Current Epidemiological Understanding of Citrus Huanglongbing*

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Abstract

Huanglongbing (HLB) is the most destructive citrus pathosystem worldwide. Previously known primarily from Asia and Africa, it was introduced into the Western Hemisphere in 2004. All infected commercial citrus industries continue to decline owing to inadequate current control methods. HLB increase and regional spatial spread, related to vector populations, are rapid compared with other arboreal pathosystems. Disease dynamics result from multiple simultaneous spatial processes, suggesting that psyllid vector transmission is a continuum from local area to very long distance. Evolutionarily, HLB appears to have originated as an insect endosymbiont that has moved into plants. Lack of exposure of citrus to the pathogen prior to approximately 100 years ago did not provide sufficient time for development of resistance. A prolonged incubation period and regional dispersal make eradication nonviable. Multiple asymptomatic infections per symptomatic tree, incomplete systemic distribution within trees, and prolonged incubation period make detection difficult and greatly complicate disease control.

INTRODUCTION AND ETIOLOGY

Huanglongbing (HLB): yellow shoot disease that affects citrus and is often referred to as citrus greening

Asian citrus psyllid (ACP): *Diaphorina citri*

The introduction of biota to new geographical areas has increased in direct relationship to the increase in human movement over the past few thousand years. Throughout history, humans have always been transient; first as hunters and gatherers in search of game and other food and eventually, following the establishment of agriculture, in search of natural resources, arable land, political or religious freedom, etc. Over the past few centuries, there have been exponential increases in global emigration, travel, and trade. Throughout this time period, humans have simultaneously increased the purposeful and inadvertent movement of biota: domestic animals and pest animals (rodents, reptiles, amphibians, etc.); domestic crops, ornamental plants, and noxious weeds; and pests (insect, arachnid, nematode, etc.); and disease-causing organisms (viruses, bacteria, fungi, and other microbes) of humans, animals, and plants. The number and complexity of pathways for the introduction of pests and diseases have surged with this increase and complexity of human interactions.

Citrus is grown within a broad band of approximately $\pm 40^{\circ}$ latitude of the equator. What we know as commercial citrus cultivars and varieties are composed of species of the genus *Citrus* and several other related genera, and a multitude of intergeneric and interspecific crosses, that are plagued with a diversity of pests and pathogens. From the time citrus was first recognized as an edible fruit with positive nutritional qualities, it has moved with man, and so too have its pests and diseases.

The most devastating and feared disease of citrus is huanglongbing (HLB), a Chinese name meaning yellow shoot disease and often referred to as citrus greening in English-speaking countries. The earliest description of HLB-like symptoms was from central India in the 1700s and was referred to as dieback (19). Perhaps the best early description of the symptoms was by Husain & Nath (67) who described a decline and death of citrus in the Punjab. They attributed the decline to psyllid feeding damage, but it was most likely HLB, especially considering their description of "insipid fruit," which is consistent with our modern interpretation of a bitter, acidic flavor of fruit from HLB-infected trees. This was also the first report of an insect, the Asian citrus psyllid (ACP), *Diaphorina citri*, being associated with the problem, which we now recognize as the major insect vector of the disease. Several reports of a similar malady subsequently emerged from southern China where Lin (72, 73), in the Chaoshan district of Guangdong Province, eventually described the disease as a transmissible agent and gave it the name huanglongbing, which describes the diagnostic shoots of yellow, chlorotic, mottled foliage expressed in the spring and fall. Thus, it is likely that HLB may have become established in India before spreading to China. A similar disorder was reported by citrus farmers in South Africa in 1929 (81, 92), and it is in the northeast of South Africa where researchers began calling it greening because of the poor color development of the stylar end of affected fruit (92). The vector, *D. citri*, has been present in Brazil for over 60 years (56). The psyllid has since spread into other South and Central American countries and the Caribbean, and was discovered in Florida in 1998, Texas in 2001, Southern California in 2008, and Arizona in 2009 (30, 55, 70). HLB was discovered in São Paulo, Brazil in 2004 and in South Florida in 2005. The disease has not yet been found in California, Texas, or Arizona; however, it was recently discovered in the Yucatan, the western states of Mexico, Belize, and multiple countries in the Caribbean. In both Brazil and Florida, the disease has spread rapidly throughout commercial and residential citrus plantings. The introduction of both ACP and HLB is believed to be the direct result of human movement of plant material and now threatens nearly all commercial citrus-producing areas worldwide.

Although there are recent reviews of HLB (17, 24, 25, 41), a brief overview of the salient features of the pathosystems, especially in light of some new research findings, is pertinent to an

in-depth discussion of the epidemiology of this complex pathosystem. The disease manifests as severe chlorosis of foliage and dieback, it can result in eventual tree death, and it is associated with increasing crop loss due to disease-induced fruit drop. Fruit become misshapen and develop an undesirable flavor, seeds abort, and the proportion of unmarketable fruit increases in prevalence as the disease progresses in severity within individual trees (17, 24, 25, 41).

The disease is associated with three bacteria: *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter africanus (Laf), and *Candidatus* Liberibacter americanus (Lam). The ranking *Candidatus* is assigned to these bacteria by the International Committee on Systematic Bacteriology because the three bacterial species cannot be maintained in bacterial culture. These bacteria are associated with three unique HLB pathosystems of citrus. Las, which is associated with Asian HLB (greening), is the most prevalent bacteria, and Asian HLB is the most prevalent disease and has been found worldwide, including in the Western Hemisphere since 2004. Until recently, Lam was found only in Brazil (74) but has now been reported in Hunan, China as well. In Brazil, Lam initially constituted a much greater proportion of the total bacterial population there than Las, but this proportion has reversed since 2004, and Las is now the most prevalent species. Laf is related to what is known as African greening (HLB) and is found on the African continent, predominantly in South Africa, as well as Saudi Arabia, and on a few islands in the Indian Ocean (17, 18).

While the ACP, *D. citri*, is by far the most prevalent HLB vector worldwide, the African citrus psyllid, *Trioza erytreae*, transmits Laf in Africa, parts of Arabia, and some Indian Ocean and Atlantic Ocean islands, although either psyllid can transmit any of the three bacterial species (J. Bové, unpublished data). Recent attempts at culturing the bacteria have shown some promise (27, 89); however, durable cultures have so far not been forthcoming. In addition, Koch's postulates have not been completed for any of the bacteria associated with any of the HLB diseases. The full sequence of the genome of Las has now been completed, and the genome sequences of Laf and Lam are nearly complete (28). Interestingly, bioinformatic analyses of the Las genome have indicated that the Las bacteria has a small genome compared with the majority of plant bacterial pathogens and that it does not have all the necessary genes to code for all housekeeping pathways and some other metabolic pathways (28). This could be indicative of why axenic culturing of these bacteria has been difficult as their survival may be dependent on a compulsory association with other microbes. The discovery of the metabolic limitations of Las has led to alternative hypotheses that individual Liberibacters are not capable of causing HLB independently but may require additional endophytic microflora to provide the missing metabolic pathways and perhaps to elicit the full disease syndrome.

CHANGING PERSPECTIVES OF THE ORIGINS OF *CITRUS*, PSYLLID VECTORS, AND LIBERIBACTERS

Although discussion of the origins of *Citrus*, psyllid vectors, and Liberibacters would appear to be somewhat esoteric, from an epidemiological perspective such a discussion may be very important, explain some of the quantitative epidemiological findings, and help place these findings in proper perspective. Prevailing conjecture is that the genus *Citrus* originated in Southeast Asia between India and China or perhaps southward through Malaysia (29, 93) and that HLB originated in citrus in the same general region. However, recent molecular taxonomic analysis of the family Rutaceae subfamily Aurantioideae provides evidence that *Citrus* more likely evolved in Australasia. *Citrus medica* (citron), the first described *Citrus* species, long considered to be native to India, was shown to originate in Australasia (12, 13). An eloquent new hypothesis based on molecular evidence suggests the genus most likely evolved in Australasia (14, 15). This hypothesis states that during the breakup of Gondwana and

Polyetic: describes plant disease epidemics that continue from one growing season to the next, often over multiple years

the separation of Australasia from Antarctica, westward equatorial currents carried buoyant citrus fruits to Southeast Asia approximately 37 mya to 40 mya.

It was Beattie et al. (14) who noted the long overlooked publication by Husain & Nath (67) that presents the first description of the damage caused by *D. citri* in 1915 and 1920, in what is now believed to be the first evidence of HLB in Southeast Asia. Beattie also presented that modern psyllids (Diaphorinineae and Triozinae) probably evolved in Gondwanda (5, 14, 58, 59, 94) but did not arrive in Southeast Asia until the late 1800s, perhaps with Portuguese Maritime traders. Thus, coevolution of the host, vector, and pathogen are highly unlikely. In fact, the disease is a very recent arrival on the scene. When we combine this with the near absence of any resistance or tolerance of the disease within the genus *Citrus* and its near relatives, it would appear that the host-pathogen combination is a very recent occurrence in evolutionary time. Additionally, Las, with its reduced genome, multiplies in and colonizes the hemolymph of the psyllid, presumably concomitant with other insect endosymbionts that provide the end products of its missing metabolic pathways, with minimal detrimental effect to the psyllid's lifecycle. Thus, Las and the other Liberibacters are most likely of animal (insect) origin, and probably insect endophytes. The concept put forward by this author is that we may be witnessing host and pathogen species not previously in contact with each other prior to approximately 100 years ago. When ACP arrived, its Liberibacter endophyte was given its first contact with citrus phloem via the insect's feeding activities. The result was a devastating and lethal new disease of citrus, caused by a pathogen of animal origin (alone or possibly in combination with other microbes), with no innate resistance within citrus to ward off the infection. Therefore, one interpretation from an evolutionary perspective is that this new combination is leading to what would be a severe species or genus culling event, if citrus was still a plant residing only in its natural ecosystem.

TEMPORAL INCREASE OF HLB EPIDEMICS

Because HLB is a polyetic, i.e., multiyear, disease, it has been difficult to conduct quantitative epidemiological studies on HLB. Commercial citrus producers are reluctant to allow the disease to progress without intervention of control activities. This is because the devastating effects of HLB are linked to a severe negative impact on citrus yield and quality and the associated fear of commercial citrus industries to allow uncontrolled inoculum sources to exist in regions near susceptible plantings. Therefore, it is difficult to locate study sites where epidemics can be followed over multiple years to fully understand the epidemic potential (41). From the few existing estimations of epidemic progress, the relative increase of the HLB disease appears slow compared with other vectored plant diseases such as vegetable and field crop virus diseases, even when vector populations are high and inoculum sources are prevalent. However, taking into account the perennial nature of citrus plantings and examining HLB epidemics in the context of other arboreal diseases, HLB epidemics would be considered to increase rapidly. Because of the general lack of data sets where disease incidence closely approaches asymptotic levels, analyses of epidemics are often restricted and incomplete and focus on low to moderate disease incidence. Therefore, the exponential, logistic, and Gompertz models have all been applied to adequately describe disease progress over time (7, 39, 48). A diversity of host, pathogen, vector, and environmental conditions have been reported and thus estimated rates of HLB increase can vary greatly. Disease incidences in various orchards have been reported or estimated to reach high asymptotic levels over a range of 3 to 13 years after the first symptom onset (5, 7, 22, 31, 38, 39, 41, 49). The rate of disease progress is influenced by (*a*) extent of the inoculum reservoir, (*b*) local vector populations, and (*c*) age of the grove at first infection and numerous environmental factors. Where the disease is endemic or there is no effective control by reduction of bacteria inoculum and psyllid vectors, in young

Table 1 Example of HLB epidemic rates

Location	Pathosystem	Terminal age of planting (years)	Terminal incidence of symptomatic trees	Logistic rate (R_L) of disease per year[a]	Reference
South Africa	*L. africanus/T. erytreae*	5	98%	2.89	(22)
Reunion Island	*L. asiaticus/D. citri*	7	96%	0.83	(5, 39)
China (Guangxi)	*L. asiaticus/D. citri*	13	>98%	1.97	(39)
China (Guangxi)	*L. asiaticus/D. citri*	9	100%	0.99	(39)
Indonesia (Bali)	*L. asiaticus/D. citri*	1.75	76%	9.14	(18)
Vietnam	*L. asiaticus/D. citri*	3	96.3%	8.53	(31)
Brazil (São Paulo) (34 plantings)	*L. asiaticus*+ *L. americanus/D. citri*	3	26–37%	0.22–4.62	(7)
Florida (8 plantings)	*L. asiaticus/D. citri*	3–4	15.2–23.6%	1.37–2.37	(Gottwald & Irey, unpublished data)

[a]Logistic rates of disease increase R_L calculated by linear regression of transformed disease incidence.

plantings (up to three years old) disease can reach more than 50% incidence in three to five years, whereas in older groves the disease will not reach such high incidence for five or more years (**Table 1**).

In order to understand the explosive potential of HLB, it is instructive to examine some of the more recent data sets from Vietnam, Brazil, and Florida. In the Mekong River region of South Vietnam, the high level of inoculum and high psyllid vector populations were the cause of a very fast epidemic in orchards established in 2003 (31). Three years after planting, incidence of HLB reached 0.96 in the orchard managed without any insecticide. Even orchards receiving conventional insecticides reached an HLB incidence of 0.74, and those receiving monthly trunk applications of systemic insecticide reached 0.24 HLB incidence, three years after planting (31). In São Paulo, Brazil, the incidence of HLB-symptomatic trees in four citrus blocks that were surrounded by older heavily HLB-affected blocks varied from 0.004 to 0.20 after two years and reached 0.26 to 0.40 the third year. At the same farm, two other citrus blocks increased from 0.24 to 0.70 incidence in one year and 0.06 to 0.274 in 9 to 10 months, respectively (7, 8) (**Figure 1**). In a large commercial planting in South Florida, HLB increased from 0.002 to as much as 0.39 disease incidence in 10 months (41, 46, 68). If we consider the perennial nature of citrus plantings (expected investment payback seven years after planting and economic life span >50 years), HLB epidemics are comparatively rapid, and it would be a rare instance that a planting would be allowed to progress to a high disease incidence before it became nonproductive and was removed.

CROP LOSS

The evolution of symptom severity can be very fast, resulting in a rapid prevalence of severe symptoms distributed throughout the tree canopy. Severe symptoms in trees have been observed one to five years after onset of the first symptoms, depending on the age of the tree at the time of infection, but also on the number of infections per tree, which are often multiple (3, 39, 73, 88). As disease severity increases, the yield is reduced and fruit quality degrades. Yield reduction is mainly due to early abortion of fruits from affected branches and can reach 30% to 100%, depending on the proportion of the canopy affected (5, 9, 10, 22, 87). Despite premature drop, some fruit from diseased trees can be harvested and have fair quality. However, as the HLB severity increases, the percentage of

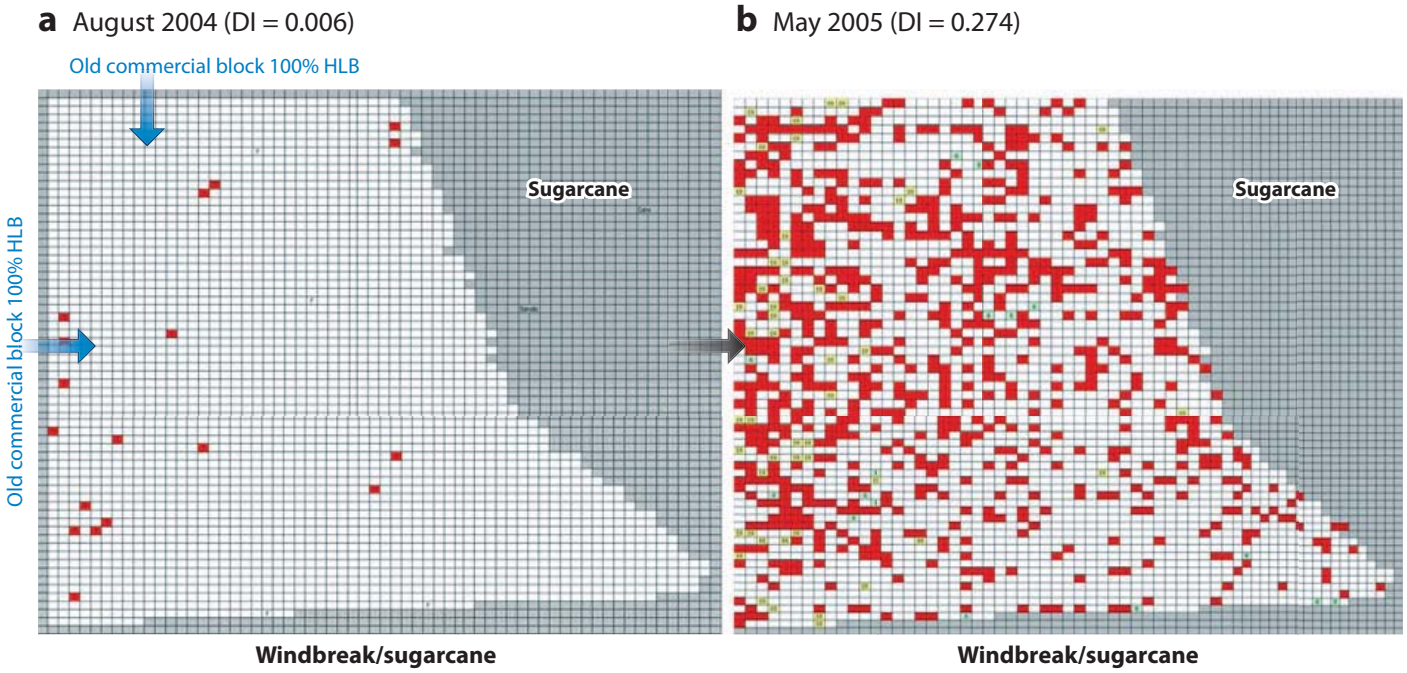

Figure 1

Example of rapid increase and spatial spread of HLB in a commercial citrus planting in São Paulo, Brazil. Planting adjacent to older completely infected blocks to the north and west, and noncitrus (sugarcane) to the east and south. Note higher incidence within the first few rows of trees along the eastern edge of the planting indicating an edge effect of increased incidence. Healthy trees, white blocks; HLB-symptomatic trees, red blocks; trees removed to attempt eradication/management, yellow blocks; DI, disease incidence. Adapted from figure provided by R. Bassanezi.

affected fruit that remains on the tree increases as well and can reach more than 40% of the fruit harvested (9, 22). These affected fruit are smaller, lighter, very acidic, and have a reduced Brix ratio. As HLB severity increases, the percentage of juice and soluble solids per box also decreases, and juice quality can become affected (10). Because of this rapid disease progress, combined with yield and quality reduction, an affected orchard can become economically infeasible within seven to ten years after planting (2, 5, 38, 84). Brazilian researchers modeled the impact of HLB on citrus yield and demonstrated that without HLB control, citrus blocks infected at one- to five-years-old would have high yield reduction two to four years after the appearance of the first symptomatic trees, whereas for citrus blocks older than five years, a significant yield reduction was more often observed five to ten years after the first symptomatic tree was observed (6).

Brix: sugar to acid ratio. A measurement variable used to define quality of fruit

Polymerase chain reaction (PCR): a technique to amplify single or few copies of a piece of DNA by several magnitudes

WITHIN TREE DISTRIBUTION OF LAS: INFECTION VERSUS PCR DETECTION

The presumed causal agent, Las, is incompletely distributed within the vascular system of infected trees (3, 25, 41). When budwood or graft wood is taken from HLB-infected trees, not all trees grown from propagations result in new HLB-infected trees. The proportion of propagations that result in new infected trees varies greatly depending upon the intensity of systemic infection of the mother tree. In a recent study, two four-year-old trees, each visually showing the first initial stages of HLB infection, were uprooted and their root systems and all aboveground parts dissected into approximately 20 cm long sections. Each section was assayed independently by polymerase chain reaction (PCR) for Las, and a numbering system was devised such that the position of each segment within the dendritic structure of the

canopy was recorded (71). A computer program was used to calculate vascular distances between PCR-positive sections, both above- and belowground. The vascular distances were used to generate frequency distribution relative to distance between PCR-positive sections to the nearest 20 cm. Interestingly, PCR-positive sections were found throughout the entire canopies and root systems, although not every section was PCR positive, indicating a diffuse systemic infection throughout the trees (50). An intriguing aspect of these frequency distributions was the repeating peaks that occurred within the frequency distribution relative to distances of approximately 200, 400, 800, 1000, 1200, and 1300 cm. The discontinuous nature of the systemic infection perhaps indicates that the bacterial titer in some sections of the tree is below the threshold for PCR detection, yet those presumptively PCR-negative sections may indeed be infected, and although largely visually asymptomatic, the phloem throughout the tree has sufficient bacterial titer for psyllid vectors to acquire and transmit the bacteria, i.e., spread the infection.

INCUBATION, LATENCY, INFECTIVITY, AND DETECTION

The incubation period for HLB can be quite variable; from a few months to one or more years (1, 21, 39, 79, 95, 96). Additionally, monitoring the occurrence of visual symptoms can be problematic because of the variability in time between psyllid vector transmission and the onset of visual symptoms among trees of the same age. Highest psyllid populations and migrations occur when new flush is available (1, 20, 21). However, infected adult psyllids and nymphs are found in citrus plantings throughout the year with no correlation between the percent of infected psyllids and higher psyllid populations (78). Thus, trees expressing the onset of infection at the same time may have been infected at different times in the past. Data from large commercial orchards in Florida under extensive psyllid management and HLB-diseased tree removal programs have indicated that 2 to 2.5 years are required to reduce the backlog of asymptomatic infections before these management strategies begin to show any effect (M. Irey, unpublished data). This is indirect but strong evidence of a 1 to 2.5 year incubation period within established commercial orchards 7- to 10-years-old. Younger orchards will express symptoms within 6 to 12 months after planting, indicating that young, rapidly growing trees, much smaller in canopy volume, have a shorter incubation period. Observations of trees over 10 years of age indicate even slower symptom development. Although not quantitative, the above observations, taken together, paint a picture of a highly variable temporal incubation window that can range from a few months to multiple years. Incubation period and latency are two concurrent and related temporal processes, both beginning at infection. Latency ends when infectivity begins and is usually followed by the end of the incubation period, when the plant becomes symptomatic.

HLB latency is also highly variable and apparently greatly affected by tree age, horticultural health, and other factors. It is likely that an asymptomatic tree may have acted as a source of infection for numerous other trees, but disease symptoms were visually subclinical in the source trees at that time. At present, owing to the limitations of PCR, infection cannot be detected for some time after vector transmission. As noted above, for trees that are displaying only very few HLB symptoms, the infection may be completely or nearly completely systemic; however, the bacterial titer is variable in individual portions of the tree and may be below the threshold of PCR detection but in graft experiments can result in new infected trees (50, 90, 91). Thus, even if a tree is infected, samples collected from a portion of the tree with low or no titer (usually asymptomatic) will yield a false negative assessment. Although PCR allows us to detect many asymptomatic infections, we are still only detecting a portion of the more recent asymptomatic infections in the planting, and an unknown number of infections exist with titers below our ability to detect. PCR is both complex and time consuming,

Incubation period: the time between infection by a pathogen and onset of symptom expression

Latency: the time between infection by a pathogen and the onset of infectivity

Infectivity: the potential of a microorganism to cause infection

RT-PCR: real time polymerase chain reaction

and at this point in time the ability to process the thousands of samples necessary to track an epidemic, with methods such as PCR, remains manpower and cost prohibitive. However, in a recent study using PCR, nearly the same number of asymptomatic but real time polymerase chain reaction (RT-PCR) positive trees within plots were found as visually symptomatic trees (69). This study indicates that if a visual inspection reveals n% of symptomatic trees, it can be assumed that there are in fact approximately 2n% infected trees present, plus an additional population of infected trees that remain subclinical relative to RT-PCR assay. This experiment was carried out in the winter, a suboptimal time to observe HLB visual symptoms. The incidence of infected/symptomatic trees probably would be much higher if the assessments were done during spring or summer. In a second recent study comparing various potential disease control strategies, it was estimated that for every symptomatic tree in the plantings studied, a mean of 13 (range 2 to 56) HLB-positive but asymptomatic trees existed in the plantings that expressed symptoms in subsequent assessments over time (T.R. Gottwald, unpublished data). Thus in HLB-infected plantings, there are potentially manyfold more HLB-infected trees than detectable either visually or via PCR.

This finding greatly affects disease management and decision-making strategies relative to the productive life span of a planting. If we know the visual disease incidence, we can estimate the subclinical incidence as well and thus the total incidence. This estimation provides a means to determine a threshold of visual disease incidence beyond which it would be more economically beneficial to remove an infected planting and replant the area with disease-free trees than to continue to attempt to manage a planting when it will likely be marginal or nonprofitable through time (41). With these caveats in mind, useful information has been gleaned from new epidemiological studies concerning how HLB disease spreads and increases, as well as the spatiotemporal processes that give rise to the disease. This information can be used to predict the economic and physical life of a given planting and is a means to investigate the influences and efficacy of possible control interventions (41).

SPATIAL DISTRIBUTION OF HLB

Understanding of the spatial distribution of HLB is valuable for developing detection methods, sampling and survey methods, and control/mitigation practices. Of course, the spatial distribution is somewhat variable and depends upon the scale at which the disease is viewed (66, 76, 85). We have already discussed the distribution within one tree (plant scale), but we can also look at the distribution of HLB among immediately adjacent trees (local scale), within one block (planting scale), within an entire plantation (plantation scale), and regionally among multiple plantations (regional or landscape scale). When larger scales are viewed, it is obvious that citrus plantings are often somewhat to highly discontinuous across most regions. This discontinuity affects the success of pathogen dispersal and infection. Since the discovery of HLB in Brazil and Florida, many maps of the disease have been compiled from visual surveys. These have been analyzed at various spatial scales to determine the spatial pattern of the disease to draw some conclusions concerning spread (8). The results of these analyses of Western hemisphere data mirror those previously found in Reunion Island and Asia.

Some evidence of aggregation (heterogeneity) of HLB among immediately adjacent diseased trees was demonstrated by the ordinary runs analysis, which is a unidirectional analysis, in all plots tested, but this was not particularly strong (8, 38, 40, 41, 77). In many cases, there was some orientation or direction to aggregation in most plots indicating that within-row aggregation (where trees are planted closer together) was slightly stronger than across-row. This directional orientation for aggregation was best demonstrated in data from Shantou, China, where a higher degree of aggregation was indicated (155/199 = 77.9% of rows tested) in the north-south direction compared with (91/205 = 44.4% of rows tested)

the east-west direction (38). This greater north-south aggregation was in the same orientation as the raised planting beds, which corresponded to predominant orchard traffic patterns. For Reunion Island and Shantou plantings, isopath maps of disease severity demonstrated higher concentrations of disease initially around the perimeter or edges of the plantings (41). This plantation edge effect is discussed more fully below. The same was true for analyses of a large number of plantings with HLB in Brazil. Aggregation among HLB-symptomatic trees was detected by ordinary runs analysis, and clustering existed in both within- and across-row directions. However, as with the Reunion Island and China data, the percentage of aggregation within and across rows was low (7).

On the local scale, the association of HLB-diseased trees within groups has been examined by Beta Binominal Analysis (BBD) as an overall assessment of heterogeneity of disease incidence (65, 77). Aggregation at the group scale was demonstrated for all plots at all locations and all quadrat (group) sizes for the majority of years (38, 39). In Brazil, the binomial index of dispersion for various quadrat sizes suggested aggregation of HLB-symptomatic trees for approximately 40% of the plots tested (7), whereas aggregation was indicated for all plots tested in a large commercial plantation in South Florida (48).

It is possible to perform a general analysis encompassing a large number of plantings simultaneously, by employing the binary form of the Taylor power law (TPL) (64), which relates the observed variance (V_{obs}) and the expected binomial variance (V_{bin}) for a random distribution of binary data. In this case, $\log(V_{obs}) = \log(A) + b\log(V_{bin})$, where A and b are parameters. Using the binary form of the TPL, least squares regression is performed using counts data from multiple plantings. Recently, data collected in both Brazil and Florida from a large number of individual plantings were parsed into various quadrat sizes and analyzed via TPL. Fitting the equation above to HLB data sets, all estimates of b and A were statistically different from 1 ($P < 0.05$), which indicated a general and significant pattern of aggregation of symptomatic plants within all quadrat sizes tested. Values of b higher than 1 also indicated that the degree of aggregation was a function of incidence. Thus, when all data from both Brazil and Florida and earlier data from Reunion Island and China are considered together, HLB spatial patterns at the field or planting level are nearly always aggregated.

Taylor power law (TPL): an assay for heterogeneity

To examine the relationship among groups of trees over longer distances, spatial autocorrelation has been used to examine data from China, Brazil, and Florida (8, 51, 77, 80; T.R. Gottwald, unpublished data). In all cases, discontinuous clusters were indicated at various distances within row, across row, and at oblique angles. Considering all of the data analyzed to date, these reflected clusters are quite variable in size, encompassing 8 to 572 trees, and are located 25 to 70 meters from the main clusters of disease, indicating psyllid vector movement resulting in transmission both to nearby trees, causing clusters, and to trees at considerable distance, initiating new foci of infection. Such a pattern of widely spaced foci perhaps indicates a spatial mechanism associated with longer distance vector movement. That is, when psyllid vectors move, either naturally, owing to crowding, in search of new feeding opportunities, or when disturbed, they occasionally do so to other than nearby trees.

EVIDENCE OF EDGE EFFECTS

As previously mentioned, data from China, Reunion Island, Brazil, and Florida all indicated occasional higher than expected incidence of HLB-positive trees at the periphery of plantings (38). Closer scrutiny of the distribution patterns revealed that HLB-diseased tree accumulations are also associated with roads, canals, ponds, and other geographical features within citrus plantations. Each of these features can be defined as an interface of some void of trees immediately adjacent to areas with dense citrus. It is within the citrus planting immediately adjacent to these voids where a higher

IPF: inverse power function

Bacteriliferous: a condition of insect vectors indicating that they are infected with and can transmit pathogenic bacteria

than expected number of HLB-diseased trees accumulate. Examination of the georeferenced data of HLB distributions in large citrus plantations in south Florida provided evidence of this edge effect. When the perimeters of entire plantations were examined as a whole, there was a strong decreasing curvilinear relationship with distance described well by an inverse power function (IPF). The IPF, often used to describe disease gradients of other pathogens, demonstrated a rapid decrease in HLB incidence with distance from the perimeter toward the interior of the plantation (45). The potential effect of internal planting roads, irrigation ditches, ponds, and other voids was also examined. The majority demonstrated a pronounced edge effect and fit well by the IPF model (45).

These results provide evidence that the interface of the planting with zones of noncitrus at its perimeter as well as voids internal to the planting created by roads, canals, ponds, and other features all contribute to HLB epidemics as potential linear and/or curvilinear foci of disease because HLB infections tend to accumulate in proportionally higher incidence at these interfaces. Intuitively, the spatial process behind edge effects is related to bacteriliferous psyllid transmission and thus to psyllid movement and migration. As psyllids forage for new feeding sites, between and among plantations and individual blocks, they apparently preferentially accumulate at the interface or edges of plantings and interior voids. This is not to say that they do not penetrate into plantings as well, but there is a higher than expected accumulation at the edge of this interface, indicating that a majority of the migrating psyllid population will alight within the first few trees that they encountered at the edge of a planting. Therefore, the HLB-disease distribution is an indirect indicator of psyllid migration and foraging preferences and response. By understanding this edge effect, we might be able to take advantage of it for psyllid control/disease management strategies, either by preferentially employing management strategies at the edges of plantings or using this information to design plantings with minimal edge interfaces to reduce infection.

PRIMARY VERSUS SECONDARY SPREAD: RANGE AND SPATIAL SCALE OF PSYLLID TRANSMISSION

The edge effects described above are presumably a good example of primary spread of HLB. To understand the contribution of primary spread from external sources versus secondary spread within plantings in HLB epidemic dynamics, a Markov-chain Monte Carlo simulation routine was used. The routine uses likelihood estimates of the posterior density of model parameters to parse spread into primary and secondary components to best explain the progression of HLB spatial patterns over time (7, 32–34, 49). Both Brazil and Florida plantings were examined. The overall interpretation was that there are two spatial processes that are ongoing during HLB epidemics, although not necessarily simultaneously. Three categories of results were indicated by these analyses. The first category was the rarest and demonstrated a predominance of background or primary spread of disease that originates from outside the plot areas. In this category, psyllids appear to be immigrating into the planting from outside sources and transmitting the pathogen. The second category was more common. Here, posterior density estimates provided evidence of secondary spread via predominantly midrange local interactions (which are not to nearest neighboring trees, but rather to trees that are nearby within a local area of influence) for dispersal of inoculum within the boundaries of the plots through time. This category provides evidence that psyllids are transmitting the disease agent within the plantings, but not necessarily to the nearest neighboring plants. The third and most prevalent category indicated that HLB spread occurs as an incessant mixture of the two spatial processes, i.e., a continuous introduction of inoculum from outside the plot combined with local spread from within the plot occurring simultaneously (8, 48, 49).

REGIONAL DISTRIBUTION AND IMPLICATIONS OF SPREAD

The regional distribution of HLB has also been examined in a single case. It is rare to have complete HLB spatial point pattern data spanning numerous adjacent plantings at the plantation scale. One data set, including thousands of trees from a very large planting in south Florida, was analyzed using a modified Ripley's *K*-function. Spatial point pattern analysis for each of the five assessment dates gave strong indications of regional spread of HLB (40, 49). The range of spatial dependency (RSD) indicated a departure from complete spatial randomness (CSR) over the majority to the entire range (from 0 to 4.6 km). The RSD was estimated to be 3.32 to 3.5 km, with a median distance of 3.5 km, and was relatively stable through time. This indicated that the spatial structure of HLB-infected trees was highly related over large distances. The maximum departure from randomness ranged from 0.88 to 1.61 km, with a median of 1.58 km, and increased over time with disease incidence over the 2-year period of the test. This suggests that there is a spatial relationship that is repeated most frequently at approximately 1.58 km and may well indicate a common or average distance for psyllid dispersal of HLB regionally (48, 49).

Citrus producers voice concern about the threat of an HLB-infected tree to surrounding trees within a planting block or the entire plantation. In a recent study, survival analysis was used to address this question. Survival analysis has been used recently in botanical epidemiology to examine plant disease epidemics and the factors affecting these epidemics through time, such as the effect of roguing of diseased plants (26, 86). Recently, this was used to address the spatiotemporal contributions of short-distance transmissions of various diseases, including HLB by psyllids, and the influence they have on the overall spatial pattern of disease that develops through time (49, 53). However, for HLB the influence of distance from prior symptomatic trees in the near vicinity or even within the block in general does not contribute greatly to survival, i.e., the probability of a tree remaining disease free. Thus, this study implies that the overarching influence in HLB epidemics is the migration and transmission of Las via psyllids from outside the block, i.e., the influence of primary spread. It also indicates that attempting to control HLB locally is probably ineffective. Significant control will likely only be achieved from regional disease management strategies.

In Brazil, both Las and the newly described Lam were first discovered in 2004 near the city of Araraquara in the state of São Paulo, near the center of commercial citrus production. Subsequent surveys indicated that the highest incidence of disease is also centered in this area providing indirect evidence that this area is the most probable point of introduction. An overall assessment of the aspect of the disease in the area considering trees believed to have the oldest infections indicated that the bacteria were likely introduced into the area approximately 10 years prior to detection. Measurement of the distance from the presumed point of introduction to the advancing edge of the epidemic resulted in an estimated distance of spread of approximately 193 km (120 miles) or approximately 19.3 km (12 miles) per year (41, 48). In Florida, HLB was first discovered in 2005 by a Florida Department of Agriculture and Consumer Services scientist during a routine survey in a commercial tropical fruit nursery in Florida City. An immediate delimiting survey confirmed the distribution extended northward 193 km (120 miles). However, unlike Brazil, there is no clear indication of a point or even a limited area of introduction. Rather, one or more initial foci are presumed to have been introduced somewhere within the southeastern Florida metropolitan residential area. Simultaneous survey was undertaken of selected commercial plantings nearest to the known residential distribution along the southeast Florida coast. Measurements from the initial discovery point and the nearest residential infections to the most adjacent and newly infected commercial planting are approximately 144 km and

Range of spatial dependency (RSD): the distance over which a spatial analysis indicates a departure from spatial aggregation or heterogeneity

Complete spatial randomness (CSR): defines a disease pattern in a planting lacking any aggregation or regular distribution

88 km (90 miles and 55 miles), respectively, across the Everglades marsh devoid of citrus. This nearest infected commercial planting was both isolated and did not acquire nursery materials from outside sources. Thus, the assumption is that Las arrived in this planting via psyllid vector dissemination and transmission. Although not conclusive, it is further possible evidence that long distance movement of HLB by psyllid vectors could, in this case, be related to movement of air masses, perhaps during hurricanes or tropical storms, carrying infected vectors over a void of citrus host plants.

SURVEY AND SAMPLING IMPLICATIONS

Effective control of HLB is predicated on early detection and rapid response. We know that visual detection is inadequate due to a potentially long incubation period that leads to an underestimation of disease, i.e., false negatives. We also know that PCR, the current preferred method of disease confirmation, is subject to false negatives due to incomplete distribution of the bacteria in trees and even among cells within discrete tissues, yielding both positive and negative samples from various tissue samples originating from the same tree. Thus, there is an urgent need for more sensitive detection and confirmation methods to make survey and sampling more effective. Multiple motivations exist for sampling, e.g., identification of individual diseased trees or blocks for control/eradication, targeting mitigation strategies, estimation of incidence for economic or logistical decision making, etc. Detection is accomplished by a number of different surveys, each designed for a different purpose (75). If eradication is deemed feasible and initiated, then intensive detection surveys within the infected area are deployed to find all foci of infection for elimination. Simultaneously, outside the infected area commercial and residential sentinel surveys are often used to continually search for new outbreaks and detect them as early as possible (44, 52, 60–63). For HLB, in all cases to date, incidence and distribution of the disease have been too widespread when first detected to attempt eradication. Even so, regional surveys are highly useful to identify foci of infection and delimit the extent of the epidemic. Florida presently employs a multi-pest survey designed to repeatedly survey the approximately 222,577 ha (550,000 ac) citrus industry, while simultaneously monitoring HLB distribution and attempting to detect other citrus diseases based on a stochastic model (83). If disease can be eliminated from an area, this same approach can be used for perpetual-intermittent surveys to continually and repeatedly survey to ensure freedom from disease is maintained. An additional regional survey presently under development takes advantage of demographics and travel data. This survey places a slight bias on surveys of areas deemed at higher risk from human travel to and from countries with known infections of HLB (T.R. Gottwald & T. Riley, unpublished data).

Recently, a hierarchical sampling method, originally designed for other *Citrus* and *Prunus* arboreal pathosystems, has been adapted for HLB survey (60, 61, 63, 68). This method takes advantage of the known distribution patterns of HLB to estimate the incidence of HLB at the individual tree scale by testing a subset of all groups at the group scale by visual, PCR, or other detection methods. Knowledge of the relationship between visual disease estimates and PCR assay estimates of HLB distribution can be utilized to predict total (visual + asymptomatic) incidence by hierarchal sampling using only visual assay.

A new method that utilizes the epidemiological processes of spatial spread and host heterogeneities is currently under development to estimate HLB distribution from small subsamples. An iterative optimization approach utilizes information on the underlying host distribution and on the spatial complexities in pathogen dispersal and infection to accurately map the probability of disease at unsampled host locations. The approach is pathogen generic but is especially relevant for diseases such as HLB that exhibit strong spatial dependencies (82).

DISEASE CONTROL

At present, there is nowhere in the world where HLB is under adequate control, and where the disease does occur, it continues to increase in incidence and severity. The first line of defense for HLB has always been quarantines to ensure the bacteria is not introduced and established. However, with ever-increasing international trade, travel, and immigration, the probability of unintentional introduction continues to rise. The United Nations Development Programme Food and Agricultural Organization (UNDP FAO) conducted a multinational Southeast Asian citrus rehabilitation project during the 1980s and 1990s, the main goal of which was to promote a greater understanding of HLB in the area and development of practical disease control strategies (2). One of the most important contributions of this project was the compilation of an overall strategy for HLB management based on experience of commercial producers and agricultural agencies, which remains the cornerstone of HLB disease control today. The major recommendations resulting from this project were:

- Control of psyllid vectors in commercial plantings by chemical insecticides and, where applicable, via biocontrol to reduce transmission.
- Removal of HLB-infected trees in commercial plantings to reduce inoculum sources.
- Geographical isolation and disease certification programs for budwood sources.
- Geographical isolation of nursery production.
- Requirement that all citrus nursery production be conducted in secure insect-proof screen houses.

Chemical control of HLB psyllid vectors requires multiple sprays yearly to reduce and maintain low vector populations. Depending upon the economics of individual producers, the heavy financial burdens of spraying can be marginal to infeasible. An even greater challenge is the large population of adjacent HLB-positive residential citrus trees where regulated chemical control is not an option in the United States, but might be feasible in some countries. Moreover, many insecticides presently used to control psyllids are systemic, requiring immigrating psyllids to feed to acquire lethal levels of insecticide. This feeding can result in pathogen transmission prior to death of vectors.

The effectiveness of roguing (i.e., removing) infected trees to control HLB is directly related to latency of infection. Although diseased-tree removal is recommended, it is recognized that numerous infected but asymptomatic trees probably exist in the vicinity and that these early stage asymptomatic trees contribute to pathogen dispersal.

The incidence of HLB infections within the surrounding region greatly affects the probability and efficacy of slowing the epidemic. Recent control studies in small replicated plantings demonstrated no significant difference among combinations of insecticide treatments and roguing versus no treatment (T.R. Gottwald, M.S. Irey, J.H. Graham, unpublished data). Gatineau demonstrated only a slight decrease in the rate of disease progress from heavy use of insecticide sprays in Vietnam plantings (31). In both cases, lack of control was most likely due to continuous primary infection via immigrating bacteriliferous vectors from surrounding plantings.

A commercial producer may be able to diligently control vectors and rogue infected trees, but if surrounding infected plantings and adjacent residential trees are not as rigorously managed, the planting may be overwhelmed with immigration of bacteriliferous vectors. Additionally, the efficacy of vector control and roguing is much higher in the initial stages of an epidemic. When HLB incidence builds up in areas over time, the efficacy of even diligent HLB control efforts is much reduced. Thus, regional control has been proposed. Some producers are forming groups to collaboratively spray entire regions in an attempt to suppress vectors, thereby reducing both primary and secondary spread.

One of the most logistically difficult and costly portions of the citrus industry to

SIR: class of epidemic models considering variables for susceptible (S), infected (I), and removed (R) individuals

manage is the nursery propagation. Collectively, the industry must secure all budwood and nursery operations by migrating from the industry norm of field nursery operations to protected insect-proof screen or glass houses. Sufficient HLB-free replacement trees must exist for normal turnover and increased demand owing to increasing HLB-infected tree removals. If not, the result will be a reduction of viable citrus production areas as diseased trees are continuously removed at a higher rate than replacement, which quickly affects production and downstream marketing.

Biological control has been attempted by the importation of hymenopterous ectoparasites of ACP. Some success via biological control of vector populations has been achieved in Reunion Island, where it is believed that the hyperparasites reduced the psyllid populations significantly and lessened the damage of HLB (4, 20, 23, 30). Elsewhere, biocontrol via introduction of parasites has also been attempted but has had very limited success (4).

Recently, attention has been given to the use of systemic acquired resistance (SAR) compounds such as salicylic acid and phosphite combined with micronutrients to sustain infected trees in a productive state, especially by industry producers of infected plantings who believe their options are limited. However, in some cases, there was not a significant difference between treated versus nontreated HLB-positive trees, relative to tree decline, fruit drop, yield, and quality. Additionally, there was no significant difference in Las titer between treated versus untreated trees, indicating that such a practice would hazardously preserve inoculum sources (T.R. Gottwald, J.H. Graham, M.S. Irey, B. Wood, unpublished data).

A control method under examination is the use of repellent plants. Vietnamese growers in the Mekong Delta discovered that citrus plantings survived several years longer when interplanted with guava (*Psidium guajava*). Vietnamese/Australian/Japanese collaborative studies and additional studies in Indonesia have shown pronounced initial reductions in rates of disease increase in citrus/guava interplantings compared with citrus monocultures, apparently due to volatiles given off by the guava that repel psyllids (14, 43, 57). The guava effect is now under study in Florida to determine if it is compatible with western citriculture practices as well.

A final approach is to simultaneously maximize efficacy, fiscal, and manpower inputs. Presently, a stochastic epidemic SIR (susceptible, infected, removed) model is under development that will allow policy makers and pathologists to examine a range of what-if control/eradication scenarios for HLB and other disease pathosystems. The epidemic model has as few parameters and variables as possible and initially considers three classes, healthy trees susceptible to infection (*S*), infectious trees (*I*) both infected and capable of infecting other trees, and trees that die or are removed (*R*). Other components can be added such as a dispersal kernel (α, estimates how far inoculum is dispersed by vectors), transmission (probability of infection when inoculum contacts a susceptible host) characterized by primary (ε, spread from outside the area) and secondary (β, tree to tree spread) transmission rates, and duration of infectiousness ($1/\mu$). Various scenarios can be tested by stochastic simulation to estimate posterior probability distributions using Markov-chain Monte Carlo methods (35–37). Policy makers can use the model to identify an optimal strategy, which may require weighting several variables, including not only the total costs of control, but also the duration of the epidemic, the total number of trees needed to be removed, manpower, and the socioeconomic implications of perhaps a slightly longer eradication program but one more tolerable to producers, homeowners, etc.

The ultimate control of HLB is the development of resistant citrus cultivars. Unfortunately, no readily available genes or source of resistance has yet been identified for use with either conventional breeding or transgenic improvement systems. However, it is hoped knowledge acquired from both the newly sequenced Las pathogen and citrus host species,

combined with bioinformatic studies, will point to resistance genes or pathogen vulnerabilities and thus paths for future incorporation of disease resistance mechanisms.

SUMMARY POINTS

1. HLB is the most destructive of all citrus pathosystems worldwide, and nowhere in the world where it exists is it under adequate control. To date, there has been a decline in all commercial citrus industries that have faced the disease.
2. Temporal rates of HLB increase are related to psyllid vector population prevalence and spatial spread and are quite rapid compared with other arboreal pathosystems.
3. The dynamics of HLB can result from multiple spatial processes. Introduction and subsequent spatial spread can be related to human movement of plant materials, establishing new foci of HLB. Once established, spatial spread is also quite rapid and regional in character, suggesting that psyllid vector movement is a continuum from local area, but not necessarily nearest neighbor, to very long distance, i.e., up to multiple kilometers, and can be augmented by severe weather patterns that can transport insect vectors long distances. This provides justification for the efficacy of regional psyllid vector control.
4. Evolutionarily, HLB appears to not have originated as a plant disease, but rather as an animal (insect) endosymbiont that has moved into plants (i.e., *Citrus* species and other Rutaceous *Citrus* relatives) within the past century or two. Lack of prior exposure of citrus species to Liberibactor species also precludes any selective pressure toward the development of resistance genes, whereas the Las, Laf, and Lam bacteria apparently infect the psyllids *D. citri* and *T. erytreae* with no apparent deleterious effects, suggesting an evolutionarily advanced symbiotic association.
5. Recent completion of the sequence of the Las genome and subsequent analyses have determined that the Las genome is small and incomplete, apparently lacking some housekeeping genes. This suggests that Las is an obligate parasite and/or auxotroph requiring the missing gene products to be supplied by other microbes (perhaps other endophytes) or a citrus host. Potentially, this explains why Las has resisted culturing and why recent reports of culture success were only as cocultures with other bacteria, and these cultures were not durable and soon lost. Obligate dependency on microbial or host-gene end products may provide clues to potential control strategies.
6. HLB can have a long incubation period prior to symptom expression. The length of the incubation period is influenced by tree age and health. When assessing the disease visually, there can be two- to manyfold more asymptomatic infections already established compared to those that are visually symptomatic. This greatly complicates disease control.
7. HLB is highly invasive. When introduced to new areas, owing to the prolonged incubation period prior to symptom expression, the disease is usually at too high an incidence level and too spatially dispersed regionally for eradication to be viable.
8. Incomplete systemic distribution of Las within trees and the period between infection and sufficient titer increase for detection makes detection and confirmation of asymptomatic infections very difficult. Trees appear to be infective (the bacteria can be acquired and transmitted by psyllid vectors) prior to titer reaching PCR detection levels, making the disease commercially difficult to control.

FUTURE ISSUES

1. The basic reproductive number (R_0) has not been calculated for HLB because of the complexities of determining precise latency and infectious periods. However, we know that latency can be variable and affected by tree age and that once infected, trees can survive and serve as inoculum sources for multiple years before they die. Logically, it follows that R_0 would be large, implying that HLB epidemics will be quite difficult to control or mitigate.
2. More work needs to be done on the apparent microbial interactions of Las and the obligate parasite/auxotrophic nature of the Liberibacters.
3. Modeling the effect of vector-repellent volatiles from intercrop species such as guava as a control for HLB versus current chemical and horticultural control strategies needs to continue. Plants that produce insect inhibitory or repellent volatiles have a distinct advantage compared with traditional control methods such as insecticides. Repellent volatiles preclude vector feeding and thus inhibit transmission and infection, whereas chemical control necessitates feeding to acquire the insecticide prior to mortality providing the opportunity for pathogen transmission and subsequent infection. The effect on the epidemic rate needs to be determined.
4. Quantitative epidemiological analyses will be required in future studies to determine the contribution of insect vector control versus inoculum reduction by roguing in HLB epidemics.
5. It is important to determine the quantitative contribution of the citrus host at various stages of disease—asymptomatic but infected trees versus newly symptomatic trees versus trees with long-standing infections—to the rate of HLB epidemic progress and spatial spread.
6. Host/pathogen/vector interactions need to be examined on a seasonal scale. Determine the seasonal contribution of HLB-infected trees to act as inoculum sources. The proportion of bacteriliferous vectors within the total population is dynamic and varies temporally. Determination of the relative rates of transmission of vector populations through time will help to target and maximize control efforts to those time periods when transmission is the highest.
7. It is important to develop economic models integrated with epidemic models to establish disease thresholds that trigger plantation rotation cycles for maximized yield, quality, and profit.

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Errata

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