PROGRESS AND PERSPECTIVES OF RESEARCHS ON HLB AND ITS VECTOR *Diaphorina citri*

Silvio A. Lopes, Nelson A. Wulff, Pedro T. Yamamoto, and Marcelo P. Miranda

Fundecitrus, Av. Adhemar Pereira de Barros, 201, Vila Melhado, Araraquara, SP, CEP 14807-040, Brazil, slopes@fundecitrus.com.br

Abstract

Recent progress on HLB and *Diaphorina citri* researches are presented, and the implications of the research findings for understanding of the HLB problem, for improving management practices, and for developing new technologies or strategies to control the disease are discussed.

Introduction

For a long time, the HLB occurrence was restricted to Africa and Asia, the Arabian Peninsula, and the Reunion and Mauritius Islands. More recently the disease reached the Americas. It was first reported in the State of São Paulo (SP), Brazil, in 2004, then in Florida (FLA), USA, in 2005, Cuba in 2007, and Dominican Republic in 2008 (Halbert, 2005; Teixeira et al, 2005; Llauger et al, 2008; Matos et al, 2009). The presence of HLB in these locations has caused apprehension to the other states and neighboring countries, especially those where the vector is already present, as is the case of Texas and California (Da Graça et al, 2008; Polek et al, 2008), and Mexico in North America (Robles et al, 2008), and the remaining states in Brazil and countries in South America.

Brazil and FLA have faced a dramatic increase in incidence and in geographic distribution of the disease overtime. In Brazil, the number of affected municipalities increased from 15 in October 2004 (all located in SP) to 251 in May 2009 (216 in SP, 1 in Minas Gerais and 34 in Parana States). In FLA, 34 counties were affected by the disease in December 2008 (Halbert et al, 2008). In USA, the disease also was reported in Louisiana in 2008 and South Carolina in 2009.

In Brazil and FLA, the fast spread of HLB in SP has been associated with severe economic losses, leading growers and private and governmental agencies to take actions in an attempt to suppress disease progress locally, and to protect the areas still free of the disease. These actions include (i) spontaneous or law enforced elimination of symptomatic trees or entire blocks to reduce inoculum sources, (ii) applications of insecticides to reduce as much as possible vector populations, (iii) implementation, where it doesn’t exist, of a system to produce nursery trees in screened houses, and (iv) implementation of an education program to call grower attention to the HLB problem. At the same time, private and governmental agencies have made available significant amounts of financial resources to stimulate and engage researchers in the investigation of the several aspects of the disease. The purpose is to generate information to improve the current disease management practices or, preferably, to find new and more sustainable technologies to control the disease.

In the last 5 years, a significant progress was made in our understanding of the complex interactions involved in the HLB pathosystem. Three new organisms were found associated with the HLB symptoms: *Candidatus* Liberibacter americanus and a phytoplasma closely related to the pigeon pea witches’-broom phytoplasma (Teixeira et al, 2008a) in Brazil, and an additional phytoplasma showing 100% DNA identity with *Candidatus* Phytoplasma asteri in China (Chen et al, 2009). Considerable improvements in Liberibacter detection was obtained allowing a more precise HLB diagnosis and bacterium quantification in the host plant and in the insect vector.

The ornamental orange jasmine was found to naturally host both *Ca.* L. americanus and *Ca.* L. asiaticus in citrus farms and urban areas (Lopes et al, 2005 and 2006). The basis for the competitive advantages of *Ca.* L. asiaticus over *Ca.* L. americanus in SP has been elucidated

(Lopes et al, 2009 and 2009a). An overall distribution of Liberibacter in the aerial parts of HLB affected individual trees as well as in the root system has been demonstrated (Gottwald et al, 2008; Tatineni et al, 2008), providing direct evidence of their downward movement in the citrus phloem and an explanation for the lack of success of pruning in removing all infected tissues (Lopes et al, 2007). Concerning the vector, the environmental and biological factors governing insect multiplication, survival and death, and attraction and repellency to visual cues and plant host have been elucidated (Liu and Tsai, 2000; McFarland and Hoy, 2001; Étienne et al, 2001; Parra et al, 2006; Nava et al, 2007, Hall, 2008; Rouseff et al, 2008; Sétamou et al, 2008; Torres and Parra, 2008; Zaka and Zeng, 2008; Qureshi et al, 2009; Wenninger et al, 2009). Epidemiological studies are demonstrating the importance of infected trees within and outside the orchards for disease progress and, at the same time, providing indirect information on insect movement and Liberibacter transmission (Gottwald et al, 2007). Finally, in the management area, a better procedure for field inspection, including the use of platforms, was developed (Belasque, 2006).

In this short review we try to present these research findings and discuss their benefits and perspectives in the management of the disease. Proceedings of recent workshops or meetings, and articles published in phytopathological and entomological journals were the main source of information.

Progress on HLB research

1. HLB diagnosis

The Liberibacters are the bacteria most commonly found associated with HLB. Until recently no culture media were known to support axenic growth of these organisms outside of the host tree and insect vector. Therefore, HLB diagnosis has been totally dependent on indirect evidences of the presence of the associated bacteria in the suspect tree. The methodologies most used in the past included electron microscopy (EM) (Garnier et al, 1984), thin layer chromatography (TLC) (Schwarz, 1968), and biological indexing (BI) by grafting buds from the suspected material onto the trunk of susceptible citrus cultivars. These methodologies are laborious and costly (EM), unspecific (TLC), or require a large set of test plants and long intervals to produce the results (BI). Currently they have been of very limited use.

With the sequencing and characterization of DNA fragments of the different HLB associated bacteria (Jagoueix et al, 1994; Teixeira et al, 2008), and the development of primers for use in PCR or PCR-based techniques (Li et al, 2006; Teixeira et al, 2008), a considerable improvement in HLB diagnosis has been obtained. Although the cost of HLB diagnosis has not yet been resolved, the accuracy provided by the use of specific primers has been very helpful in disease diagnosis and HLB research. The uncertainties faced even by trained personnel when confronted with trees showing yellow leaves, especially in areas where the disease has not occurred or occur in very low incidences, can now be easily resolved through PCR. Progress in the genomic studies and sequencing of Liberibacter genomes (Duan et al, 2009; Wulff et al, 2009) will provide not only additional targets for PCR-based detection techniques, but also accelerate the studies of host-pathogen-insect interactions.

A further improvement in this area was obtained with the development of the quantitative real-time PCR (qPCR, RT-PCR) which increased the sensitivity 10 to 100 fold when compared to conventional PCR (Li et al, 2007; Teixeira et al, 2008). Also, with the use of primers specific for known copy number of the target DNA, qPCR has allowed indirect estimations of the amount of bacterium DNA in the plant or insect and, consequently, reliable estimations of bacterial titers (Teixeira et al, 2008b; Lopes et al, 2009 and 2009a). Crucial information for our understanding of the complex interactions involved in the HLB problem has been obtained with the use of qPCR. Rate of bacterium distribution in field and in experimental trees, temperature influence on Liberibacter multiplication in plant tissues, and rate of pathogen acquisition and transmission by the insect vector are some examples.

2. Liberibacter multiplication in citrus trees

In Brazil, field and experimental data accumulated over the years suggested that *Ca.* L. asiaticus and the newly described *Ca.* L. americanus have contrasting competitive behavior. In July 2004 both Liberibacters were present in SP but, at that time, *Ca.* L. americanus was, by far, the most prevalent species (Wulff et al, 2006). From August 2004 to January 2005, of the 137 samples in which Liberibacter was detected at the Fundecitrus lab,131 (95.6%) were positive for *Ca.* L. americanus and only 4 (2.9%) for *Ca.* L. asiaticus (Lopes et al, 2009a). The higher proportion of *Ca.* L. americanus at that time led to the assumption that Ca. L. americanus had a competitive advantage over *Ca.* L. asiaticus (Gottwald et al, 2007). In the succeeding years, however, a dramatic shift in Liberibacter prevalence has been observed. From August 2007 to January 2008, of the 1,288 samples in which Liberibacter was detected, 253 (19.6%) were positive for *Ca.* L. americanus, and 1,024 (79.5%) for *Ca.* L. asiaticus (Lopes et al, 2009a). In December 2008, the situation had changed even more with Ca. L. asiaticus being detected in 96% and *Ca.* L. americanus in 4% of the analyzed samples (Wulff, unpublished data). Farms that initially were affected almost exclusively by *Ca.* L. americanus are today almost exclusively affected by *Ca.* L. asiaticus.

Since the first report of the presence of HLB in SP, several studies using graft inoculations have been carried out in insect-proof greenhouses. This method consists of grafting young 6 to 12 month-old trees, with 2 to 4 cm long budsticks removed from symptomatic shoots of naturally-infected field trees (Lopes and Frare, 2008). Data from several experiments has invariably shown higher transmission percentages for *Ca.* L. asiaticus than for *Ca.* L. americanus (Lopes et al, 2009a). Quantitative real-time PCR was carried out with DNA from a large number of graft-inoculated and naturally-infected field grown trees. Average bacterial titers, in log cells per gram of leaf midrib, were 6.42 for *Ca.* L. asiaticus and 4.87 for *Ca.* L. americanus in the graft inoculated experimental plants, and 6.67 for *Ca.* L. asiaticus and 5.74 for *Ca.* L. americanus in the naturally-infected field trees used as the source of inoculum. The differences in bacterial titers has now been confirmed in field trees with a larger number of samples, collected during the summer and winter from distinct geographic regions in SP (S. Lopes, unpublished data).

The results of qPCR analyses strongly suggests that the shift in Liberibacter prevalence and the higher graft transmission efficiencies of *Ca.* L. asiaticus is presumably associated with higher bacterial multiplication in the citrus phloem. Higher titers would increase the chances of pathogen acquisition and transmission by the insect vector, a phenomenon similar to the observed for the xylem-limited bacterium *Xylella fastidiosa* in grapevines transmitted by sharpshooters (Hill and Purcell, 1997). This hypothesis has in fact been confirmed in recent transmission experiments using *D. citri* (Yamamoto, unpublished data).

3. Liberibacter sensitivity to high temperatures

The abilities of Liberibacters to multiply in greenhouse and field citrus trees were further investigated in growth chambers experiments (Lopes et al, 2009), where the temperature could be controlled with precision. These studies initiated in 2004 only with *Ca.* L. americanus but later included also *Ca.* L. asiaticus for comparison. The work was motivated: (i) by the fact that nothing was known with respect to *Ca.* L. americanus; (ii) by the knowledge that *Ca.* L. asiaticus and *Ca.* L. africanus differ in sensitivity to higher temperatures, explaining their distribution in countries like South Africa and Reunion Islands (Bové et al, 1974); and (iii) by the observation, at that time, of an uneven spatial distribution of HLB in SP, i.e., regions with hotter summers (North and Northwest SP), being less affected by the disease. Although the disease has reached the hotter regions, the disease incidence remains very low. HLB has moved faster and longer distances towards the south, despite the presence of the vector and susceptible citrus trees providing conditions favorable for disease spread (Lopes et al, 2009).

The question of why the north and northwestern are less prone to HLB progress will probably be resolved with the experiments that are underway. Presently, the growth chamber studies demonstrate differential responses of *Ca.* L. americanus and *Ca.* L. asiaticus to temperatures of

32°C and above. While *Ca.* L. asiaticus is affected only by the daily temperature regimen of 9h at 24°C and 6 h at 38°C, for 60 days (the highest temperature condition tested), leading to a decrease in bacterial titer from estimated averages of log 7 to log 3 cells per gram of tissue, *Ca.* L. americanus is drastically affected by a less elevated temperature regimen of 5h at 27°C and 12h at 32°C. Under this condition *Ca.* L. americanus is eliminated from the previously affected trees, and a complete and permanent symptom remission is observed.

4. Liberibacter distribution in citrus trees

Knowledge on the distribution patterns of Liberibacter in individual citrus trees is important for the understanding of mechanism underlying pathogenicity and to improve the HLB management practices (Tatineni et al, 2008). Liberibacter multiplies in the phloem sap at the inoculation site and moves in downward or upward directions, to other parts of the plant. This assumption was based on electron microscopy studies and on observations of symptom progress in naturally-infected field and experimentally-inoculated trees. The symptoms appear initially at the tip of one or more branches. They progress with time, and eventually involve the entire tree canopy, at which time the fruit productivity is severely reduced (Bassanezi et al, 2008).

Downward and upward movement of the bacterium has been demonstrated recently with the use of qPCR. *Ca.* L. asiaticus was detected in all portions of graft inoculated trees, including bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in endosperm and embryo (Tatineni et al, 2008). Downward movement and systemic distribution of *Ca.* L. asiaticus was demonstrated also in 5 and 7 year-old naturally-infected trees displaying symptoms only at the tip of one or two branches (Gottwald et al, 2008). This downward movement and overall distribution also was demonstrated for *Ca.* L. americanus in the hundreds of field trees in a pruning experiment carried out in SP (Lopes et al, 2007). The trees were pruned by removing only the symptomatic shoots (by cutting them at the trunk level), or by removing the entire canopy (by cutting the trunk 15 to 20 cm above the graft line). Mottled leaves reappeared on most symptomatic (69.2%) and also on some asymptomatic (7.6%) pruned trees, regardless of age, variety, and pruning procedure.

5. Orange jasmine as a Liberibacter alternative host

In Brazil, hosts of Liberibacters include all commercial citrus species and cultivars (with sweet oranges and mandarins showing higher susceptibility levels than limes and lemons), and orange jasmine (*Murraya paniculata*), known in Brazil as ‘murta’, a very popular ornamental tree (Lopes et al, 2005 and 2006). The first orange jasmine tree suspected to host Liberibacter was found in October 2004, in the citrus farm most affected by HLB at that point. The tree was showing yellow leaves and shoot dieback throughout the canopy. Leaves were collected and adults of *D. citri* captured from two parts of the plant. The pathogen (*Ca.* L. americanus) was detected by PCR in both leaf samples and in one of seven lots of 10 psyllids. In a survey conducted later in urban areas, *Ca.* L. americanus was detected in 11.4% and *Ca.* Liberibacter asiaticus in 0.5% of the 550 symptomatic trees sampled in 17 SP municipalities (S. Lopes, 2008, unpublished data). Like citrus, affected trees showed yellow leaves and/or shoot dieback in one or more sectors of the canopy. Contrary to citrus, the characteristic blotchy mottling was not present on the leaves and which complicates the identification of the affected trees. Citrus and orange jasmine showed, however, similar patterns of pathogen distribution in the canopies. In a few trees tested, PCR-positive results were only obtained from leaf samples collected from symptomatic shoots (Lopes et al, 2006a).

Progress on *D. citri* research

1. *D. citri* feeding behavior and Liberibacter transmission

A technique called electrical penetration graphing (EPG) (McLean and Kinsey, 1964) has been used to investigate feeding behavior of *D. citri* and the process of Liberibacter transmission by the insect. The technique is based on establishment of an electrical circuit between the insect and the testing plant. When the insect inserts the stylet into the citrus leaf, the circuit closes leading to specific changes in the amplified signals (waves) recorded. The distinct wave forms are related to the following stylet activities: (i) penetration of the stylet into the intercellular parenchyma, (ii) contact of the stylet with the phloem sieve tube (iii) salivation, (iv) phloem sap ingestion, and (v) xylem sap ingestion (McLean and Kinsey, 1964; Tjalingii, 1988). The average time for the stylet to reach the phloem was 154 min. Phloem sap ingestion continued for average 206.1 min over an 8 hour recording period.

Developmental stage of the citrus leaf influences feeding behavior and Liberibacter acquisition efficiency by *D. citri*. Nymphs and adults prefer younger and tender tissues than older mature leaves. EPG analysis indicated that 80% of the insects that were allowed to feed for 96 hours on young tissues reached the phloem, versus 20% of those that were allowed to feed for the same time interval on older leaves (Bonani et al, 2008). Efficiency *Ca.* L. asiaticus acquisition after insect feeding was 54% for young leaves, and just 10% for old leaves.

Acquisition and transmission efficiencies are also related to the developmental stage of the insect. Nymphs are more efficient than adults (Vichin et al, 2008). Insects that complete their lifecycle on an infected shoot are much more likely to acquire the bacteria then those individuals that feed on the infected shoot as adults only (Ebert et al, 2008; Miranda, unpublished data).

2. Life cycle and population dynamics of *D. citri*

D. citri females lay their eggs on young new flushes. During the entire adult phase at 25°C, each female deposits on average, 626 eggs (Liu and Tsai, 2000). The lifecycle includes 5 nymph stages with the duration of each stage being strongly influenced by temperature. The entire cycle varies from 14.1 days at 28°C, to 49.3 days at 15°C (Liu and Tsai, 2000), and 12.1 days at 18°C, and 43.5 days at 32°C (Nava et al, 2007). It has been demonstrated that *D. citri* can withstand very low temperatures. Temperature of -1.9°C for up to 10 hours killed a relatively low percentage of adults, while -5.0 to -5.5 °C for 4 hours or longer killed 95 to 100 % adults (Hall, 2008). Relative humidity (RH) also affects female survival. After 30 hour exposure at 25ºC with a RH of 33%, only 20% of the adults survive; the survival rate increases to 60% at 97% RH (McFarland and Hoy, 2001).

Under favorable environmental conditions and the presence of young new flushes *D. citri* populations may be abundant. However, considerable natural mortality of *D. citri* has been observed in some areas due to the attack of several natural enemies including insects and fungi. *Tamarixia radiata* seems to be the most common parasitoid. *T. radiata* produces a high level of parasitism in association with high rates of field dispersal and establishment capacity (Torres and Parra, 2008). Natural parasitism of 27.5 to 80.0% was observed in SP (Parra et al, 2006), and up 56% in FLA (Qureshi et al, 2009). These high parasitism rates have not been enough, however, to limit pathogen dissemination. Plans exist to massively release *T. radiata* in citrus plantings to reinforce natural populations (Hoy and Nguyen, 2000; Parra et al, 2006). So far, Reunion Island is the only country where this strategy has succeeded (Étienne et al, 2001). The current heavy application of insecticides in attempts to control *D. citri*, and of miticides and fungicides to control pests and other vectors and citrus pathogens, reduces natural parasitism and interferes with the use of this strategy (Yamamoto and Miranda, 2009).

3. *D. citri* attractants and repellents

Field observations, yellow sticky trap insect monitoring, and Y-tube olfactometer experiments have shown that the flight activity of *D. citri* might be governed by visual cues and by the odors released by plants. They are attracted to light, green and yellow colors, and to volatiles released by the new flushes of citrus and orange jasmine trees (Sétamou et al, 2008; Wenninger et al, 2009), and are repelled by volatiles released by guava (Zaka and Zeng, 2008).

Attraction to light was demonstrated in Texas (Sétamou et al, 2008). Populations were 3 to 4 times higher on yellow sticky traps maintained during the day (with picks from noon to 15:00 pm) than on those maintained during the night. During the night, illuminated traps captured 5 times more insects than non illuminated traps. Light also affected other insect activities. In potted plants it increased plant colonization and egg deposition by females.

Investigations on the potential repellent effect of guava to *D. citri* started after observations in Vietnam that the HLB incidence and *D. citri* populations were much lower in blocks where the citrus trees were interplanted with guava than in those where citrus trees were planted alone (Beattie et al, 2006; Ichinose et al, 2008). A possible repellant effect of a volatile compound released by guava was later investigated in free choice testes, and in caged citrus and guava plants (Zaka and Zeng, 2008). Higher numbers of insect were found on citrus shoots caged alone than on those caged with guava. Young and old guava shoots showed the same response. Y-tube olfactometer confirmed the repellant effect of guava to adult psyllids, that the repellency was dose dependent, and that affected male and females similarly. Chromatographic analysis of wounded guava leaves revealed the presence of dimethyl disulfide (DMDS), known to be toxic to insects, as one of the components responsible for the repellant activity of this plant species against *D. citri* (Rouseff et al, 2008).

A study of cases on HLB management in SP

Four years after the first report of HLB in SP, a study of cases was carried in SP to determine the most important factors associated to the success of disease control. Data from 20 citrus farms was submitted to multiple regression analysis. Overall, the study showed better chances of success in larger farms with old trees, located away from neighboring farms not practicing HLB control. Most important for successful management is the early and aggressive implementation of HLB control practices when the disease incidence is still very low (Belasque et al, 2008).

Benefits and perspectives of HLB and *D. citri* research findings on HLB management practices

1. Symptomatic tree elimination

As indicated, elimination of symptomatic trees to reduce inoculum sources is the primary procedure recommended for the HLB management. Survey and detection of affected trees is dependent almost exclusively on symptom observations, namely leaf yellowing and mottling, fruit deformation, and leaf and fruit drop. The symptoms expressed by trees infected with Liberibacter or phytoplasma in SP are indistinguishable in the field (Teixeira et al, 2008). Therefore, Symptomatic trees have been eliminated without exact knowledge of the associated bacterium. This precludes precise estimation of the distribution and incidence of this recently discovered phytoplasma agent.

The development of PCR and qPCR provided higher sensitivity in Liberibacter detection, and consequently greater accuracy in HLB diagnosis (Li et al, 2007; Teixeira et al, 2008). Since the symptom expression may vary depending on the season and citrus species (Lopes et al, 2009), the use of PCR has been very useful for validation of field inspections. However, PCR is costly and time consuming. Therefore, its use has been limited to training inspectors for symptom recognition, and for confirmation of HLB in leaf samples with questionable symptoms.

The existence of an inexpensive, easy to handle and accurate field diagnostic test would be of great help during tree inspections. An iodine test was developed with this objective (Takushi et al, 2007). The test is based on the accumulation of starch in the parenchyma cells of leaves of HLB affected trees and on the fast starch and iodine reaction in water, producing a darkish solution. Its accuracy has not yet been totally evaluated in Brazil. In FLA, the iodine and qPCR tests agreed for 76% of the samples (Chamberlain and Irey, 2008). It produced better results for mottled leaves and from samples collected in February and July than in June. The authors found the iodine test useful for field diagnosis but considered unsuitable as a substitute for PCR confirmation.

The task of finding, by walking, the HLB affected trees in fields of large adult plants, affected by one or more other diseases, pests or mineral deficiencies, is challenging, even for a well trained inspectors. From the ground, the view of the top of the tree is compromised and, consequently, symptomatic trees may escape detection. A solution to this problem has been the use of platforms, which consist of metal structures that are coupled to tractors. On these platforms, the inspector can thoroughly examine the top of large trees. In an experiment in SP, groups of 2 to 4 trained inspectors detected, with the use of platforms, on average 60% of the symptomatic trees, compared to 48% when the inspection was made by walking (Belasque, 2006). Platforms are currently used in several farms in SP. Platforms have not only increased inspection efficiency but also reduced the labor intensity of the inspection process. This study also confirmed the necessity of carrying out frequent inspections in areas where HLB is present. Frequent inspection increases the chances of finding the symptomatic trees that escaped detection in previous passages and also those trees that developed HLB symptoms later.

Frequent field inspections have shown that HLB incubation period is long and variable. Therefore, at any given time in an infected orchard there will be (i) trees not infected by the HLB bacteria, (ii) trees infected but not showing any symptoms, (iii) symptomatic trees that were not detected during inspections, and (iv) infected trees showing unequivocal and obvious symptoms, which will certainly be detected in the following inspection. While graft and insect transmission studies have clearly demonstrated the importance of symptomatic branches as sources of inoculum for the healthy trees, nothing is known in this regard for the role of the asymptomatic branches or trees for the HLB epidemics.

The normative instruction regulating symptomatic tree elimination includes citrus and orange jasmine, and was written based on research information demonstrating that (i) most affected trees would not be cured by pruning and, therefore, would serve as source of inoculum for the healthy trees, and (ii) on the demonstration that orange jasmine may harbor not only *D. citri* but also the two HLB associated Liberibacters. Currently, a campaign is underway in SP aiming to inform, especially mayors, of the risk of exposure of orange jasmine in urban areas to the nearby citrus plantings. In Paraná State, a resolution of the State Secretary prohibits the production and commercialization of this plant species. In SP, as well as in Paraná, some local municipalities already replaced orange jasmine with other ornamental plants.

Data on field surveys conducted in 2004, 2007, 2008 and 2009 have shown higher rates of HLB increase in the South and Center of SP, than in the North and Northwest regions. In fact, in the Northwest the disease was found only in 2008 and in very low incidence. Although several factors may account for these results, the temperature may be playing a role. Coincidently, the North and Northwest regions are characterized by summer days hotter than in the other regions. In growth chamber programmed with daily temperature regimens representative of the summer days prevalent in those regions, both the multiplication of the Liberibacters and the expression of the leaf symptoms were negatively affected. Current experiments aim to determine if the temperature will affect Liberibacter multiplication in the vector as well. A better understanding of this issue might be important to improve the existing control practices, or to create new control strategies. If the temperature limits HLB progress in a given geographic area, facilitating disease management, this area could be looked as a promising place for starting new, or expansion of existing citrus plantings.

2. Reduction of vector populations

Reduction of vector populations is the second complementary procedure to manage HLB in the field. Unfortunately, the high mortality rates of *D. citri* observed naturally in the field has not been enough to reduce vector populations, and to suppress Liberibacter dissemination and disease spread. Therefore, reduction of vector populations has relied on the use of insecticides. However, this approach is costly as it requires frequent applications mainly during the spring and summer months when the insect reaches peak populations due to the availability of young new flushes,.

To estimate the need for chemical applications, the presence of psyllids in the orchard has been monitored by visual scouting or with the use of yellow sticky traps (Yamamoto et al, 2008). In Texas, green-sticky traps were, however, more efficient than yellow-sticky traps to attract adult psylla (Sétamou et al, 2008), which has been confirmed in SP (Yamamoto, unpublished data). Green-sticky traps should replace yellow-sticky traps for monitoring *D. citri* in SP.

If *D. citri* is present in the field, the grower can choose one of a set of insecticides, systemic or of foliar application, available in the market and demonstrated in several tests to be effective for killing *D. citri* (Yamamoto et al, 2008). The systemic ones are the most effective, with residual periods of 60-80 days, but they are also the most expensive and, for this reason, are applied to young nursery trees (before leaving the nursery), and in field trees up to three years of age. The insecticides for foliar applications are cheaper but less effective, with residual periods of no longer than 20 days. They have been sprayed on trees older than three years, as well as on younger trees during the dry seasons. To reduce costs, some growers combine foliar-applied insecticides with other chemicals (miticides, fungicides) or micronutrients.

The practices for vector control have, so far, been less benefited by the recent research findings than the practices related to symptomatic tree detection and elimination. However, studies involving the use of Y-tube olfactometer, chromatography, EPG and qPCR techniques are showing promise for providing important new information to improve vector control. EPG applied in the assessment of *D. citri* feeding behavior on insecticide-treated and non-treated, healthy and infected plants, will be helpful to determine the role of the insecticide in preventing Liberibacter transmission, an important question not yet fully answered. The Y-tube olfactometer, gas chromatography, and mass spectrometry have been helpful in the detection and identification of volatiles compounds as repellants or attractants to *D. citri*. The expectation is to have one or more synthetic repellant compounds for use in the future as new commercial products to prevent the vector from feeding on citrus trees.

The qPCR technique has allowed Liberibacter detection in psylla in areas free of the disease well before the appearance of the first affected trees (Manjunath et al, 2008). This has allowed for the ellucidation of existence of a seasonal variation in the frequency of insects carrying *Ca.* L. asiaticus (Ebert et al 2008; Sadoyama and Takushi, 2008). Therefore, qPCR may help to track the movement of infectious insect, and to determine the optimum time to apply insecticides. This, in turn, could lead to a reduction in the use of insecticides and their impacts on beneficial parasitoids and the environment.

3. Planting of healthy young trees

There is no guarantee for the disease freedom of young citrus trees in new plantings, if they have not been produced in nursery structures, protected from psylla carrying one of the HLB associated bacteria, or other insect vectors carrying other disease agents. Fortunately, in SP there has been a law since 2003 that requires the propagation of all young trees only in screened nurseries. This system has provided for the continual reduction in the incidence of citrus variegated chlorosis, a disease caused by *X. fastidiosa*, a sharpshooter-vectored, xylem-limited bacterium. Since screening the nurseries also protects the young trees from the access of psylla, this system has been crucial for preventing, since 2003, the movement of HLB to new areas by movement with nursery trees. A similar protected nursery production system has now been implemented in FLA (Irey et al, 2008).

The system adopted in Brazil requires that all steps involved in young tree production, except seed production, be carried out inside the screened nursery. New research data has suggested that *Ca.* L. asiaticus might be seed transmissible (Graham et al, 2008; Hartung et al, 2008; Shatters, 2008; Zhou et al, 2008). If confirmed, the trees used for seed production will have to be kept under screen as well.

Conclusions

As shown in this short review, a substantial amount of new information has been developed since the first report of HLB occurrence in the Americas, the principal area for citriculture for orange juice produced in the world. Three additional bacteria have been found associated with the HLB-like disease symptoms; more sensitive detection methods have been developed; a new alternative host was discovered in Brazil; the competitive relationship of *Ca.* L. americanus and *Ca.* L. asiaticus was defined; the multiplication and distribution of the Liberibacters in citrus trees were demonstrated; the feeding behavior, biology and population dynamics of the insect vector, as well as it attractiveness or repellency to visual cues, and to volatiles released from host and non host plants, were determined.

All of this information is providing for greater understanding of how the host-pathogen-vector relationship works. This will help to improve management practices, and expectation for the development of new ones. In this regard, the most promising areas are the development of volatiles that repel *D. citri*, and the development of transgenic citrus trees that resist pathogen attack or to prevent vector transmission. Considerable research efforts are currently being expended on these issues.

At the same time, the disease is spreading very fast, causing severe economic, social and environmental losses. There is an overall perception that the way we are dealing with HLB, with constant removal of trees and applications of tons of costly insecticides, is not sustainable in a long term. Therefore the research community is racing against the time. Hopefully we will be able to find, in time, a way to better interfere in the HLB disease cycle to suppress the HLB epidemics.

Acknowledgements

We thank Dr. James H. Graham from Citrus Research and Education Center, Lake Alfred, Florida, for proofreading the final version of the manuscript.

References

Bassanezi, R. B., Montesino, L. H., Amorim, L., Gasparoto, M. C. G., and Bergamin Filho, A. 2008. Yield reduction caused by Huanglongbing in different sweet orange cultivars in São Paulo, Brazil. Pages 237-238 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Beattie, G. A. C., Holford, P., Mabberley, D. J., Haigh, A. A., Bayer, R., and Broadben, P. 2006. Aspects and insights of Australia-Asia collaborative research on Huanglongbing. Pages 47-64 in International Workshop for prevention of Citrus Greening Disease in Severely Infested Areas Ishigaki, Japan.

Belasque Junior, J. 2006. Detecção de plantas com HLB em pomares. Page 52 in Proceedings of the Huanglongbing-Greening International Workshop, Ribeirão Preto, SP, Brazil.

Belasque Junior, J., Bassanezi, R. B., Yamamoto, P. T., Lopes, S. A., Ayres, A. J., Barbosa, J. C., Tachibana, A. Violante, A. R., Tank Jr., A., Giorgetti, C. L., Di Giorgi, F., Tersi, F. E. A., Menezes, G. M., Dragone, J., Catapani, L. F., Jank Jr., R. H., and Bové. J. M. 2008. Factors associated with control of huanglongbing in Sao Paulo, Brazil: a case study. Pages 296-300 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Bonani, J. P., Fereres, A., Appezzato-da-Gloria, B., Garzo, E. I. Miranda, M. P., and Lopes, J. R. S. 2008. Characterization of electrical penetration graphs of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) in citrus. Page 194 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Bové, J. M., Calavan, E. C., Capoor, S. P., Cortez, R. E., and Schwarz, R. E. 1974. Influence of temperature on symptoms of California stubborn, South African greening, Indian citrus decline and

Philippines leaf mottling diseases. Pages 12-15 in Proceedings of the 6th Conference of the International Organization of Citrus Virologists. Riverside, CA.

Chamberlain, H. L. and Irey, M. S. 2008. Comparison of a starch-based field test for Huanglongbing to results from real-time PCR testing of field samples from symptomatic trees in Florida. Page 130 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Chen, J., Pu, X., Deng, X., Liu, S., Li, H., and Civerolo, E. 2009. A phytoplasma related to *Candidatus* Phytoplasma asteri' detected in citrus showing huanglongbing (yellow shoot disease) symptoms in Guangdong, P. R. China. Phytopathology 99:236-242.

Da Graça, J. V., Skaria, M., Sétamou, M., Kunta, M., Arredondo, M., Salas, B., and Parker, P. E. 2008. Texas steps up surveys for huanglongbing and the Asian citrus psyllid. Pages 102-103 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Duan, Y.P., Zhou, L.J., Hall, D., Li, W.B., Liu, L., Gottwald, T. Genome sequencing of *Candidatus* Liberibacter asiaticus, the causal agent of citrus huanglongbing (greening) in Florida, 2008. Proceedings of the 11th International Citrus Congress, Wuhan, China.

Ebert, T. A., Rogers, M. E., Brlansky, R. H. 2008. Seasonal occurrence of *Candidatus* Liberibacter asiaticus in Asian citrus psyllids in Florida. Pages 229-230 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Étienne, J., Quilici, S., Marinal, D., and Franck, A. Biological control of *Diaphorina citri* (Hemiptera: Psyllidae) in Guadeloupe by imported Tamarixia radiata (Hymenoptera: Eulophidae). Fruits, 56: 307-315, 2001.

Garnier, M., Danel, N., and Bové, J. M. 1984. The greening organism is a Gram negative bacterium. Pages115-124 in Proceedings of the 6th Conference of the International Organization of Citrus Virologists. Riverside.

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

Gottwald, T., Parnell, S., Taylor, E., Poole, K., Hodge, J., Ford, A., Therrien, L., Mayo, S., and Irey, M. 2008. Within-tree distribution of *Candidatus* Liberibacter asiaticus. Pages 270-273 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Graham, J. H., Irey, M. S., Dawson, W. O., Hall, D., and Duan, Y. 2008. Assessment of transmission of Liberibacter asiaticus from seed to seedlings of 'Pineapple' sweet orange and Carrizo citrange. Pages 174-175 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

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.

Halbert, S. E. 2005. The discovery of huanglongbing in Florida. Page 50 in Proceedings of 2nd International Citrus Canker and Huanglongbing Research Workshop, Florida Citrus Mutual, Orlando.

Halbert, S. E., Manjunath, K. L. and Brodie, M.W. 2008. Large-scale distribution of *Diaphorina citri* Kuwayama and citrus huanglongbing in Florida. Page 107 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Hall, D. G. 2008. Effects of freezes on survival of *Diaphorina citri*. Page 192 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Hartung, J. S., Halbert S., and Shatters, R. 2008. Can *Ca.* Liberibacter asiaticus be transmitted through citrus seed? Page 166 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Hill, B. L. and Purcell, A. H. 1997. Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. Phytopathology 87:1197-1201.

Hoy, M. A. and Nguyen, R. 2000. Classical biological control of Asian citrus psylla. Citrus Industry, 81: 48-50.

Ichinose, K., Band, D. V., and Dien, L. Q. 2008. Better management for citrus greening: chemical-uses or guava-interplanting? Pages 308-309 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Irey, M., Gottwald, T., Stewart, M., and Chamberlain, H. 2008. Is it possible to replant young groves in an area with endemic HLB – a hierarchical sampling approach to determine infection? Pages 108-109 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Llauger, R., Luis, M., Collazo, C., Peña L., González, C., Batista, L., Teixeira, D. C., Martins, E., Perdomo, A., Casín, J. C., Peréz, J. L., Cueto, J. R. and Bové, J. M. 2008. Current situation of citrus huanglongbing in Cuba. Pages 97-100 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Li, W., Hartung, J. S., and Levy, L. 2006. Quantitative real-time PCR detection and quantification of *Candidatus* Liberibacter species associated with citrus huanglongbing. Journal of Microbiology Methods 66:104-115.

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

Liu, H. Y and Tsai, J. H. 2000. Effects of temperature on biology and life table parameters of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae). Annals of Applied Biology, 137: 201-201.

Lopes, S. A., Martins, E. C., and Frare, G. F. 2005. Detecção de *Candidatus* Liberibacter americanus em Murraya paniculata. Summa Phytopathologica 31: 48-49.

Lopes, S. A., Martins, E. C., Frare, G. F. 2006. Detecção de Candidatus Liberibacter asiaticus em Murraya paniculata. Fitopatologia Brasileira 31:303.

Lopes, S. A., Frare, G. F., and Martins, E. C. 2006a. Hosts of Liberibacter in Brazil. Pages 25-26 in Proceedings of the Huanglongbing-Greening International Workshop, Ribeirão Preto, SP, Brazil.

Lopes, S. A., Frare, G. F., Yamamoto, P. T., Ayres, A. J., and Barbosa, J. C. 2007. Ineffectiveness of pruning to control citrus huanglongbing caused by *Candidatus* Liberibacter americanus. European. Journal of Plant Pathology. 119:463-468.

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

Lopes, S. A., Bertolini, E., Frare, G. F., Martins, E. C., Wulff, N. A., Teixeira, D. C., Fernandes, N. G., and Cambra, M. 2009. Graft transmission efficiencies and multiplication of '*Candidatus* Liberibacter americanus' and '*Ca.* Liberibacter asiaticus' in citrus plants. Phytopathology 99:301-306.

Lopes, S. A., Frare, G. F., Bertolini, E., Cambra, M., Fernandes, N. G., Ayres, A. J., Marin, D. R., and Bové, J. M. 2009a. Liberibacters associated with citrus huanglongbing in Brazil: '*Candidatus* Liberibacter asiaticus' is heat tolerant, '*Ca.* L. americanus' is heat sensitive. Plant Disease 93:257-262.

Manjunath, K. L., Halbert, S. E., Ramadugu, C., Webb, S., and 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.

Matos L., Hilf, M. E., and Camejo, J. 2009. First Report of '*Candidatus* Liberibacter asiaticus' Associated with Citrus Huanglongbing in the Dominican Republic. Plant Disease 93(06):668.

McClean, D. L. and Kinsey, M. G. 1964. A technique for electronically recording aphid feeding and salivation. Nature 202:13581359.

McFarland, C. D. and Hoy, M. A. 2001. Survival of *Diaphorina citri* (Homoptera: Psyllidae), and its two parasitoids, Tamarixia radiata (Hymenoptera: Eulophidae) and Diaphorencyrtus aligarhensis (Hymenoptera: Encyrtidae), under different relative humidities and temperature regimes. Florida Entomologist 84: 227-233.

Nava, D. E., Nascimento, A. M., Stein, C. P., Haddad, M. L., Bento, J. M. S., and Parra, J. R. P. 2007. Biology, thermal requirements, and estimation of the number of generations of *Zaprionus indianus* (Diptera: Drosophilidae) for the main fig producing regions of Brazil. The Florida Entomologist 90: 495-501.

Parra J. R. P., Nava D. E., and Gómez Torres M. L. 2006. Biologia e Técnicas de Criação para Controle Biológico de *Diaphorina citri*. Page 32 in Proceedings of the Huanglongbing - Greening International Workshop, Ribeirão Preto, SP, Brazil.

Polek, M. and Luque-Williams, M. J. 2008. The State of California implements its action plan for Asian Citrus Psyllid. Page 322 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Qureshi, J. A., Rogers, M. E., Hall, D. G., and Stansly, P. A. 2009. Incidence of invasive *Diaphorina citri* (Hemiptera: Psyllidae) and its introduced parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) in Florida citrus. Journal of Economic Entomology 102: 247–256.

Robles, G. P., Sanchez, A. H., and Mendez L. O. 2008. National plan for detection of HLB in Mexico. Page 104 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Rouseff, R. L., Onagbola, E. O., Smoot, J. M., and Stelinski, L.L. 2008. Wounding of guava (*Psidium guajava* L.) leaves produces defensive sulfur volatiles. Page 278 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Sadoyama Y. and Takushi T. 2008. The seasonal incidence of *Candidatus* Liberibacter asiaticus infection in the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae) in Okinawa, Japan. Pages 223-224. In Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Shatters Jr., R. G. 2008. Detection of *Candidatus* Liberibacter asiaticus in citrus seedlings germinated from Florida seed. Page 173 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Schwarz, R. E. 1968. Indexing of greening and exocortis through fluorescent marker substances. Proceedings of 4th IOCV. 118-124.

Sétamou, M., Sanchez, A. Patt, J., and Louzada, E. 2008. Diurnal patterns in flight and effect of light on host finding behaviour of the Asian Citrus Psylla. Pages 225-227 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Tjallingii, W. F. 1988. Electrical recording of stylet penetration activities. Pages 95-108 in A. K. Minks & P. Harrewijn (eds.), Aphids, their biology, natural enemies and control, v. 2B. Elsevier, Amsterdam, The Netherlands, 364 p.

Takushi, T., Toyozato, T., Kawano, S., Taba, S., Taba, K, Ooshiro, A., Numazawa, M., and Tokeshi, M. 2007. Scratch method for simple, rapid diagnosis of citrus huanglongbing using iodine to detect high accumulation of starch in the citrus leaves. Journal of Phytopathology 73: 3-8.

Tatineni, S., Sagaram, U. S., Gowda, S., Robertson, C. J., Dawson, W. O., Iwanami, T., and Wang, N. 2008. In plant distribution of ‘*Candidatus* Liberibacter asiaticus’ as revealed by polymerase chain reaction (PCR) and real-time PCR. Phytopathology 98:592-599.

Teixeira, D. C., Ayres, J., Kitajima, E. W., Tanaka, F. A. O., Danet, L., Jagoueix-Eveillard, S., Saillard, C., Bové, J. M. 2005. First report of a huanglongbing-like disease of citrus in São Paulo State, Brazil and association of a new Liberibacter species, “*Candidatus* Liberibacter americanus”, with the Disease. Plant Disease 89:107.

Teixeira, D. C., Dane, J. L., Eveillard, S., Martins, E. C., Jesus, W. C., Jr., Yamamoto, P. T., Lopes, S. A., Bassanezi, R. B., Ayres, A. J., Saillard, C., and Bové, J. M. 2005a. 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.

Teixeira, D. C. Saillard, C., Couture, C., Martins, E., Wulff, N.A., Yamamoto, P.T., Eveillard-Jagoueix, S., Ayres, A.J., Bové, J.M. 2008. *Candidatus* Liberibacter americanus, agent of huanglongbing disease of citrus in Sao Paulo State, Brasil: Distribution and quantification of the Liberibacter in leaves of an affected sweet orange tree as determined by PCR methods. Molecular and Cellular Probes 22:139-150.

Teixeira, D. C., Wulff, N. A., Martins, E. C., Kitajima, E. W., Bassanezi, R., Ayres, A. J., Eveillard, S., Saillard, C., and Bové, J. M. 2008a. A phytoplasma closely related to the pigeon pea witches’-broom phytoplasma (16Sr IX) is associated with citrus huanglongbing symptoms in the state of São Paulo, Brazil. Phytopathology 98:977-984.

Torres, M. G. and Parra, J. R. P. 2008. Bioecology of *Diaphorina citri* and *Tamarixia radiata*: zoning for citrus groves of the State of São Paulo. Page 275 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Vichin Neto, R., Nascimento, F. E., Balbinotte, J., Coletta-Filho, H., Lopes, J. R. S. 2008. Comparação da capacidade de aquisição de *Candidatus* Liberibacter asiaticus em citros por ninfas e adultos de *Diaphorina citri* Kuwayama (Hemiptera:Psyllidae). In 16º. Simpósio Internacional de Iniciação Científica - USP, Piracicaba, São Paulo, Brazil.

Yamamoto, P. T., Felippe, M. R., Beloti, V. H., and Rugno, G. R. 2008. Efficiency of insecticides to control *Diaphorina citri*, vector of huanglongbing bacteria. Page 285 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Yamamoto, P. T. and Miranda, M. P. 2009. Controle biológico na cultura dos citros. in XI Simpósio de Controle Biológico, Bento Gonçalves, Rio Grande do Sul, Brazil.

Wenninger, E. J., Lukasz, L. S., and Hall, D. G. 2009. Roles of olfactory cues, visual cues, and mating status in orientation of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) to four different host plants. Environmental Entomology. 38: 225-234.

Wulff, N. A., Eveillard S., Foissac, X., Ayres, A. J., and Bove, J. M. 2009. Ribosomal RNA operons and genome size of *Candidatus* Liberibacter americanus, a bacterium associated with citrus huanglongbing in Brazil. International Journal of Systematic and Evolutionary Microbiology (in press).

Wulff, N. A., Teixeira, D. C., Martins, E. C., Leite, A. P. R, Padiar, N. R. H., Mariano, A. G., Carmo, A. U., Abrahão, D. P., Sousa, M. C., Ayres, A. J., and Bové, J. M. 2006. Huanglongbing diagnosis. Page 17 in Proc. Huanglongbing–Greening International Workshop, Ribeirão Preto, Brazil.

Zaka, S. M. and Zeng, X. N. 2008. Repellent effect of guava odor against adult of citrus psyllid, *Diaphorina citri*. Pages 276-277 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.

Zhou, L.-J., Benyon, L. S., Powell, C. A., Gottwald, T., and Duan, Y.-P. 2008. Improved detection of low-titer, non-lethal, seed transmitted *Candidatus* Liberibacter asiaticus in citrus, periwinkle and dodder using nested PCR. Pages 112-113 in Proceeding of the International Research Conference on Huanglongbing, Orlando, Florida.