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Distribution and quantification of *Candidatus* Liberibacter americanus, agent of huanglongbing disease of citrus in São Paulo State, Brasil, in leaves of an affected sweet orange tree as determined by PCR

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ABSTRACT

Huanglongbing (HLB), an insect-transmitted disease of citrus, known for many years in Asia and Africa, has appeared in the state of São Paulo State (SSP), Brazil, in 2004, and the state of Florida, USA, in 2005. HLB endangers the very existence of citrus, as trees infected with the bacterial pathogen, irrevocably decline. In the absence of curative procedures, control of HLB is difficult and only based on prevention. Even though not available in culture, the HLB bacterium could be shown to be Gram-negative and to represent a new candidate genus, Candidatus Liberibacter, in the alpha subdivision of the *Proteobacteria*. Three *Candidatus* (*Ca.*) L. species occur: *Ca.* L. africanus in Africa, *Ca.* L. asiaticus in Asia, SSP, and Florida, and *Ca.* L. americanus in SSP. The liberibacters occur exclusively in the phloem sieve tubes. On affected trees, HLB symptoms are often seen on certain branches only, suggesting an uneven distribution of the Liberibacter. Occurrence of *Ca.* L. americanus, the major HLB agent in SSP, has been examined in 822 leaf samples from an affected sweet orange tree by two conventional PCR techniques and a newly developed real time (RTi) PCR, also used for quantification of the Liberibacter in the leaves. Even though RTi-PCR was able to detect as few as 10 liberibacters per gram of leaf tissue (l/g), no liberibacters could be detected in any of the many leaf samples from a symptomless branch, while in blotchy mottle leaves from symptomatic branches of the same tree, the Liberibacter titer reached values as high as 10^7 l/g. These results demonstrate the uneven distribution of the Liberibacter in HLB-affected trees.

1. Introduction

Huanglongbing (HLB) or yellow-shoot-disease is a destructive, newly emerging, century-old malady of citrus [1]. Already mentioned in southern China in the late 19th century, the disease has been present for many years in three major regions of the world: (i) Asia, including the Indian subcontinent, China, and South East Asia, (ii) East Africa and Cameroun in West Africa, as well as Madagascar, Reunion and Mauritius islands, and (iii) the south-western Arabian Peninsula. HLB has now been reported for the first time in two of the major citrus growing regions of America: São Paulo State, Brazil, and Florida State, USA, respectively, in 2004 and 2005. Today, only the Mediterranean basin, the Middle East, Australia and New Zealand, and North- and South-Pacific islands are still free of the affection. HLB is feared worldwide, because citrus trees, once infected, will irrevocably decline. There are no effective curative procedures, and thus control of HLB consists of preventing trees from becoming infected.

Many features of HLB were already known when the disease entered America. Lin Kung Hsiang of the South China agricultural University transmitted the disease by graft-inoculation, and thus proved for the first time the infectious nature of the disease agent [2]. However, his results remained essentially unknown to the western World for almost 10 years. In 1965, McClean and Oberholzer [3,4] confirmed the work of Lin by transmitting South African HLB (greening) by graft-inoculation, and they showed the African citrus psyllid, *Trioza erytreae*, to be a vector of the HLB agent in South Africa. The Asian citrus psyllid, *D. citri*, was found to be a vector of the HLB agent in 1967 in India [5] as well as in the Philippines [6]. Biological control of the two psyllid vectors by

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Tamarixia spp. was achieved in Reunion Island [7,8]. The most characteristic leaf symptom of HLB, blotchy mottle, well known in China [2], was also stressed in other parts of the world [9–11].

The HLB agent, seen by electron microscopy for the first time in 1970 by Laflèche and Bové [12], is a Gram-negative bacterium [13,14]. The HLB bacterium is exclusively restricted to the phloem sieve tubes, not only in citrus, but also in two non-citrus, experimental hosts, periwinkle (*Catharanthus roseus*) and tobacco (*Nicotiana xanthi*) plants, to which the HLB bacterium was transmitted from HLB-affected citrus by dodder (*Cuscuta campestris*) [10,15]. The HLB agent has never been obtained in culture, and had to be characterized by DNA-based techniques. Comparisons of 16S ribosomal DNA (16S rDNA) sequences [16], as well as 16S/23S intergenic region sequences [17], confirmed that the HLB agent was a Gram-negative bacterium and, more precisely, a member of a new lineage in the alpha subdivision of the *Proteobacteria*. The trivial name liberobacter, later replaced by Liberibacter [18], was given to the HLB bacterium. The *Liberibacter* strains in Africa could be distinguished from those in Asia on the basis of temperature sensitivity, DNA hybridizations and genomic properties, and serology. For these reasons, they represent two different species: *Candidatus* L. africanus in Africa and *Candidatus* L. asiaticus in Asia, the term *Candidatus* (*Ca*) indicating that the organism has not been cultured and has been characterized essentially by DNA-based techniques [19]. A subspecies of the African liberibacter, *Ca.* L. africanus subsp. capensis, has been characterized in an ornamental rutaceous tree, *Calodendron capense*, in the Western Cape province of South Africa, where Ca. *L. africanus* was detected in 1996 [18,20]. In São Paulo state, a third liberibacter species, *Ca.* L. americanus, has been identified in 2004 [21–23]. The *nus*G–*rpl*KAJL–*rpo*BC gene clusters of the Asian and African liberibacters were obtained and sequenced [24,25]. The same gene cluster has recently been obtained from the American liberibacter (Teixeira, unpublished). Additional liberibacter genes, including the *omp* gene, have been isolated by using Random Amplified Polymorphic DNA (RAPD) [26]. The *omp* gene was used to study the genetic variability of the Asian liberibacter [27]. Several monoclonal antibodies (MAs) recognizing Asian or African strains of the HLB agent have been obtained [28,29]. They revealed the existence of various serotypes of the HLB agent, but they were too strain specific for detection purposes [11,28]. MA 10A6, against an epitope of an Asian HLB strain (Poona strain, India), was used in immunoaffinity chromatography to purify the Poona HLB bacterium and visualize it for the first time out of its cellular habitat. The length of the filamentous bacteria varied from 1 to 4 μ, with an average length of 2 μ and an average width of 0.2 μ [28,30].

From 1970 to the early 1990s, electron microscopy (EM) was the first, reliable technique to detect the HLB bacterium in suspicious citrus trees and to confirm HLB symptoms [11,31]. The first molecular detection technique was DNA hybridization. DNA probe In-2.6 [32] was specific for the Asian liberibacter, and DNA probe As-1.7, for the African liberibacter [25]. EM and DNA hybridization have been used to detect the liberibacters in several hundred leaf samples from about 30 different countries [11,31,33,34]. Only the African liberibacter was detected in Africa, and only the Asian liberibacter in Asia. Liberibacters were seen by EM exclusively in the sieve tubes. Probe In-2.6 was also used to detect the Asian liberibacter on crush blots of *Diaphorina citri* psyllids [33].

The first PCR method for detection of the HLB liberibacters became available in 1996, and was based on 16S rDNA amplification with forward primers OI1 and OA1, and reverse primer OI2c [35]. With these primers, no amplification was obtained with the novel American liberibacter, and new primers, f-GB1 and r-GB3, specific for 16S rDNA amplification of the American HLB agent, were developed [22]. A second PCR detection method, based on the sequence of the *rpl*KAJL gene cluster coding for ribosomal proteins K, A, J, and L, was worked out for the detection of the African and the Asian liberibacters [36]. The two PCR methods gave similar results, in particular with 95% of 428 samples tested from Indonesia, South Africa and Mauritius Island.

HLB has been reported in the state of São Paulo (SSP) in March 2004. The disease in Brazil was found to be caused by two liberibacters: *Ca.* L. asiaticus [22,37], present in about 4% of the trees, and a new liberibacter, *Ca.* L. americanus [22], which infected most of the trees; some trees were infected simultaneously with the two liberibacters. Since 2006, an increase in the percentage of trees infected with the Asian liberibacter has been witnessed. An HLB control program has been developed in SSP, and conventional, 16S rDNA-based PCR has been used to confirm that trees with HLB symptoms were indeed infected with the HLB agent(s). The PCR test has been applied to several thousand samples of symptomatic leaves from many different citrus farms. Practically, all leaves with blotchy mottle symptoms gave positive PCR reactions. However, many HLB-affected trees do not show symptoms uniformly; some branches having symptomatic leaves and fruits, others being free of symptoms. Also, leaves with blotchy mottle symptoms are not always available, and the possibility of using leaves with other symptoms, such as zinc deficiency patterns, or even symptomless leaves, should be envisaged. Finally, detection of the liberibacters in recently infected trees, but showing no symptoms yet could be hampered by irregular distribution of the HLB agent. Therefore, we have studied the distribution of *Ca.* L. americanus, the major liberibacter in SSP, in the various branches of a "Westin" sweet orange tree by collecting 822 leaf samples, and analysing the samples for liberibacter detection by conventional PCR, nested PCR, and a newly developed SYBR Green real time PCR (RTi-PCR) with quantification for *Ca.* L. americanus. The results of this work are presented here.

2. Materials and methods

2.1. Plant material, insects, and DNA extraction

Westin sweet orange leaves with blotchy mottle symptoms were collected on several trees from an orchard in the Araraquara region of the state of São Paulo (SSP), and used to identify the infecting liberibacter species by conventional 16S rDNA PCR with primer pair GB1/GB3 for *Ca.* L. americanus and primer pair OI1/OI2c for *Ca.* L. asiaticus [22].

One 4-year-old, HLB-affected, Westin sweet orange tree infected with *Ca.* L. americanus was selected for studying the distribution and quantification of the liberibacters in the leaves throughout the canopy. The position of each leaf sample was recorded and materialized on the tree with a label carrying the sample number (Fig. 1). A total of 822 leaf samples were collected. For each sample, only leaves with one type of symptom were taken, and the type of leaf symptom (blotchy mottle, zinc deficiency, no symptoms) was recorded.

Leaves from a given sample (~5–10 leaves) were washed under running tap water, blotted dry on filter paper, and their midribs were recovered and cut into small pieces. Five hundred milligram of midribs were ground in a Homex 6 homogenizer (Bioreba AG, CH-4153 Reinach BL1, Switzerland). Total DNA was extracted using the CTAB (cetyl trimethyl ammonium bromide) method [38]. DNA preparations were adjusted at 100 ng DNA/μl and kept at −20 °C. Healthy leaf DNA was prepared in Bordeaux (France) from greenhouse-grown, sweet orange seedlings.

Adults of *Diaphorina citri*, the psyllid insect vector of HLB in SSP, were collected on trees with or without symptoms of HLB, and used for DNA extraction by the CTAB method. The DNA preparations from 105 batches of five psyllids each were adjusted with water to a final volume of 30 μl and used in conventional rpl-PCR with new rpl primers (see 2.3.1) and in RTi-PCR.