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Letter to the Editor

Huanglongbing solutions and the need for anti-conventional thought

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Abstract

Citrus huanglongbing (HLB) has been recognized for a century yet control and management remain elusive despite over 90 years of research. The bacterial pathogen is an insect endosymbiont that was most likely inadvertently introduced into citrus where it found a compatible environment for growth in citrus phloem cells and therefore jumped from the animal to plant kingdom. Because the genus citrus did not coevolve with the bacteria it has no resistance and little tolerance to it and the resulting vascular disease is severe. The winged insect vector of the bacteria, the Asian citrus psyllid (ACP), is an exotic introduced species in its own right, prolific, and difficult to control even on a regional spatial scale. The resulting disease has a long latent period prior to symptom expression and a challenging cryptic period during which detection by convention PCR and other methods can be elusive. The result is an unusually rapid increase and spread of the resulting disease. This article offers some nonconventional perspectives to examine this unusual and devastating pathosystem to stimulate thought toward improved control/mitigation.

Keywords: Huanglongbing, Liberibacter asiaticus, Asian citrus psyllid, endosymbiont, reproductive rate, cryptic infection, latency, detection

Much has been written about huanglongbing (HLB) and the devastation it causes to citrus industries where it occurs. HLB was likely present in China in the 1800s, but wasn't described until 1927 in India (Husain and Nath 1927). Over time, HLB has spread throughout the Eastern Hemisphere into most major citrus growing regions of the world with the exception of Australia, most of Africa, and the Mediterranean (Bové 2006). Currently, hundreds of researchers worldwide are working on HLB, its bacterial vector, and a multitude of bacteriological, genetic, and epidemiological aspects of this challenging and unique pathosystem. However, with over 90 years of research, control of the disease remains largely ineffective and a cure elusive. The research community generally agrees that disease resistant germplasm is the most likely ultimate solution and many breeders and molecular biologists are diligently pursuing this goal, with some promising advances. But even if resistance was in hand today, it would require decades to determine the horticultural suitability, quality, etc. of the new cultivar, propagate sufficient nursery stock, repopulate sufficient plantings, and bring them into production to revitalize declining citrus industries.

Our conundrum involves 3 diametrically opposed components: First, a clonally propagated, perennial crop that requires multiple years to bring into production/profitability. Second, an 'inadvertent' disease agent, *Candidatus* Liberibacter asiaticus (*CLas*), which is a bacterium (bacteria are highly problematic because they are the most mutagenic and adaptable organisms on the planet). The inadvertent nature of *CLas* as a disease agent is discussed below. Third, the *CLas* vector, Asian citrus psyllid (ACP), which once established is prolific and probably impossible to eradicate. Therefore, not only do we need resistance, we must actually breed citrus with multigenic resistance (a combination of multiple genes each imparting different resistance mechanisms) to ensure that the newly planted resistant trees won't be overcome by a new *CLas* mutant. That is, the breakdown in resistance may not be seen sooner, but later, i.e. it may breakdown prior to newly developed trees reaching profitability or soon thereafter, rendering the transient resistant trees of limited value. Numerous other research approaches to HLB control/mitigation are simultaneously underway such as cultural practices (i.e. soil acidification and other horticultural remedies), antimicrobial/antibiotics, nano-zinc compounds to reduce or eliminate *CLas* populations, RNAi technologies to reduce the survival of ACP and/or *CLas* in the vector and/or citrus, thermal therapy, and biocontrol, to name a few. But whether we are investigating genetic modifications to citrus or the aforementioned technologies, standard research approaches have been slow to yield noteworthy solutions.

New genres of anti-conventional thought are needed to overcome challenges we face from this unique pathosystem. Although there are many, 2 examples of historical scientific figures who impacted the world with anti-conventional thought are Leonardo Da Vinci (1452-1519) and Galileo Galilei (1564-1642). Da Vinci was a profound scientist, observer, anatomist, and inventor as well as 1 of the world's greatest artists and sculptors. His unique thought processes laid the groundwork for the airplane, parachute, bicycle, helicopter, war machines (i.e. armored car (tank), modified trebuchet, etc.), scuba gear, and revolving bridge. Galileo, despite living during the Roman Inquisition, was a consummate physicist, mathematician and anti-conventionist, whose studies of falling bodies contributed greatly to the understanding of gravity by subsequent researchers. Galileo also revised the Copernican theories on heliocentrism (theory that the sun, not the earth, is the center of the solar system), which led to admonishment by the church in 1615. He was subsequently tried by the Inquisition in 1633 and found "vehemently suspect of heresy", forced to recant his writings and spent the rest of his life under house arrest at his villa near Florence. These 2 Renaissance scientists exemplify nonconventional innovators and deep theoretical thinkers whose works have been the underpinnings of innumerable inventions, scientific endeavors, and discoveries over succeeding centuries. Perhaps analogous departures from mainstream scientific thought could be useful in our struggle against HLB. Simply given the premise that a reexamination of our HLB conundrum in anti-conventional ways could be instructive, the following are a few alternative perspectives with which to examine the HLB-pathosystem.

Host vs. vector: In humans, bacteria outnumber human cells by a ratio of 1.3:1; for about 40 trillion bacteria reside within an average human body, the vast majority in the human gut (Sender et al. 2016). Recent findings indicate that enteric gut bacteria are the basis of our immune system and serve many recently discovered functions for human health (Maynard et al. 2012). Humans are nearly sterile of enteric bacteria at birth, but acquire them intergenerationally from maternal transmission via nursing and contact (Funkhouser and Bordenstein 2013). Insect endosymbiotic bacteria such as *CLas* are also acquired intergenerationally. The ACP adults transfer *CLas* and other endosymbionts to plants while feeding, and lay eggs in same location. Developing ACP nymphs acquire these endosymbionts by feeding on the same infected plant tissues as adults.

In the case of *CLas* and ACP, conventional host and vector roles are reversed and the psyllid is the 'host' and the plant (citrus) is merely the 'vector' for intergenerational transmission between psyllids. This likely happens in a broad multitude of crop and natural plant species where plants simply serve as the reservoir for intergenerational symbiotic bacteria. This is a common pathway for phytophagous sucking insect species to transfer endosymbionts between generations (Jeyaprakash and Hoy 2000). It is not uncommon to find insect endosymbionts in plants. *Wolbachia* sp. is another example of an insect endosymbiont that is frequently encountered in plants (Wielkopolan and Obrępalska-Stęplowska 2016; Zug and Hammerstein 2012). However, *CLas* is a unique insect endosymbiont that inadvertently causes disease in citrus as does *C.* Liberibacter solanacearum (*CLsol*), the presumed causal agent of Zebra-chip of potato (Morris et al. 2017) (Fig. 1).

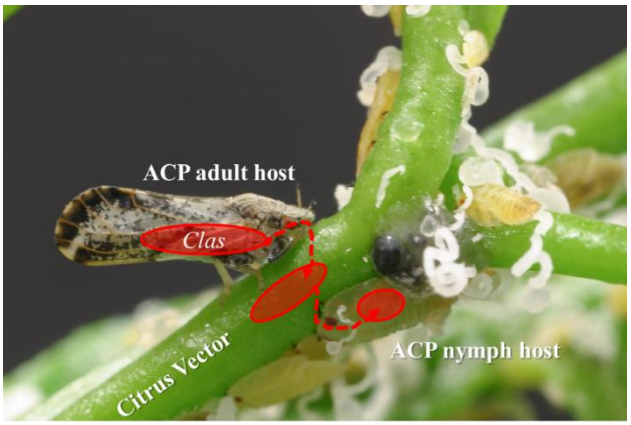

Fig. 1. Adult psyllids inadvertently deposit *CLas* bacteria into shoot tips of citrus by regurgitating saliva while feeding. Citrus acts as a survival reservoir for the insect enteric endosymbiotic bacteria. Psyllid nymphs acquire the *CLas* bacteria by feeding in the same location as the adults deposited, completing the 'intergenerational' transmission (Photo courtesy of D Hall, modified by T Gottwald).

The reason that *CLas* and *CLsol* have succeeded in jumping hosts from the animal kingdom to include the plant kingdom, specifically *Citrus* spp. and *Solanum tuberosum*, and causing disease is not understood. In a genetically outcrossing population of native plants, this would likely merely be a natural selection event, the survivors being the evolutionarily more fit and eventual progenitors of succeeding generations. The issue comes when the bacteria become severely pathogenic to horticultural species grown as huge clonal populations with nearly uniform susceptibility, such as used throughout modern agriculture, and particularly for citrus. When these clonal monocultures are long-lived tree species, the problem is compounded many-fold because they cannot be quickly replaced and brought into productivity as can be done in annual crops species. Thus what would be a potential natural selection event becomes the equivalent of a species culling event for the new citrus host of *CLas*.

***CLas* transitional states:** As indicated above, *CLas* is first and foremost an insect endosymbiont, and not a plant pathogen. It is estimated that *CLas* has evolved and adapted as an ACP endosymbiont over several million years (Beattie et al. 2006; Beattie et al. 2008). Over this time, *CLas* has achieved an optimal nutritional and population balance within the insect such that it does not tax the metabolism or vigor of the insect host nor does its population deleteriously overwhelm the insect host. However, when transmitted by ACP feeding into citrus

phloem cells, the bacterium finds itself in a challenging new and perhaps somewhat hostile environment. It must survive in this new environment, i.e. reservoir, until such time as it can be reacquired by the next-generation of ACP nymphs, i.e. its evolutionary natural host. As indicated above we estimate that *CLas* probably first came in contact with citrus within the last 100-200 years in Southeast Asia most likely near India, and has not evolved or adapted to citrus as a host. Thus when regurgitated by a psyllid into a citrus phloem cell, *CLas* finds itself in a completely new environment to which its metabolism and genetics are misaligned. Immediately after transmission into citrus *CLas* metabolism and genetic pathways are still completely adapted for enteric life within ACP. Thus *CLas* cells that are deposited into citrus phloem must transition to metabolic processes and genes up and down regulated as necessary to survive and flourish in citrus phloem.

Adaptation and the cryptic state: In general, when bacteria adapt to new environmental niches, it is usually through population shifts in their gene expression and metabolic states. As the bacteria reproduce by binary fission, successive generations begin to migrate toward adaptation to their new environment. For example, the citrus canker bacterium, *Xanthomonas campestris* pv. *citri* (*Xcc*) can be cultured easily as a saprophyte in petri dishes on traditional agar media. In axenic culture *Xcc* often loses its pathogenicity. After multiple generations of life in the petri dish, when inoculated back into the citrus host, it often requires multiple successive infection cycles on citrus leaf tissue before the bacteria regains its natural pathogenicity and aggressiveness. In another example, bacterial populations can be stimulated to develop antibiotic resistance by creating a bacterial lawn in a petri dish upon which is placed a small cotton filter paper disc impregnated with the antibiotic. A zone of inhibition will be seen around the antibiotic laced disc. By serially selecting bacteria growing immediately proximal to the antibiotic laced disc, an antibiotic resistant bacterial strain can be selected. In both cases it is not the entire bacterial population that is reacquiring pathogenicity or adapting to a new environment laced with antibiotic. Rather it is a very few members of the population which have adapted to the new environmental niche and through this adaptation have become the progenitors of the new generation of bacteria that propagate and populate the new environment.

Relative to the misaligned metabolic and genetic status, the *CLas* population must shift and change state as it adapts to the citrus phloem environment. In successive generations only a few or perhaps a single *CLas* bacterial cell will adapt toward the requisite metabolic and genetic status to transition successfully from life in an ACP to life in citrus phloem and the rest languish or perish. Several *CLas* bacterial generations may be required to transition to an optimal or near optimal status. This change of state (metabolic/genetic status) may require considerable time and undoubtedly is involved in the cryptic phase of the disease during which time the infection is either nearly quiescent or is slowly adapting toward its new environment. It is after this adaptation takes place that the bacterial populations can propagate and eventually become both systemic and achieve sufficient population size and metabolic interaction with host to result in a symptomatic response.

***CLas* titer, detection and population dynamics:** Currently, *CLas* infections in citrus can only be confirmed by detection of *CLas*-specific DNA sequences via PCR technology. Most frequently, detection of *CLas* DNA is based on quantitative polymerase chain reaction (qPCR). Detection of a *CLas* 16S rDNA fragment (Li et al. 2006) is perhaps the most widely reported qPCR method in the published literature and is part of the USDA, APHIS protocol for *CLas* diagnostics, although multiple additional PCR primers have subsequently been developed.

Considerable evidence has been reported to support that: 1) the sensitivity of qPCR for detection of *CLas* 16S rDNA is in the range of 1-9 target copies (McCollum et al. 2017; McCollum et al. 2014a), and that 2) detection of *CLas* 16S rDNA is highly specific. In addition to sensitivity and specificity of qPCR, there is a linear relationship between Log target copy number and Ct value between 10^0 and 10^7 copies per assay making qPCR ideal for estimation of *CLas* titer.

CLas infections are never uniformly distributed within the citrus canopy, coupled with the "loading capacity" (amount of tissue equivalents that can be tested in a single assay) of qPCR (approximately 1 mg tissue equivalent per assay), sampling becomes the overriding limitation of qPCR for detection of *CLas* infections. Citrus petioles are the tissue of choice for *CLas* diagnostics because they have a high proportion of phloem (*CLas* is phloem-limited) compared to other tissues. However, a moderate size citrus tree will have thousands of leaves. In the absence of suspect HLB symptoms, CLas infections within the canopy are most likely rare and of low titer. Therefore, determining where to sample is problematic and there is a low probability of randomly or even systematically selecting rare infected tissue and thus detecting *CLas*.

Detection of *CLas* infection via qPCR in samples with suspect HLB symptoms cannot be considered "early" detection, especially if the objective is to curtail the development of an HLB epidemic. Following infection of a citrus tree with *CLas*, there is a latency period of at least several months, and perhaps longer, prior to the appearance of HLB symptoms depending on the age of the tree (Gottwald 2010). Symptom expression in potted greenhouse trees can be more rapid (Fig. 2). During the latency period, *CLas* populations increase within infected shoots, and can serve as inoculum for subsequent infections. For each tree that is infected with *CLas* and eventually develops HLB symptoms, there are likely multiple neighboring trees infected *CLas*, but are in the cryptic phase, i.e. not yet HLB symptomatic (Gottwald 2010). Therefore, to be considered "early" detection,

CLas infection must be confirmed prior to the appearance of HLB symptoms.

Movement of *CLas*-infected ACP into new areas is far from uniform at the regional or orchard level (Parry et al. 2014; Gottwald et al. 2010), this contributes to the heterogeneous distribution of infections both within regions and among and within trees. At the time when *CLas*-infected ACP first invade an area, the distribution of nascent infections in citrus is highly erratic both among and within individual trees (Gottwald et al. 2010, Bassanezi et al. 2005).

CLas-infected ACP and young citrus shoot tips (flush) must be present synchronously for *CLas* infections to occur. ACP feed preferentially and lay eggs exclusively on citrus flush where by far the majority (maybe ALL) of *CLas* transmission occurs. Although there are periods of the year when flush is more abundant than others (Hall and Albrigo 2007), production of flushes is never synchronous within or among trees. This non-synchronous pattern of flushing contributes to the non-uniform distribution of *CLas* infections. The non-uniform distribution of *CLas*-infected ACP along with potential asynchrony between citrus flushes and presence of ACP means that in the absence of HLB symptoms it is essentially impossible to predict where on a tree to sample for *CLas* diagnostics.

CLas-infected adult ACP feed and lay eggs on non-infected emerging shoots (flush) and in the process *CLas* is transmitted into citrus; however, transovarial transmission is apparently not a significant route for transmission (Pelz-Stelinski et al. 2010). Nymphs feed in close proximity to where their mothers presumably fed during egg laying and in the process acquire *CLas*. These *CLas*-infected nymphs then develop into *CLas*-infected adults which move to new flush where the cycle is repeated. ACP nymphs are very efficient at acquiring *CLas*, and this is perhaps the only way that *CLas*-infected ACP are generated (Pelz-Stelinski et. al. 2010).

Two key concepts here: 1) ALL *CLas* infections in the field result from *CLas*-infected ACP adults feeding on citrus flush; 2) The preponderance of *CLas*-infected ACP adults result from acquisition that occurs in the nymphal stage. It is possible that there are never any populations of ACP that are free of *CLas* infection, supporting the idea of the bacterium being an insect endosymbiont. *CLas* may persist at low titers within the ACP population. Because *CLas* can be transmitted through citrus to nymphs even when the titer in the insect is less than the minimum infectious dose, HLB never develops. It is only when *CLas* is triggered to produce high titers in the insects do the infections become of epidemiological significance vis. HLB. To date there is no evidence to contradict this hypothesis, but there is considerable evidence to support it. Of course, ACP adults can acquire *CLas* when they are forced to feed on *CLas*-infected mature citrus leaves (Pelz-Stelinski et al. 2010) but this is unlikely to occur in nature. Not only is ACP feeding and egg laying (and the transmission of *CLas*) confined to citrus flush, mature leaves have a barrier that makes it difficult for ACP to access phloem (George et al. 2017) and thereby reduces the likelihood of transmission of CLas into mature leaves.

Not all *CLas* infections lead to HLB. We have conducted experiments in which *CLas*-infected ACP are confined with potted citrus trees and it is not uncommon to detect *CLas* in citrus within 3 weeks of exposure to *CLas* inoculative adult ACP. However, this is rare in the orchard where earliest infections are confirmed about 6-9 months after planting with young trees becoming qPCR positive over a 24-36 month period (Lin et al. 2017; McCollum, unpublished; Gottwald 2010).

Fig. 2. Lemon, mandarin and orange trees were exposed to *CLas*-infected ACP for 3 weeks, then disinfested and thereafter maintained free of ACP. For each scion, 6 weeks after ACP exposure (left) tested *CLas*-negative and (right) *CLas*-positive. However, symptoms on trees on the left began to express only after ~4 months post exposure. Photographs were taken 9 months after exposure to *CLas*-infected ACP.

Following exposure to *CLas*-infected ACP for as little as 14 days it is not uncommon to detect *CLas* in greenhouse potted citrus within 3 weeks thereafter. In some experiments we have found essentially 100% of the trees test *CLas*-positive, albeit with low titer, within a few weeks following exposure, but find that even after a year of incubation that less than 20% of the plants continue to test *CLas*-positive. As titer increases, *CLas* moves systemically and HLB symptoms develop. It could be argued that the original leaves that tested *CLas*-positive contained all of the *CLas* present in the tree and removing them for assay removed the source of inoculum. Here we propose an alternative hypothesis for the low incidence of HLB in plants that at 1 time tested *CLas*-positive. If adult ACP are placed onto *CLas*-infected plants, eggs are laid and nymphs develop, there this is a high proportion of the nymphs that are *CLas*-positive and those nymphs develop into *CLas*-positive adults. Placing ACP adults that had acquired *CLas* as nymphs onto plants not infected with *CLas* results in a new generation of *CLas*-infected nymphs. Therefore, even if none of the plants test positive for *CLas*, but ACP nymphs test positive, the *CLas* infected adults must have transmitted *CLas* through citrus for the nymphs to acquire it.

We are accumulating increasing evidence to support the hypothesis that there is a "minimum infectious dose" of *CLas* that is required in the *CLas*-infected ACP if *CLas* is to proliferate in citrus and eventually lead to the development of HLB symptoms. It appears that the probability of HLB developing following exposure to *CLas* inoculative ACP, is strongly correlated with the proportion of ACP population with *CLas* titers that exceed ca. 10^5 copies per insect. Thus, when CLas titer in ACP is less than 10^5 copies per insect, the pathogen may be transmitted to citrus and even acquired by ACP nymphs, but does not proliferate, move systemically and cause HLB.

In addition to a minimum infectious *CLas* dose for HLB development, there is a minimum *CLas* titer that is required for HLB symptom expression. Based on results with samples collected by scouts (McCollum et al. 2014a, b; McCollum et al. 2016) as well as with controlled inoculations of citrus with *CLas*, either by ACP or grafting, HLB symptoms do not become evident until *CLas* titers reach ca. 10^4 copies per mg of citrus petiole. In addition, *CLas* titers greater than 10^7 copies per mg of citrus petiole are never seen. This may indicate that the *CLas* population ceases to proliferate when titer approaches 10^6 copies per mg.

The concept of a minimum infectious dose for *CLas* proliferation/HLB disease development has significant implications. First, it may confound efforts to develop detection technologies other than qPCR. If there are systemic signals (transcriptome, proteome, metabolome, microbiome, volatile organic compounds) induced by even nascent infections, if the infection has to be confirmed by qPCR, if HLB symptoms are not visible, we are back to the sampling problem, and if *CLas* cannot be found any alternative detection technology is dismissed. However, absence of confirmation (by qPCR) is not confirmation of absence.

Rates of increase: Spread of *CLas* bacteria by psyllids and the ensuing HLB disease occurs at an unprecedented rate for an arboreal pathogen. Entire plantings can become 100% infected within 2 to 4 years, although disease expression (visual symptoms) can occur over many years (Fig. 3). To describe this increase, epidemiologists use a calculation known as the Basic Reproductive Number, R_0. In medical terms, R_0 is the number of cases that 1 case generates on average over the course of its infectious period, in an otherwise uninfected population (Ex. R_0 for Ebola, Small pox, and Measles are 1.5-2.6, 5-6, and 12-18, respectively). For HLB, R_0 is the number of trees 1 tree can infect over the course of its infectious period (lifespan of infected tree). This metric is difficult to calculate and dynamic because R_0 changes over time. For most plant pathogens, R_0 ranges from 2-50 (Ex. R_0 for stripe rust of wheat, a particularly rapid disease, is 50-60), however, the estimated R_0 for HLB based on Florida observations ranges from 1-200 or perhaps even greater under some circumstances. This is because ACP is highly prolific and an efficient vector and infected trees are long lived. Once infected, the insect is infected for life and can transport the *CLas* bacterium over short distances that can result in multiple infections within a canopy to over very long distances, establishing new epicenters, and rapidly spreading the epidemic. Additionally infected trees can survive many years continually acting as reservoirs of infection. This makes HLB incredibly difficult to control and/or mitigate. Considering the propensity for rapid infection (Fig. 4), rapid generation time, and rapid spread combined with long-lived inoculum sources; the key to HLB control is early detection and rapid response (culling = removal of infected trees) to minimize disease spread as much as possible.

Early detection: Current detection of HLB relies on a combination of visual assessment and polymerase chain reaction detection, which due to sampling issues described above can lag months to years behind *CLas* infection. In field studies, when trees become infected, PCR assay results are generally negative or inconclusive for 9 to 12 months or longer before confirmation of the infection. This is known as the *cryptic period*, when the plant is infected but we are unable to detect infection. Recently, early detection methodologies such as metabolomics, proteomics, volatile organic compound detection, canine detection, etc., have been used to directly or indirectly explore this cryptic period to a greater extent. It is the authors' belief that it is critical to detect the disease further back in time into the cryptic period in order to adequately control HLB. Epidemiological models demonstrate that early detection followed by hasty removal of infected trees in the cryptic (asymptomatic) stage is highly advantageous and requires tree replacement of only 2-3% per year capable to sustain viable production at very low disease incidence (Cunniffe et al. 2015).

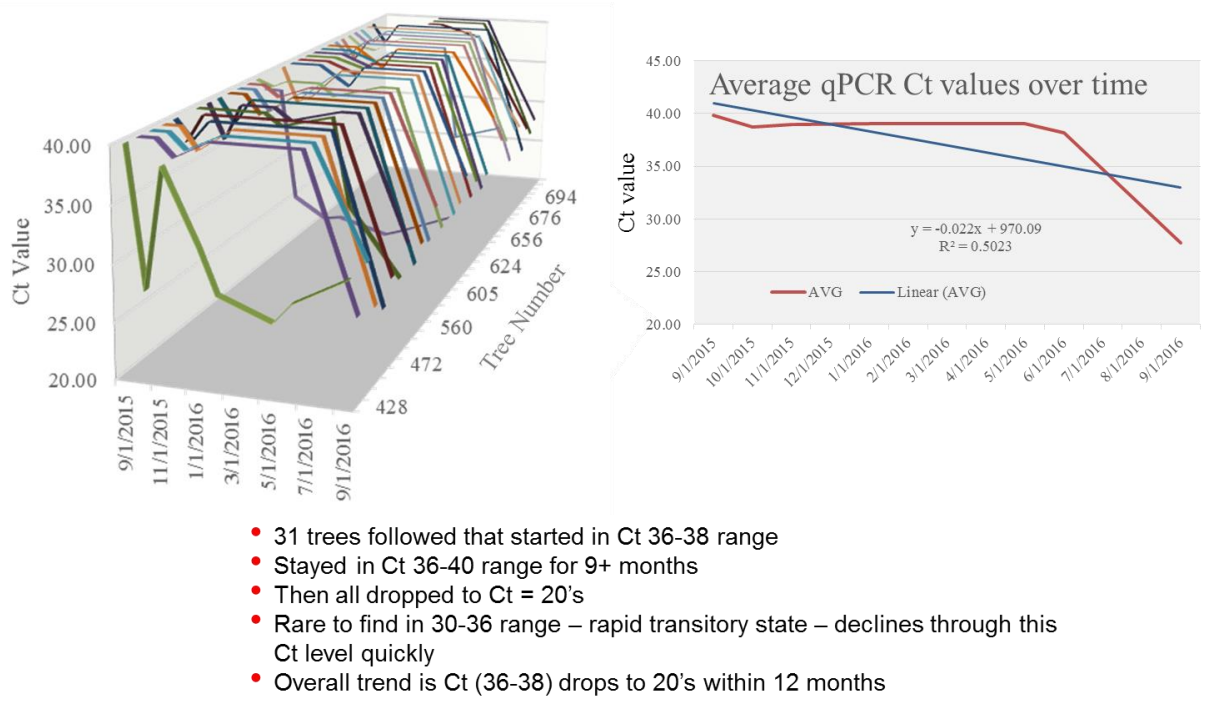

Fig. 3. qPCR Ct progression over time from a Florida commercial citrus block. Left panel indicates Ct values over time for 31 individual trees. Right panel indicates average Ct value for the 31 trees and regression analysis for the population.

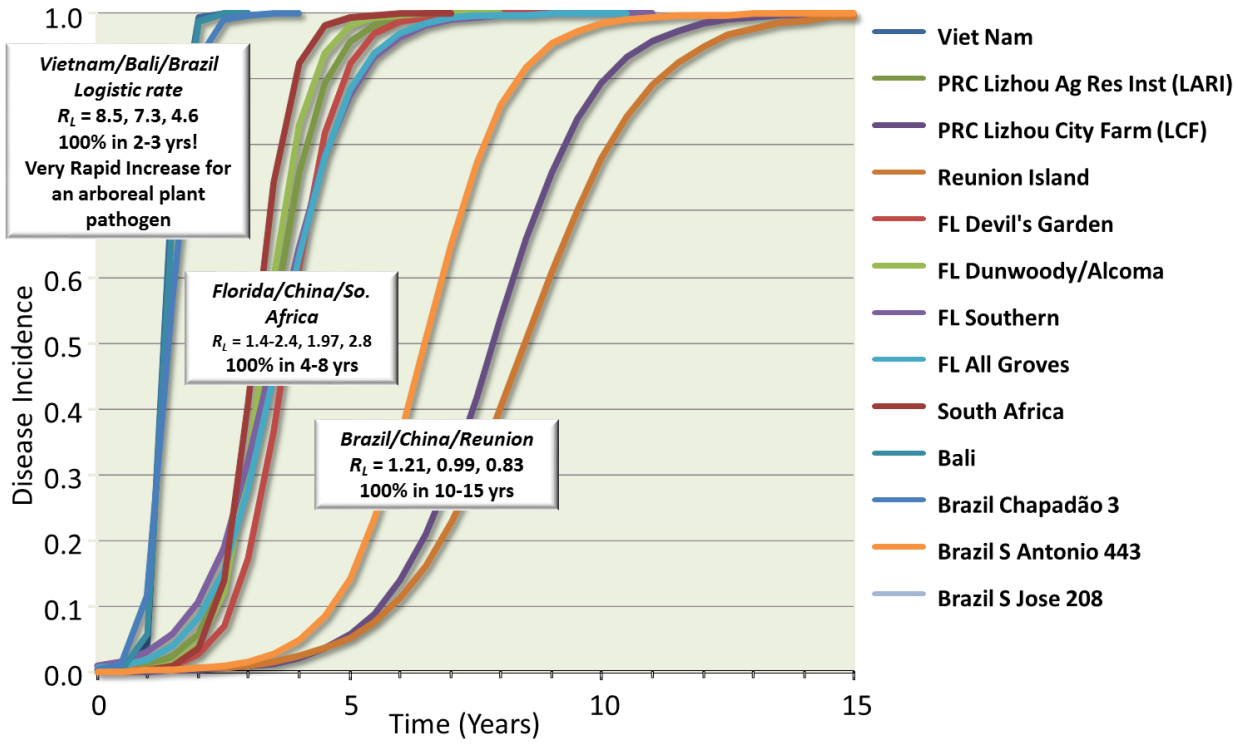

Fig. 4. HLB temporal data from 13 international locations. R values are logistic model rates of disease progress.

The Holy Grail: Effective tools to combat HLB are quite limited currently. As discussed above, the holy grail of HLB control is undoubtedly disease resistance, but few potential resistant candidates exist and field testing to assess resistance and propagation to fill the need for resistant trees in commercial HLB ravaged areas will likely require several years. Therefore, until such time as resistance becomes commonplace, citrus growers must continue to rely on the 3-pronged approach developed by the UNDP-FAO Citrus Rehabilitation Project in Southeast Asia in the mid-1980s: 1) disease-free planting material produced in psyllid-proof quarantine greenhouses, 2) ACP vector control via chemical insecticides, and 3) continual survey for HLB detection followed by diseased-tree removal 'culling' to remove *CLas* inoculum. Those few places in the world where all 3 have been diligently applied have survived longer with endemic HLB than anywhere else. However, the third prong (survey and removal) can be greatly augmented by risk-based surveys that target the most likely places for the disease to occur, thereby optimizing manpower and fiscal resources as well as early detection methods to find infected trees during the cryptic period. Risk based surveys have been deployed in Florida California, Texas and Arizona to put survey personnel in proximity to where vector and disease is anticipated for early detection as well as for continual monitoring of vector and disease dynamics (Gottwald et al. 2013).

Antimicrobial, horticultural, early detection, and epidemiological survey methods comprise the greatest departures from mainstream scientific thought in our struggle against HLB. At this time, nowhere in the world is HLB under adequate control. Thus, we have an urgent need to continue to explore these HLB-pathosystem concepts while simultaneously applying unconventional (scientifically heretical) thought to conjure up other anti-conventional approaches in search of alternative Holy Grails.

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