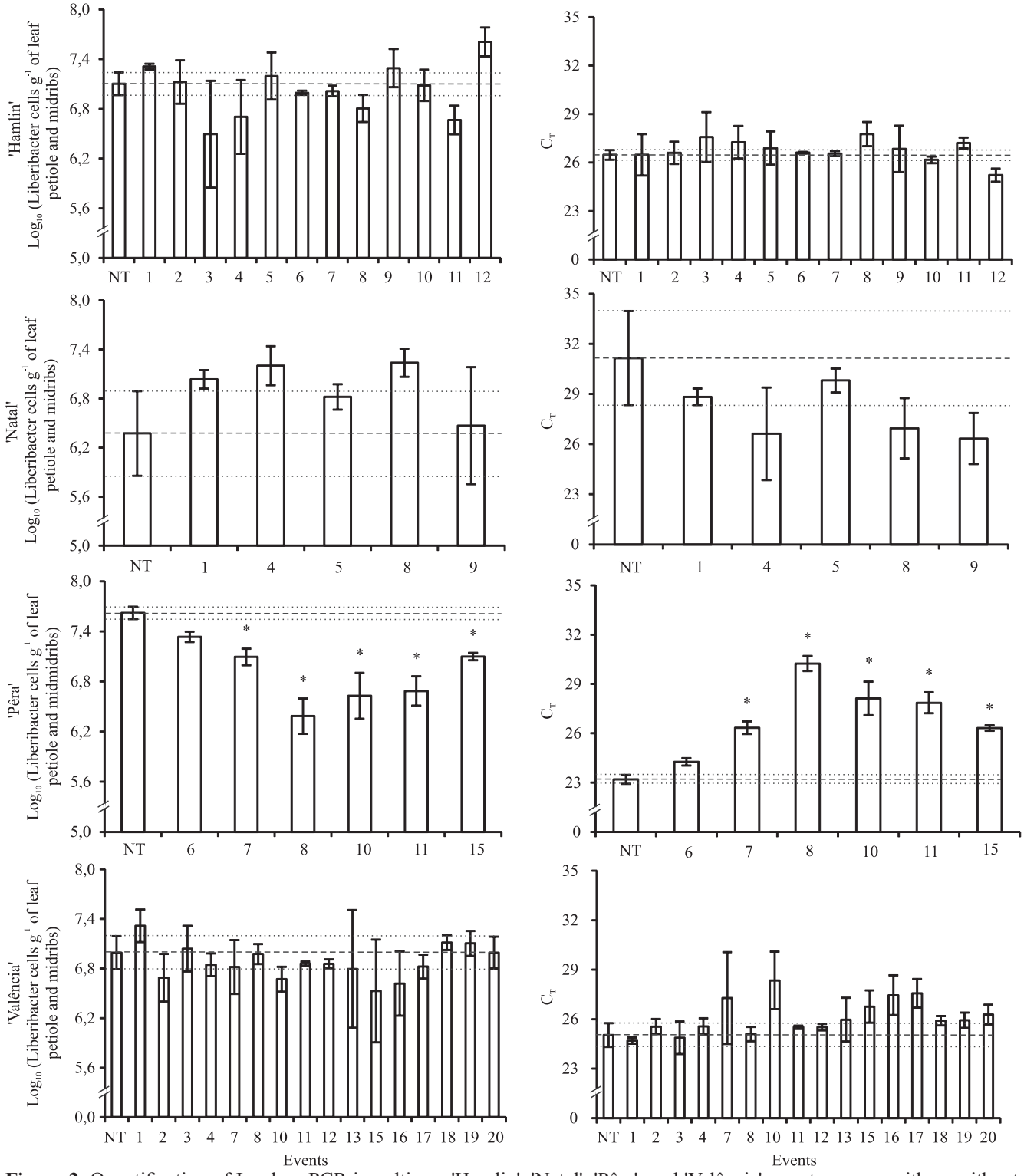

Figure 2. Quantification of Las by qPCR in cultivars 'Hamlin', 'Natal', 'Pêra', and 'Valência' sweet oranges with or without the *att*A gene, eight months after inoculation by infected buds. Log_{10} (bacterial cells g^{-1} of leaf petiole and midribs): mean values obtained from leaf bacterial titers. C_T, mean threshold cycle values. Number of evaluated plants per treatment varied according to the number of PCR positive plants (Table 1). Vertical bars indicate standard errors. Horizontal lines indicate the upper, middle, and lower limits for nontransgenic plants. NT, nontransgenic. *Different by Dunnett's test, at 5% probability. The results represent four experiments, one for each cultivar.

the causal agent of citrus variegated chlorosis disease in line P7. The action of *attacin* A on Las and *X. fastidiosa* was apparently less effective than on *X. citri* subsp. *citri*. This difference in efficacy is probably due to the apoplastic location of *X. citri* subsp. *citri*. The *attA* gene construct used in the present work encodes a signal peptide that carries *attacin* A to the apoplast (Boscariol et al., 2006), which is the colonization site of most plant pathogenic bacteria (Bové & Garnier, 2003). However, HLB bacterium is intracellular (Bové & Garnier, 2003). Despite the mode of action of *attacin* A, in the present research five transgenic lines showed reduced Las titers compared to the Las titer observed in nontransgenic plants. The effect that this decrease in titer could have on HLB management or HLB spread in the field is not known at this moment. Lower titers could provide lower chances of bacterium acquisition by *D. citri,* in a fashion similar to the recently observed by Lopes et al. (2013) in Las-affected plants, or when Lam and Las transmission rates were compared (Lopes et al., 2009). In the first work, the decrease in Las titer was the result of exposure of Las-affected plants to higher temperatures. This phenomenon was assumed to be the responsible for the lower rates of HLB spread in the northern portion of São Paulo state, where temperatures during the hot summers could reduce rates of Las multiplication in the new flushes, the feeding sites of *D. citri*. In the second work, Las reached at least ten times higher titer than Lam in Liberibacter-affected plants. The result helped to explain the considerably lower transmission rates of Lam, and the shift in Liberibacter prevalence overtime in the state of São Paulo. As indicated, in this work, some transgenic lines of 'Pêra' sweet orange contained significantly less bacteria than the nontransgenic plants. Whether this reduced titer would affect Las transmission by *D. citri* remains to be determined. Current research on the development of other gene constructs involving the *attA* gene, without the signal peptide to direct the gene product to the extracellular space, are underway with promising results to even better efficiency of this peptide against Las, due to its probably better location within plant tissue.

Conclusions

1. HLB leaf symptoms and Las titers are present in nontransgenic and in transgenic plants expressing the *attacin* A gene of four sweet orange cultivars, eight months after bacteria inoculation by grafting.

2. Five transgenic lines of 'Pêra' sweet orange expressing the *attacin* A gene indicate significantly lower Las titers as compared to nontransgenic plants of this cultivar.

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