Prevalence of *Candidatus* Liberibacter spp. in HLB-diseased Citrus Plants in São Paulo State, Brazil

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ABSTRACT. Citrus Huanglongbing (HLB) disease was reported in São Paulo State, Brazil, in June, 2004, with two different species of Liberibacter associated with the disease, *Candidatus* Liberibacter asiaticus and *Ca*. L. americanus. Initial data obtained from HLB symptomatic samples collected during 2004 showed a higher frequency of *Ca.* L. americanus, as determined by PCR. The data shown here was obtained from citrus leaves with HLB-like symptoms that were collected from a broad range of citrus growing region from São Paulo from 2005 to 2007 reaching 8,000 samples. Total DNA was obtained from the petioles of leaves and the Asian and American forms of Liberibacter were detected by PCR using specific primers. In general, there was a strong reduction in frequency of *Ca*. L. americanus in the HLB diseased plants during this period. The American form of the bacterium was present in almost 90% of HLB symptomatic samples in 2005, decreasing to 60% in 2007. The mixture infection remains almost constant (5%) during the period. Consequently there was an increase of samples infected with *Ca.* L. asiaticus, from 5% (2005) to 35% (2007). However, the frequency of the Asian form has increased strongly in the last years in all citrus regions of São Paulo State affected by HLB. Studies have been carried out concerning the influence of temperature, multiplication in both plant and vector, and vector transmission.

Citrus huanglongbing (HLB) is a major threat to citriculture due to its rapid spread throughout orchards and severe loss of production of infected trees (6, 8). This disease is associated with phloem-limited and psyllid-vectored bacteria named *Candidatus* Liberibacter spp. (1, 5). Until 2004 this disease was restricted to parts of Asia and Africa but it was subsequently described in the two largest citrus growing areas in the world, the state of São Paulo (Brazil) (3, 12), and Florida (USA) (7). In São Paulo, HLB is associated with two different species of Liberibacter, *Ca*. L. asiaticus (Las) and *Ca*. L. americanus (Lam) (3, 12). The outbreak started in a few municipalities in the central area of the State, and by December 2007, three and a half years later, HLB-diseased trees could be found in almost 150 other municipalities in a wider area.

The citrus belt of São Paulo is located in four geographic areas (North, Northeast, Southeast, and Southwest) which represent 99.7% of all citrus production totaling 570,000 ha (Fig. 1) (11). The North region is characterized by a hot climate (average maximum temperature of 38ºC), in contrast to the Southeast region which has an average maximum temperature of 32ºC. Effects of temperature can have significant influence on HLB symptoms expression as reported for HLB caused by *Ca*. L. africanus (2), as well caused by *Ca*. L. americanus (9). The objective of this report was to study the prevalence of both the American and Asian species of the HLB-associated bacteria in infected orchards in São Paulo. Leaf samples were collected according to state regulations by the CDA (State Agricultural Defense System – http://www.cda.sp.gov.br) and sent to the Centro APTA Citrus Sylvio Moreira (http:// www.centrodecitricultura.br) for analyses.

Fig. 1. Map of citrus growing regions from State of São Paulo, Brazil, from which the samples were collected and sent for HLB analysis.

MATERIAL AND METHODS

Plant material and PCR-based diagnosis. Petioles were collected from leaves displaying blotchy mottle symptoms and total DNA was extracted using the CTAB methodology of Murray and Thompson (10) with minor modifications. A duplex polymerase chain reaction (PCR) amplification was carried out in 25 µl containing 2.5µl of 10X buffer, 1.5 mmol $MgCl_2$, 2.5 mmol of each dNTP, 1 U of *Taq* DNA polymerase and 5 µl of total DNA extract ranging from 10 to 30 ng/ µl. Primers for both *Ca*. L. asiaticus (LPas - TGGTGATAGGGTGGATTTAG/RPas – CAACCTCGAAGAAAACAGAC) and for *Ca*. L. americanus (LSg2F-TTAAGT TAGAGGTGAAATCC/LSg2RCAACTTA TGATGGCAAATA) were used in the same amplification at concentration of 12.5 ng each (4).

Amplification was performed in a PT 100 thermocycler (MJ Research, Waltham, MA, USA), with pre-denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min completed with a final extension at 72°C for 5 min. For all DNA extraction and PCR, citrus tissues infected with both species of Liberibacter were used as positive controls and tissues from shot-tip grafted healthy plants maintained under greenhouse conditions were used as negative controls. Ten microliters of each reaction was loaded in a 1.2% agarose gel and DNA was visualized by staining with ethidium bromide. Positive amplifications for Las showed an amplicon of 960 base pars (bp), for Lam of 545 bp, and both amplicons are present in a mixed infection (Fig. 2).

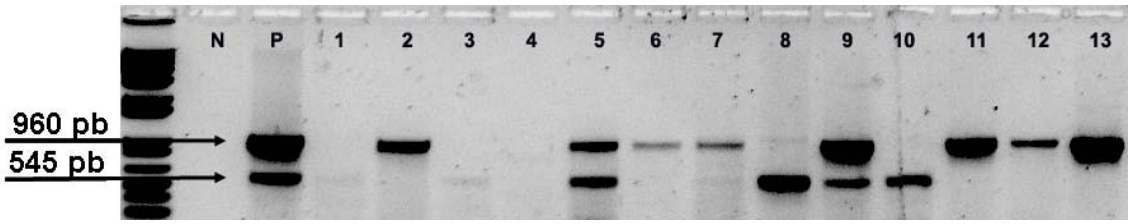

Fig. 2. Agarose gel electrophoresis DNA amplified with set of primers for *Ca*. Liberibacter asiaticus (960 bp) and for *Ca*. L. americanus (545 pb). Molecular marker - 1KB DNA size Ladder plus (Invitrogen). N - negative control. P - positive control infected with both liberibacters. 1, 3, 8, and 10 – samples infected with CLam. 2, 6, 11, 12, and 13 - samples infected with CLas. 5, 7 and 9 – samples infected with both bacteria, 4. non-infected sample.

RESULTS AND DISCUSSION

Our laboratory in the "Centro APTA Citros Sylvio Moreira/IAC" is in responsible for the analysis of citrus samples officially collected in campaign against the HLB in São Paulo. The ongoing process started in first half of 2005 with the objective of locating, diagnosing and eliminating infected tress from the entire São Paulo citrus belt. During the first tree years (2005, 2006, and 2007) over 8,000 samples were analyzed by PCR and 400,000 samples by visual blotchy mottle symptoms. These analyses involved trees from almost 160 different municipalities throughout the citrus belt of São Paulo State. It is important to emphasize that each PCR amplification corresponded to a different citrus block in different properties. Lam was present in 88.6% and 92.5% of the analyzed samples in 2005 and 2006, respectively, followed by a significant reduction to 60% in 2007. Consequently, the presence of Las increased from almost 5% in the first two years to 35% in the third year. Co-infection was always low (≈ 5%) during these 3 yr of analyses (Fig. 3). A similar ratio of infection by these two bacteria was determined in the other studies. Teixeira et al. (13) analyzed 1500 samples using specific primers for Lam and Las, and showed that 92.5% of trees with blotchy mottle leaf symptoms were PCR positive for Lam, 5.4% for Las, and 2.1% for both bacteria. In 2006 a larger number of samples showed that the proportion of Las increased to 21.4%, and consequently, Lam dropped to 77.2 % of all positive PCR samples analyzed (14). Our data confirm the increasing frequency of Las in HLB-diseased trees in São Paulo State. In spite of this, 3 yr after the first detection of HLB, Lam is still the prevalent causal agent of the disease, but with strong increase of HLB caused by Las.

The prevalence of both Lam and Las in each of four different citrus growing regions of São Paulo State (North, Northeast, Southeast, and Southwest) has shown a reduction of American species in all the four regions, and an increase of the Asian HLB bacteria (Table 1). The change is clearly seen in Fig. 4, where the Lam:Las ratio dropped from almost 20 in 2006 to 3 in 2007 in all regions. Lopes et al. (9) showed that temperatures above 27 ºC for 8h

could have a significant effect on reduction of symptoms of Lam-affected trees, that could explain the low incidence of disease in the orchards of North region where the annual hours above 32ºC is at least five times higher than in the Southeast region. Our data shows that although the prevalence of Lam has decreased in the North region, this bacterium still is present in 60% of HLB-diseased trees there. However, the low incidence of the disease in the North region could possibly be associated with the lack of sufficient inoculum for a quickly spread of bacteria by the psyllid as well for the strategies of intense management of disease in the field, like removing rouging of diseased plants and vector control. A higher initial inoculum source could have contributed to the quick spread of disease in the orchards of Southeast and Southwest regions. It has been noticed that the control of sharpshooters associated with *Xylella fastidiosa* transmission has been more effective in the North in comparison to South region. However, in general, this control has indirectly contributed to maintain lower psyllid populations in the North than in the South.

Finally, although the presence of Lam has been dropping recently, this bacterium is still the most prevalent in the HLB-affected trees at the time that these data was analyzed. We can only speculate that Las is taking over niches previously occupied by Lam. The concentration of Las was always higher than Lam in samples from symptomatic trees as measured by real-time PCR (data no shown). Perhaps the higher titer of Las could give it a competitive advantage during transmission by psyllids, thus explaining the increase of its frequency in orchards independent of the geographic region.

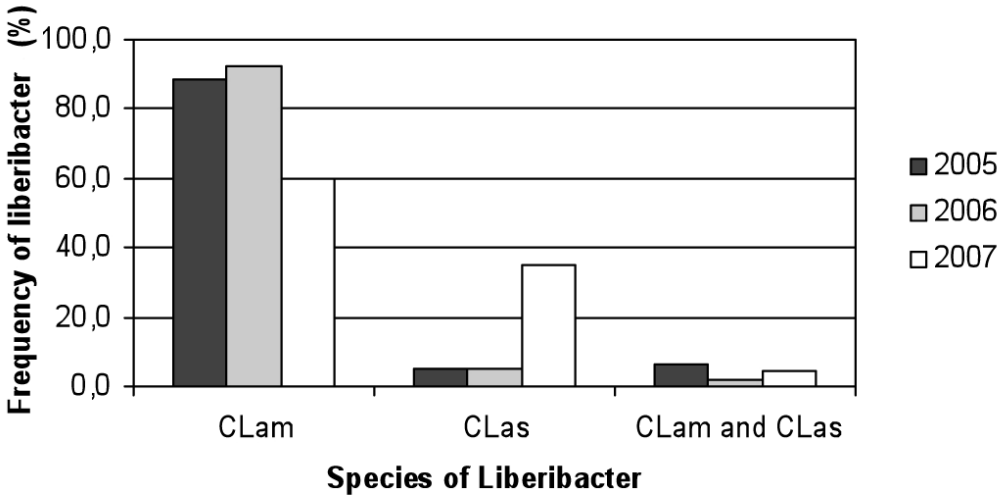

Fig. 3. Frequency of *Ca.* Liberibacter americanus (Lam), *Ca.* L. asiaticus (Las), and both species in the HLB diseased samples collected from throughout the State of São Paulo during 2005, 2006, and 2007.

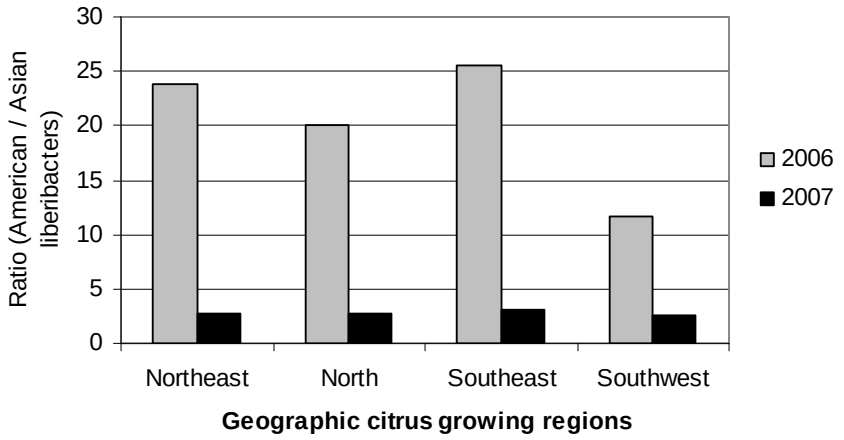

Fig.4. Ratio between *Ca.* Liberibacter americanus (Lam) and *Ca.* L. asiaticus (Las) in the citrus growing regions of the São Paulo State (Brazil) in 2006 and 2007.

TABLE 1
PREVALENCE OF *CANDIDATUS* LIBERIBACTER SPP. IN THE ORCHARDS OF CITRUS FROM DIFFERENT REGIONS OF SÃO PAULO STATE IN 2006 AND 2007

Region	2006		2007	
	Lam (%)	Las (%)	Lam (%)	Las (%)
North	95.80	4.20	64.41	35.58
Northeast	95.00	5.00	64.61	35.38
Southeast	96.09	3.91	67.17	32.82
Southwest	91.40	8.60	61.73	38.27

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