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Abstracts of Presentations

Abstracts presented at the 4th International Research Conference on Huanglongbing in Orlando, Florida, USA, February 9–13, 2015. The abstracts are arranged alphabetically by the first author's name. The abstracts are published as submitted. They were formatted but not edited by the Journal of Citrus Pathology. Recommended format for citing abstracts, using the first abstract below as an example, is as follows: Alabi OJ, Kunta M, Dale J, Sétamou M. 2015. Survey and detection of '*Candidatus Liberibacter asiaticus*' in a citrus nursery facility in South Texas [abstract]. J Cit Pathol. iocv_journalcitruspathology_30222.

Survey and detection of '*Candidatus Liberibacter asiaticus*' in a citrus nursery facility in South Texas

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Huanglongbing (HLB) disease, associated with '*Candidatus Liberibacter asiaticus*' (CLAs), is primarily spread via infected citrus nursery trees and by infective Asian citrus psyllid, the insect vector. Recently, the Texas Department of Agriculture initiated regulations requiring commercial and retail citrus nurseries in Texas to transition from traditional open-field to enclosed facilities with insect-resistant screens to mitigate the risk of nurseries serving as sources of CLAs. Although several nursery production facilities have adopted this regulation, non-enclosed nurseries continue to exist and pose a significant threat to the citrus industry as potential sources of CLAs during this transition period. A systematic survey for HLB was embarked on in a semi-open nursery facility in South Texas in April 2014. Leaf tissue samples taken from 94 trees representing 5% of the total number of potted trees in that nursery were tested for CLAs by quantitative and conventional PCR assays. Of 94 trees tested, 3.2% (3 trees) tested positive for CLAs using both assays. The presence of CLAs in the PCR-positive samples was confirmed by multi-locus sequence analyses. The results represent the first report of HLB in a nursery facility in Texas and underscore the need for more intensive surveillance for HLB in citrus nursery stocks as an integral component of HLB mitigation efforts in Texas.

HLB-infected citrus tree physiology and plant growth regulator effects on preharvest fruit drop

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Since HLB has become widely spread through most Florida citrus trees, preharvest fruit drop has become a major problem for the past two harvest seasons. Trees declining from HLB have greater drought stress and higher percentage fruit drop than healthier trees even though they have fewer leaves. Declining trees had 8 to 12 bars more water stress and 10 to 14% more fruit drop than healthier, full canopy trees. In the 2013-14 harvest season GA3, Citrus Fix (2, 4-D), ProMaxa (NAA), Retain (AVG), 1-MCP, and s-ABA were evaluated for their ability to reduce preharvest fruit drop. No treatment significantly reduced fruit drop from the Control level, although GA3 plus 2, 4-D often had numerically less drop than the Control, usually 5 to 7% less. In the 2014-15 season, larger plots of GA3 plus 2, 4-D were applied as well as plots of the strobilurin Headline. Results are reported.

Spectral sensitivity of the Asian citrus psyllid, *Diaphorina citri*

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The Asian Citrus psyllid, *Diaphorina citri*, as a vector of the bacteria causing citrus greening, is considered one of the most important citrus pests globally. Movement of infected psyllids onto uninfected young citrus remains a key concern for the maintenance of citrus production.

Attraction of dispersing psyllids is the foundation for the use of colored sticky traps surveillance by sticky traps. Conversely, the use of UV-reflecting mulch provides protection of young trees from infestation. Detailed knowledge of the visual system of these insects can provide the basis for enhanced sensitivity by surveillance traps and a better understanding of the potential for visual cues as repellents. Using electroretinograms, the spectral sensitivities of male and female citrus psyllids were determined. Regions of maximal sensitivity were matched by theoretical pigment absorption curves to determine the composition of photopigments present and related to behavioral responses of psyllids. This information can be used to enhance visual surveillance traps' effectiveness and optimize use of visual deterrents.

Strategies for monitoring of plant epidemics in trade networks

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The global movement of plants and plant products across the globe has increased in recent years, triggering an increase of introduced plant pathogens. Tree nurseries importing material from abroad may play a role in the introduction and spread of tree diseases. One example is the spread of sudden oak death in North America and Europe. A more recent example suggests that ash dieback could have been introduced into the UK through nursery trade, as well as through aerial spread. Huanglongbing disease is also believed to be the direct result of human movement of plant material. The economic, environmental, and social cost associated with the spread of invasive pathogens become considerably larger as the incidence of the pathogen increases. To control the movement of pathogens across the plant trade network, it is crucial to develop efficient sampling and monitoring programmes at key points of the network. By detecting the introduction of invasive pathogens at low incidence, the control and eradication of an epidemic is more likely to be successful. Here, we develop a simple epidemiological model to detect and trace the dynamics of an invasive plant pathogen in a plant nursery. Using statistical methods, we can predict the epidemic incidence given that a detection of the pathogen has occurred for the first time. Equally, we trace forward the probability of pathogen movement into another part of the trade network. We explore the effect of these results on data for Ash dieback in the UK and Huanglongbing disease in Florida. These results provide new insight for the design of monitoring strategies at key points of the trade network.

Assessment of the supply of metal micronutrients on HLB-infected trees

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The occurrence of the Huanglongbing (HLB) in citrus has caused significant fruit losses because of the severity of the disease and the lack of effective control. Spray of nutrients to the leaves and more recently the application to the soil have been employed in an attempt to mitigate the effects of the disease on citrus groves. In this context, this study evaluated the growth of young sweet orange trees infected with

'*Candidatus Liberibacter asiaticus*' (Las) by over-grafting and healthy ones. Plants were supplied either with foliar applications or via substrate (check non-fertilized, Cu, Mn, Zn or Cu + Zn + Mn) with copper hydroxide, manganese sulphate, and/or zinc sulphate solutions. The nutrient applications were at intervals of 30 days in a period of 12 months, and the treatments effects were evaluated at 180, 270, and 360 days after inoculation (DAI) with Las. The nutritional status of plants was evaluated, also the production of dry matter of leaves, branches and roots, leaf area, specific leaf weight, starch, chlorophyll contents, and concentration of bacteria in the leaves by PCR. Diseased plants produced lower amounts of total dry mass and root compared to healthy ones, except for leaves. Visual symptoms of the disease were manifested in the leaves at the end of the experiment. Increased specific weight of the leaves of diseased plants compared to those healthy was detected. Although the applications of micronutrients have affected the nutritional status of plants, there was no contribution to increased tolerance or resistance to HLB, since the concentration of the bacteria increased in the periods evaluated. A second part of the experiment studied the Las acquisition by the Asian citrus psyllid (ACP) from trees treated with the micronutrients. The Cu + Zn + Mn treatment impaired the bacteria acquisition by either adult or nymph as revealed by PCR analysis.

Replication of *Candidatus Liberibacter asiaticus* in its psyllid vector *Diaphorina citri* (Hemiptera: Liviidae) following various acquisition access periods

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The Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), is the primary vector of *Candidatus Liberibacter asiaticus* (CLAs) implicated as causative agent of citrus Huanglongbing (citrus greening), currently the most serious citrus disease worldwide. CLAs is transmitted by *D. citri* in a persistent circulative manner, but the question of replication of this bacterium in its psyllid vector has not been resolved. Thus, we studied the effects of the acquisition access period (AAP) by nymphs and adults of *D. citri* on CLAs acquisition and transmission as well as on CLAs replication in this psyllid vector. *D. citri* nymphs or adults were caged on CLAs-infected citrus plants for an AAP of 1 or 7 days then transferred weekly to healthy citrus or *Murraya* plants for 5–6 weeks, and sampled weekly for RT-PCR analysis (of individual psyllids). Our results indicate that following 1–7 day AAP as nymphs, 49–59% of the psyllids became CLAs-infected, whereas only 9–24% were infected after 1–7 day AAP as adults. RT-PCR also indicated that the CLAs titer in *D. citri* (relative to that of the psyllid S20 ribosomal protein gene) was: (1) generally higher following acquisition as nymphs compared to that following acquisition as adults, (2) higher with longer AAP, and (3) higher as post-acquisition time on healthy plants increased between 1 and 21 days but seemed to level off or decline after that. Implications of these findings on the concept of CLAs replication in *D. citri* nymphs and adults are discussed.

Differences in stylet sheath occurrence and the fibrous ring (sclerenchyma) between *Xcitroncirus* plants relatively resistant or susceptible to adults of the Asian citrus psyllid *Diaphorina citri*

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The Asian citrus psyllid (ACP, *Diaphorina citri*, Hemiptera: Liviidae), is the principal vector of the phloem-limited bacteria strongly associated with Huanglongbing (HLB), the world's most serious disease of citrus. Host plant resistance may provide an environmentally safe and sustainable method of controlling ACP and/or HLB. Two *Xcitroncirus* accessions (hybrids of *Poncirus trifoliata* and *Citrus* spp.) that are relatively resistant (UN-3881) or relatively susceptible (Troyer-1459) to ACP adults were compared in relation to ACP feeding behavior and some structural features of the leaf midrib. It was previously shown that ACP feeding on UN-3881 plants resulted in shorter adult life span

compared to that on Troyer-1459. In our present study, using ACP adults caged on the central leaflet, fewer stylet sheaths were formed in the midrib and fewer stylet sheath termini reached the vascular bundle (phloem and/or xylem) in UN-3881 compared to Troyer-1459 plants. Furthermore, in midribs of UN-3881, the fibrous ring (sclerenchyma) around the phloem was significantly wider (thicker) compared to that in Troyer-1459. Our data indicate that feeding and/or probing by ACP adults into the vascular bundle is less frequent in the more resistant (UN-3881) than in the more susceptible (Troyer-1459) accessions. Our results also suggest that the thickness of the fibrous ring may be a barrier to stylet penetration into the vascular bundle, which is important for successful ACP feeding on the phloem and for transmitting HLB bacteria. These results may help in the development of citrus plants resistant to ACP, which in turn could halt or slow the spread of the HLB-associated bacteria by this vector.

Within-grove edge effects of the azimuth of the sun on *Diaphorina citri* adults

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Huanglongbing (HLB) has been considered the most devastating disease of citrus. The bacterium and vector associated with HLB in Florida are '*Candidatus Liberibacter asiaticus*' and *Diaphorina citri* (Asian citrus psyllid), respectively. *D. citri* is positively phototropic, and higher populations have been found along edges of groves exposed to the sun. An experiment was designed to determine if *D. citri* adult populations along edges of groves varied according to time-of-day and time-of-year in relation to the azimuth of the sun. The experiment was conducted twice. Citrus groves, each divided into nine sampling areas, were surveyed for *D. citri* via stem-tap sampling. Groves were sampled three times per day (near sunrise, solar noon, and sunset) and four times per year (near the summer solstice, autumnal equinox, winter solstice, and vernal equinox). Time-of-year and sampling area significantly affected psyllid counts ($p = 0.0518$ and 0.0630 , respectively). *D. citri* adults were most prevalent during the summer solstice sampling period. No overall significant time-of-day effect was observed ($p > 0.6$). Localization of adult *D. citri* in sampled citrus groves did not significantly change in relation to time-of-year ($p = 0.0907$). Linear mixed regression was used to fit a quadratic equation to log *D. citri* abundance data in relation to elevation-corrected azimuth at the time of sampling; the fitted model was significant and predicted log *D. citri* abundance to exhibit a concave-up pattern with increasing elevation-corrected azimuth.

Effects of postharvest treatments on survival of *Diaphorina citri* nymphs on infested curry leaves

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Studies were conducted to evaluate treatments that reduce survival and attachment of *Diaphorina citri* nymphs on infested curry leaves. Experiments were conducted three times. Decontamination of curry leaves infested with *D. citri* in relation to disinfectant (none or Pro-San), temperature (0, 40, and 50°C), and treatment duration (0, 5, 10, and 20 min) was examined using a split-split plot design. Treatment duration did not significantly affect *D. citri* nymph survival or removal ($p > 0.2$). Temperature highly significantly affected *D. citri* nymph survival and removal ($p < 0.0001$). Disinfectant significantly affected *D. citri* nymph survival and removal ($p < 0.031$). The interaction of temperature and disinfectant was significant with respect to nymph survival ($p < 0.0001$) but did not significantly affect removal ($p = 0.4589$). Tissue damage was significantly affected by temperature ($p = 0.0056$), duration ($p = 0.0023$), the interaction of temperature and duration ($p = 0.0320$), and the interaction of disinfectant, temperature, and duration ($p = 0.0410$). Of the treatments resulting in 100% *D. citri* nymph mortality on infested curry leaves, 40°C for 5 min with Pro-San was accompanied with the least proportion curry leaf tissue damage (0.14 greater than untreated control, $p = 0.25$). Results from these studies may be useful in

formulation of future regulatory policies regarding trade of citrus foliage, especially those used as condiments.

RNAi feeding bioassay - Development of non-transgenic approaches to control Asian citrus psyllid and other hemipterans

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RNA interference (RNAi) comprises a natural mechanism of gene regulation and antiviral defense system in eukaryotic cells, resulting in sequence-specific degradation of RNAs. Recent scientific studies demonstrate the feasibility of use RNAi-based strategies to control insect pests in plants. This technology permits development of sprayable non-transgenic strategies to control a single insect species, with no negative effects on non-target species, like honey bees or parasitoids. Our works are focused to develop non-transgenic RNAi-based approaches to control the Asian citrus psyllid (ACP), vector of citrus Huanglongbing (HLB). In order to screen insect target genes, we develop an RNAi feeding bioassay for hemipteran, called *in plant system* (iPS), that enables the evaluation of designed insect-targeting dsRNA's delivered in a natural way by feeding from plants. Citrus flush is placed in 1.5 mL vials with 0.5 mL of water and dsRNA solution for 24 hours. Groups of 15-20 ACP adults were caged with treated flush during 15 days, and mortality scored daily. Several dsRNA's targeting selected ACP genes were screened using the iPS. Results obtained show that psyllid mortality varied, dependent upon transcript and dsRNA concentration. Greater mortality was achieved when psyllids fed on plants treated with dsRNA from genes ENRG-1, DETOX-1, and MET-1. First ACP mortality for MET-1 was 4-5 days postfeeding (dpf), whereas DETOX-1 and ENRG-1 induced mortality 5-7 dpf. Effects of dsRNAs were tested in seedlings. Insect mortality was observed in all, with high intensity in plants that received MET-1. Furthermore, on MET-1 treated plant, no offspring were observed.

Bio-products effective against the citrus Huanglongbing bacterium "*Candidatus Liberibacter asiaticus*" in Mexican lime trees

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Huanglongbing (HLB), the most destructive citrus pathosystem worldwide, is associated to *Candidatus Liberibacter* spp. and *Candidatus Phytoplasma* spp. and no cure is currently available. HLB was detected in Mexico in 2009 and has dispersed rapidly to 16 out of the 24 citrus producing States, decreasing in 301,314 tons (20.4%) of the national Mexican lime (*Citrus aurantifolia*) production from 2008 to 2013. In 2008, Colima State contributed with 48% of the national production and currently almost 100% of the lime groves (19,000 ha) are affected by HLB, with productivity reductions of 58% of tons produced and 38% of yield. In Mexico, the integrated control program in commercial orchards is limited to the chemical control of the psyllid vector, removal of HLB-PCR positive trees, and the use of certified nursery plants. Three bio-products formulated with a combination of antimicrobial agents, plant extracts, and resistance inducers were evaluated as a possible control strategy of Huanglongbing in Mexican lime trees. The experiment was conducted in a commercial grove in Colima and in potted HLB-infected trees. Bio-products were applied monthly and bacterial titer was quantified by nested TaqMan® PCR. Changes in carbohydrate metabolism (starch, sucrose, fructose, glucose, and total soluble and reducing sugars) and photosystem II (PSII) efficiency were also quantified before and 12 months post application (mpa) of bio-products. The mean CLas titer of Fitoplazmix™/Fitoxxit™ treated trees was significantly ($p < 0.05$) lower (less than 50%) than the untreated positive control trees (HLB+). The imbalance of carbohydrate partitioning associated to HLB+ trees was only observed in untreated positive control trees. In Fitoplazmix™/Fitoxxit™ treated HLB+ trees and in control HLB- trees, no accumulation of starch, sucrose, fructose,

glucose, and soluble and reducing sugars was observed. Thus, Fitoplazmix™/Fitoxxit™ had a significant effect on CLas titer, carbohydrate metabolism, and photosystem II (PSII) efficiency on Mexican lime trees with HLB.

Suitability of *Swinglea glutinosa* and leaf age of flush growth of citrus on the development of *Diaphorina citri*

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Management of Huanglongbing (HLB) requires a thorough understanding of the factors that influence spread of the disease. In this instance, we determined relationships between the development of sweet orange leaves on oviposition by *D. citri* females and survival of nymphs of the psyllid, and compared the suitability of Tabog (*S. glutinosa*) with oranges and lemons on *D. citri* development. Tabog, a citrus relative from the Philippines, is commonly planted to 'fence' citrus orchards in Colombia, but its suitability as a host of *D. citri* is poorly understood. For experiment 1, 2-year-old potted plants of 'Valencia Americana' grafted on Swingle citrumelo were pruned every week for four consecutive weeks. The plants were then divided in three groups (14 to 20 plants on each) based on the extent of flush development: less than 2-cm long with small unopened leaves (stage 1: S1), over 10-cm long with partially expanded leaves (stage 2: S2), and over 10-cm long with fully expanded (stage 3: S3) soft leaves. Two pairs of 15-day-old insects were caged for 72 hours on a single new flush maintained on each plant. For experiment 2, the insects were similarly caged on S1 flush growth present in 5-6 plants of the 'Valencia Americana', 'Rubi', 'Natal', 'Hamlin', and 'Pera' sweet oranges, or 'AB', 'Femminello', and 'Limonera' lemons, and 10 Tabog plants. Averages per flush for eggs laid, survival of nymphs, numbers of eclosed adults, and percent survival of adults were higher on flush stage S1 (46 ± 6 , 87%, 32 ± 4 , 63%) than on stages S2 (25 ± 6 , 16%, 1 ± 0.7 , 5%) and S3 (4 ± 2 , 0%, 0%). The same parameters were higher in S1 flushes of oranges (41 ± 6 , 83%, 37 ± 5 , 70%) than on S1 flushes of lemons (36 ± 5 , 51%, 14 ± 3 , 33%) and Tabog (24 ± 4 , 0.7%, 0.2 ± 0.2 , 0.43%). Our results reinforce the need to protect all citrus new flush growths, regardless of developmental stages, to reduce vector populations in citrus orchards. Although Tabog was a less suitable host for *D. citri* than oranges and lemons, its continued use around citrus orchards in Colombia will need to be carefully managed if HLB becomes established.

An innovative approach to defining components necessary for the growth of *Candidatus Liberibacter asiaticus*

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The ability to culture a bacterium affects almost all facets of phytopathology from disease diagnosis and morphological investigation to antigen development and genetic analysis. Thus, our current inability to culture *Candidatus Liberibacter asiaticus* (Las) is hindering advancements in all of these areas. Although several reports have indicated an increase in the bacterial cell number, sustained growth of Las over time has not been achieved. In an attempt to devise a culture media that could support the growth of Las, studies involving specific components critical for the development of Las were devised. These studies involved the ability of Las to utilize 190 different carbon sources, 376 different nitrogen sources, 59 different phosphorous sources, and 35 different sulfur sources. In addition, 94 different known nutrient supplements and several osmolytes were also examined. Three different inoculum sources were used to help resolve which of the individual components were critical for growth. To aid in the verification of the obtained results and provide a reference point for comparison, *Liberibacter crescens* was also tested for its capacity to utilize the same substrates. Subtractive analysis revealed the use of three different carbon sources and two nitrogen sources by both *L. crescens* and Las from all three of the different inoculum sources. Many more potential nutrient supplements were identified when mining the data for areas of overlap amongst only three of the four different inoculum sources presented. Information revealed in this analysis may be used in conjunction with other biochemical data to aid in the identification of a media capable of supporting the perpetual growth of Las.

Exploring the innate immune system of the Asian citrus psyllid, *Diaphorina citri*, through genomics and controlled bioassays

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Manipulating the innate immune system of insects as a means of inhibiting vector acquisition of pathogens is currently being explored in many insect vectors of human pathogens. To apply these techniques to hemipteran vectors of plant pathogens, the immune systems of these insects must first be understood. The two hemipterans that have been investigated, the pea aphid and brown plant hopper, have differing immune capabilities; with the brown plant hopper having a robust immune system similar to other insects; while the pea aphid has a drastically reduced immune system. To identify if immune manipulation could be a technique used to inhibit the Asian citrus psyllid (ACP) transmission of *Candidatus Liberibacter asiaticus* (CLAs), we annotated the immune genes of ACP and exposed ACP to different classes of bacteria. Genome annotation revealed a substantially reduced immune system in ACP with an absence of recognition proteins in the classes PGRP and GGBP, the majority of the IMD pathway, and few genes for antimicrobial effectors. These results suggest that ACP have a particularly reduced immune response against gram-negative bacteria. When ACP were exposed to either gram-negative bacteria or gram-positive bacteria through oral infections or cuticular punctures, significantly increased mortality was observed in response to gram-negative bacteria. Taken together, the genetic data and the controlled infection results indicate that ACP are not able to detect or eliminate gram-negative bacteria such as CLAs. Although innate immune manipulation may have limited utility for inhibiting CLAs acquisition by ACP based on these findings, microbial insecticides may prove to be an effective control technique for ACP.

Association of *Candidatus Phytoplasma asteris* with Citrus Huanglongbing disease in Mexico

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Currently, HLB is associated with *Candidatus Liberibacter* spp., although several phytoplasmas have been found in citrus showing HLB-like symptoms. The aim of this study was to determine if, in addition to *Ca. L. asiaticus* (CLAs), phytoplasma species (CP) are also associated with HLB in citrus of Mexico. Citrus plants showing HLB-like symptoms were collected in the Mexican States of Nayarit, Colima, and Sinaloa between August 2011 and September 2012. Samples were evaluated for CP and CLAs by nested-PCR and conventional PCR, respectively. For actual RFLP, phytoplasma fragments were digested with restriction endonucleases and fractionated using a QIAxcel system (Qiagen, Valencia, CA). Virtual RFLP analysis was performed on the 16SrDNA sequences using the virtual gel plotting program pDRAW32 (AcaClone). Phylogenetic trees were constructed with the Neighbor-Joining method, using the MEGA program (version 5.2.2). A total of 86 HLB-symptomatic samples of Mexican lime (*Citrus aurantifolia*, Christm., Swingle), Persian lime (*Citrus latifolia*, Tanaka), and Valencia sweet orange (*C. sinensis* (L.) Osbeck) were analyzed. Diffuse chlorosis, blotchy mottle and vein yellowing were symptoms observed, even though we were unable to clearly identify symptoms specifically associated with either bacterium alone or together. Fifty-four out of 86 citrus plants were positive for CLAs, 20 were positive for CP, seven were found in mixed infections with both pathogens, and 19 samples were negative for CLAs and phytoplasmas. Actual and virtual RFLP analyses of the 16S rDNA sequences enabled us to classify two HLB phytoplasma strains as members of the aster yellows group (16SrI) *Candidatus Phytoplasma asteris* (CPa), the subgroup B from Nayarit and subgroup S from Colima and Sinaloa, which was confirmed by phylogenetic analysis. In addition, the partial CLAs sequences were identical to the strains isolated from several countries affected by HLB.

These results confirm the association of CPa with HLB in citrus in Mexico.

Use of qPCR to predict quality of orange juice affected by HLB

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Huanglongbing (HLB), presumably caused by *Candidatus Liberibacter asiaticus* (CLAs), is a devastating citrus disease associated with off-flavor in orange juice. Relative CLAs titer was determined by analyzing the 16S rDNA gene (Li primer) and the LJ primer targeting the CLAs prophage in fresh or processed/pasteurized orange juice using qPCR. A method was developed that yielded large quantities of highly purified DNA, using only a small quantity of juice, which was then compared to the sensory characteristics of the juice using linear regression. To this end, orange juice was centrifuged and the pellet was used for qPCR analysis. After lysing the cells in an alkaline and non-ionic detergent Tris-based buffer, the initial DNA precipitation step was accomplished using hexadecyl trimethyl ammonium bromide (CTAB) and sodium chloride at low concentration to remove polysaccharides like pectin. A trained sensory panel analyzed the same juice for various descriptors for flavor, aroma, mouthfeel, and aftertaste and the chemical components of the juice were also evaluated. By using multiple dilutions of a known amount of standard DNA, a standard curve was generated for log concentration of sensory descriptors against Ct. The amount of nucleic acids in an unknown sample can then be calculated from its Ct value. For the Li primers, Ct values between 35 and 30 indicated a minor decrease in juice flavor quality, but below 30 indicated a more significant flavor decline. Values below 30 indicated flavor decline for the LJ primer in relation to sweet taste or overall orange flavor and conversely to typical HLB flavor scores ($r = 0.7$ to 0.8). This technology also worked for off-flavor causing microorganisms and human pathogens, for example *Alicyclobacillus acidoterrestris* or *Escherichia coli*, respectively, in orange juice or apple cider.

Spatial point pattern analysis of diagnostic Ct-values from Asian citrus psyllid samples: Utility in predicting infected host locations

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An opportunity to analyze spatial patterns to determine the underlying biological process has developed from the widespread sampling and testing of Asian citrus psyllids in Texas and California to locate early infections of Huanglongbing disease. The real-time polymerase chain reaction diagnostic methods used to detect the casual agents of HLB, *Candidatus Liberibacter asiaticus*, are set to run for 40 cycles. The reaction must surpass a set threshold prior to the completion of the run to be considered positive for the presence of CLAs. When diagnostic testing of ACP initially started in Texas and California, the threshold had to be reached at <32 cycles and then later was raised to <37 cycles. Currently, reactions that surpass the threshold at 37 or more cycles have proven impossible to acquire confirmatory conventional PCR bands and DNA sequence data. Thus, these samples are classified as inconclusive. The question we are trying to answer is whether information in the Ct-values between 37 and 40 is also useful for predicting locations with HLB-infected citrus plants. Analysis of 2013 data from California indicates that psyllid samples with high Ct-values tend to be clustered at close ranges and then dispersed at larger scales. Clustering of samples with Ct-values in the 38-39 range is within 1 km of samples with Ct-values below 37. In both Texas and California, spatial clustering of psyllid samples with inconclusive Ct-values have been shown to cluster around known positive HLB-infected trees.

HLB epidemics and orange yield after three years of nutritional programs

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Despite the effectiveness of area-wide inoculum reduction and Asian citrus psyllid (ACP) control to manage Huanglongbing (HLB), growers still look for alternative practices, such as nutrient sprays, to avoid removing diseased trees and to minimize losses due to HLB progress. However, clear evidences of positive effects of enhanced mineral nutrition on health and productivity of diseased trees is lacking. Therefore, in December 2010, an experiment was set up in a non-irrigated grove of 8-yr-old Valencia sweet orange trees on Rangpur lime to evaluate the effects of nutrients (boron, potassium nitrate, zinc sulfate and manganese sulfate), phosphite, and salicylic acid leaf sprayed to the trees four to five times per year during flushing periods. The experiment has eight treatments in four randomized blocks with 1,280 trees/plot. ACP has been monthly controlled in 3 of 4 blocks. At the beginning of the experiment, incidence of HLB-symptomatic trees was <2%, and 20 HLB-affected trees with mean disease severity (percentage of tree canopy area with symptoms) <3% were marked. After 3 years, there was no difference among enhanced nutritional treatments for whatever variables assessed. Nutritional treatments did not reduce the overall progress of HLB-symptomatic tree incidence nor the disease severity progress in marked trees, and they did not improve yield of HLB-symptomatic trees. Considering the yield per total plot (healthy + diseased trees), there was no difference among treatments, but the yield was decreasing each year, from an average of 2,325 boxes/ha in the first year to 1,763 boxes/ha in the second year and 1,249 boxes/ha in the third year. In June 2014, the mean HLB incidence was 15% and 36%, respectively, for plots with and without ACP control. In June 2014, the mean disease severity on previously marked trees was 37%, with or without ACP control. Compared with symptomless trees, HLB-affected trees had a mean yield reduction of 15%, 45%, and 62%, respectively, in the first, second, and third year after the beginning of the experiment.

Sivanto™, a new tool for control of Asian citrus psyllid in citrus

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Sivanto™, with its active ingredient flupyradifurone, is the first member of the new insecticide class, butenolide, and has received an IRAC classification of 4D. Sivanto™ is active on many sucking pests, including the Asian citrus psyllid (*Diaphorina citri*). This new insecticide has been shown to effectively control adult and immature stages of the Asian citrus psyllid in replicated field trials in both Florida and California. In addition, Sivanto™ has shown rapid and strong feeding cessation effects in electrical penetration graph (EPG) studies testing adult psyllids on citrus. This experiment showed that Sivanto™ stopped phloem stage feeding, the critical stage for transmission of *Candidatus Liberibacter asiaticus*. Additionally, Sivanto™ exhibits a favorable safety profile and has been designated as a Reduced Risk candidate by the US EPA in 2012 for many food use crops, including citrus. Therefore, Sivanto™ not only provides excellent psyllid control, but the proposed label also allows growers a flexible application window, including during bloom. Sivanto™ offers Citrus growers a new Asian citrus psyllid management tool.

HLB epidemics in well-managed groves result from primary dissemination from external sources

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Commercial citrus blocks under intensive HLB management were evaluated about *Diaphorina citri*'s population and HLB incidence through time. These HLB-managed blocks, in a total of 19 belonging to 3 farms, were neighbored by managed and unmanaged commercial and non-commercial citrus plants. The total number of citrus trees per block varied from 1,505 to 19,372 and 4 to 36 yellow sticky traps were distributed in each block. Yellow traps were changed each 15 days and adults of *D. citri* were counted and collected for qPCR to detect *Ca. Liberibacter* spp. Neighboring citrus plants (or areas) were also monitored with yellow traps. The average number of adults per trap were from 0.0 to 0.17 (per date) for the 19 studied blocks, from 0.0 to 0.50 in neighboring areas with some insecticide application (<10 times per year), and from 0.0 to 2.2 in areas with no insecticide sprays. HLB-

managed areas received at least one insecticide spray per month and frequent remotion of HLB-symptomatic trees (≥4 times per year). Presence of adult vectors carrying *Ca. Liberibacter* spp. were detected in some HLB-managed blocks and, at the highest proportion, in non-sprayed, HLB-symptomatic plants, in non-commercial areas. Distribution of *D. citri* adults were concentrated at the border of sprayed citrus blocks and 80% of total adults detected occurred up to 200 meters from the border of the blocks. In our study, we demonstrated that non-commercial areas maintained with HLB-symptomatic and unsprayed plants are important sources of primary inoculum for neighboring citrus areas where HLB-management practices are adopted. Also, we demonstrated that in managed groves (with eradication of symptomatic trees and chemical vector control), secondary spread is not relevant and the epidemic results from primary dissemination from external sources.

Effect of *Murraya koenigii* volatiles on the host selection behavior of the psyllid *Diaphorina citri* Kuwayama (Hemiptera: Lividae)

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Huanglongbing (HLB) or greening is considered the main disease of citrus. This disease is associated with the bacteria "*Candidatus Liberibacter* spp.". Once there are no curative measures for the disease, management is based, among other measures, on the control of the insect vector, the Asian Citrus Psyllid (ACP) *Diaphorina citri*. This insect has a host range of about 20 species of the family Rutaceae, including *Citrus* spp. However, there are a few studies related to their host preference. So, the aim of this project was to evaluate the behavior of the host choice of ACP comparing curry tree (*Murraya koenigii*) and 'Valencia' Sweet orange (*Citrus sinensis*). Therefore, we have investigated the effect of curry tree volatile on the behavior of *D. citri* through bioassays with olfactometer type 'Y'. This type of olfactometer is composed of a main pipe and two side glass tubes. Chambers containing the treatments were connected to the tubes. The treatment consisted of citrus plants and curry tree, containing shoots of the same size, and control (clean air). The attractiveness of both males and females, with defined ages, was evaluated. When citrus plants and curry tree were compared, it was observed that the females were more attracted to curry tree than citrus, which did not occur with the males. Subsequent studies will be conducted to collect the possible volatile involved on the process of the host selection of curry tree, looking for the achievement of attractiveness for *D. citri* management.

RNAi-mediated gene knock-down of '*Candidatus Liberibacter asiaticus*' induced citrus transcriptome, Cal7 and PP2, with CTV-based silencing vector to prevent phloem plugging in HLB-affected citrus

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Citrus greening or Huanglongbing (HLB) presently is the economically most important disease in citriculture. In response to *Candidatus Liberibacter asiaticus* (CLAs) infection, citrus trees up-regulate the transcription of genes that contribute to phloem plugging which leads to disruption of carbohydrate transport, excessive starch accumulation in leaf chloroplasts, disease manifestation, bitter juice quality, and eventual death of trees. Transcriptional response to CLAs infection revealed a notable accumulation of phloem protein 2 (PP2) transcripts in 'Valencia' orange leaves. The product of two genes, callose synthase 7 (CalS7) and phloem protein 2 (PP2), were identified in phloem plugging material of CLAs infected citrus. Preliminary RT-qPCR data show 2.7- and 3-fold difference in the expression of CalS7 and PP2, respectively, in the bark of infected citrus compared to healthy citrus. Therefore, we hypothesized that down-regulation of overexpressed genes; CalS7 and PP2, via RNA-interference using a CTV-based silencing vector may preclude phloem plugging and ultimately make the effect of HLB infection inconsequential. We have challenged young citrus trees expressing truncated CalS7 and PP2 dsRNA sequences, with CLAs-infected psyllids to feed *ad libitum* to establish HLB. Leaf and bark tissue samples will be collected at regular intervals to examine the differences in CalS7 and PP2 expression, and phloem plugging, using

RT-qPCR, northern blotting, and fluorescent microscopy. Results will be presented.

Molecular identification and distribution of *Candidatus Liberibacter asiaticus* in citrus species in Mexico

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HLB disease is spreading into the major producing citrus states of Mexico. The Mexican lime crop is the most affected by this disease because it reduces its yield about 50% when the HLB severity is from 75 to 100%. The objective of this work was to diagnose HLB by a molecular technique and determine its geographic distribution in Mexico. Citrus samples were collected in nine states of Mexico. DNA was extracted by the CTAB method from leaf tissue of different citrus species showing the mottling symptom. PCR was performed as described by Hocquellet et al. (1999). A total of 207 samples from different citrus species were analyzed. *Candidatus Liberibacter asiaticus* was detected in 57 samples obtained from trees of Mexican lime (*Citrus aurantifolia*), Persian lime (*C. latifolia*), sour orange (*C. aurantium*), sweet orange (*C. sinensis*), grapefruit (*C. paradisi*), and mandarin (*C. reticulata*) from the states of Baja California Sur, Colima, Jalisco, Michoacan, Nayarit, Quintana Roo, Sinaloa, and Yucatan. In the state of Michoacan, where there is the largest surface of sour citrus, *Ca. L. asiaticus* was detected in samples of Persian lime and Mexican lime. While in the state of Colima, the presence of this pathogen was detected in grapefruit and Mexican lime, and one sample of tangerine was negative. All the samples from the state of Puebla were taken from sweet orange trees and they resulted negative; however, other reports indicate the presence of HLB in this state. Despite the extended distribution of *Ca. L. asiaticus* in citrus growing areas of Mexico, the pathogen has not been detected in the state of Veracruz, which has the largest area of citrus in Mexico.

Incidence of '*Candidatus Liberibacter asiaticus*' in *Poncirus trifoliata* hybrids under field conditions

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Huanglongbing (HLB), caused by the bacterium '*Candidatus Liberibacter*' sp. is one of the most important citrus diseases worldwide. So far, there is no source of genetic resistance to HLB in *Citrus* or its relatives. However, several studies have reported *Poncirus trifoliata* and some of its hybrids to be more tolerant to the disease. The objective of this study was to report differences in the incidence of '*Ca. L. asiaticus*' (CLAs) infection in citrandarins, hybrids of *Citrus sunki* and *P. trifoliata*, under field conditions. Bacteria in the leaves and roots were detected using qPCR. These citrandarins were established about 7 years in an area with a high incidence of CLAs-infected plants. We selected two experimental areas (Area A and Area B), located approximately 10 meters apart. Area A consists of 168 trees of 'Pera' sweet orange (*C. sinensis*) grafted onto 56 different citrandarins, with three replicates. Area B consists of 275 citrandarin scions grafted onto 'Rangpur' lime (*C. limonia* Osb.), with three replicates. As a result, the incidence of CLAs-infected plants was 92% in Area A and 14% in Area B, which can be interpreted as evidence of resistance or tolerance of citrandarins as scion. However, we cannot conclude, in the case of citrandarins as scion, if that resistance is in relation to bacteria or to psyllids. As rootstock, we examined whether the citrandarins influenced HLB development in 'Pera' sweet orange scion observing the distribution of CLAs in leaves and roots of some trees. In this survey (Area A), among the CLAs-negative plants, three were grafted onto the same rootstock, and this hybrid may be promising. However, future investigation, mainly using the most promising citrandarins, will help understand the probable mechanism of defense and/or identifying compounds in *P. trifoliata* and its hybrids that are very important as strategy to combat HLB.

Citrus endophytic phytobiome and Huanglongbing

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The long-term goal of this research is to develop novel, effective, and sustainable Huanglongbing (HLB) management strategies. We hypothesize that in HLB-infected groves, a number of *Candidatus Liberibacter asiaticus* (CLAs) positive trees remain asymptomatic because the phloem-dwelling endophytic phytobiomes (or microorganisms) inhibit CLAs colonization, thereby reducing disease symptoms. We envision that such phytobiomes can be stored in "mother" trees or in long-term *in vitro* cultures, and then be incorporated into production citriculture by grafting or direct inoculation procedures, and that these HLB-inhibiting phytobiomes can be promoted and/or maintained by cultural practices. Our preliminary experiments have shown that the phytobiome is, in fact, different between HLB-positive and HLB-negative trees. High throughput sequencing of 16S rRNA genes was used to examine the phloem bacterial communities in three samples from the single HLB-positive Pummelo-lemon tree in Los Angeles, California and 15 samples from five HLB-negative 22-year-old sweet orange trees growing on Swingle citrumelo and Carrizo and C35 citrange rootstocks. A beta diversity analysis showed that the bacterial communities in the HLB-positive tree were different ($p = 0.022$) than the HLB-negative ones. There were no differences between the bacterial communities in the 15 HLB-negative samples ($p = 0.330$), suggesting that the different rootstocks had relatively little impact on the endophytic bacteria in the sweet orange scion. The dominant bacterial phylum in the HLB-positive tree was Proteobacteria (alpha and gamma), with 51.5% of the sequencing reads being CLAs. The dominant bacterial phyla in the HLB-negative trees were Firmicutes, Tenericutes, and Proteobacteria (gamma and beta), with no CLAs sequences. This preliminary finding is in agreement with our main hypothesis, that endophytic phytobiomes may play a role in HLB development.

Effects of reciprocal grafting rootstocks and scions on tree tolerance to Huanglongbing

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Huanglongbing (HLB) is a widespread and devastating disease of citrus, caused by the bacterial pathogen *Candidatus Liberibacter asiaticus* (Las). Studies have identified tolerance to Las within the species *Poncirus trifoliata* (trifoliolate orange) and some hybrids of Citrus and trifoliolate orange that are commonly used as rootstocks. Although these rootstocks may impart some tolerance to Las when grafted with commercial citrus scions, the relative Las tolerance for such grafted trees is less than desired. Edible quality scion cultivars that incorporate the trifoliolate-type tolerance to Las are being developed, but it is not clear how much tolerance they might have to Las. It is also not understood the relative importance of the scion and rootstock genotypes in determining the tolerance of a grafted tree to Las. This study describes experiments to compare the Las-tolerance of trees composed of susceptible and tolerant cultivars in both the scion and the rootstock position. Experiments were conducted in the greenhouse using the susceptible cultivars Valencia orange and Cleopatra mandarin, and the tolerant cultivars US-897, US-942, US-802, and Carrizo citrange. Reciprocal graft combinations were produced and trees were either mock-inoculated or graft-inoculated with Las. Foliar disease expression was scored in 2-month intervals over a period of 12 months and was highest in susceptible cultivars. Only rarely were symptoms observed in the tolerant genotypes, regardless of the rootstock. Las copy numbers were assessed at 6 and at 12 months after inoculation. Both scion and rootstock affected the rate with which trees became PCR positive for Las and the amount of bacteria present in leaves. Similar to the observations for foliar disease expression, the amount of bacteria was considerably lower in the tolerant scions even when grafted on susceptible rootstocks.

Gene expression patterns in leaves and roots of citrus trees composed of different scion and rootstock genotypes to elucidate control of tolerance to HLB

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Huanglongbing (HLB) caused by the bacterial pathogen *Candidatus Liberibacter asiaticus* (Las) has spread across all areas of Florida, and infection rates in most commercial citrus blocks approach 100%. Most common scion cultivars are severely damaged by infection with the

bacteria, but significant tolerance to HLB has been identified within the species *Poncirus trifoliata* (trifoliolate orange) and some of its hybrids that are commonly used as rootstocks. This study describes experiments to compare the effect of Las on citrus trees composed of susceptible and tolerant cultivars in both the scion and the rootstock position, and expression of genes associated with disease resistance in leaf and root tissue. Experiments were conducted in the greenhouse using the susceptible cultivars Valencia orange and Cleopatra mandarin, and the tolerant cultivars US-897, US-942, and US-802. Reciprocal graft combinations were produced and either mock-inoculated or graft-inoculated with Las. Both scion and rootstock affected the rate with which trees became PCR positive for Las and the amount of bacteria present in the leaves. Typically, trees with tolerant cultivars in the scion position were less damaged by Las infection than trees with susceptible cultivars in the scion position, regardless of rootstock. Differential expression of HLB-associated and other genes selected from previous gene expression studies was investigated in leaves and root tissues of non-infected and infected plants. Genes included those commonly associated with pathogenesis and disease resistance in other host pathogen systems and with carbohydrate metabolism. Notable differences were observed not only between tissue types but also between susceptible and tolerant genotypes and may be key to understanding HLB tolerance. This information may be utilized in breeding programs to preselect new rootstock and scion lines most likely to have HLB tolerance in advance of field testing.

Tracing infection sources from psyllids to natal trees using isotopic signatures

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Living organisms acquire their atomic makeup from their mother, their food source(s), and their environment. However, atoms of a single chemical element exist in multiple isotopic states due to variation in atomic mass (variation in the number of neutrons). As a result, each organism's atomic composition represents the cumulative isotopic ratios of these three sources. Since plant feeding insects acquire the vast majority of their elemental isotopes from their host plant, they represent the isotopic ratios of their host plants. Metabolically inactive tissues retain the signature isotopic ratio laid down during development, even in the face of host plant shifts. Thus, adult psyllids retain a signature of the Citrus tree they developed on. Moreover, citrus management regimes affect the isotopic composition of trees. In particular, conventional and organic regimes differ greatly in the source, and thus isotopic composition, from which elemental fertilizers originate. Because psyllids acquire HLB more readily as nymphs than adults, we capitalize on this isotopic signature to trace psyllids back to the most likely source of infection – their natal tree – through analysis of stable isotopic ratios within each psyllid. Our goal is to aid HLB survey and removal efforts by narrowing the search space for infected trees.

Transcriptomics, proteomics, and yeast-2-hybrid analyses reveal genes pathways important for *Candidatus Liberibacter* sp. circulative, propagative transmission

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Ca. Liberibacter asiaticus (CLAs) and *Ca. L. solanacearum* (CLso) are the fastidious bacterial causal agents of Huanglongbing and Zebra chip diseases of citrus and solanaceous crops, respectively. They are transmitted by psyllids in a circulative, propagative manner. Identification and functional characterization of genes involved in *Ca. Liberibacter* systemic invasion-transmission are of great interest as targets when implementing RNA-interference technology for disease management through psyllid vector control. The 110,937 and 83,231 Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama and potato psyllid (PoP) *Bactericera cockerelli* Sulc. *in silico* annotated transcripts expressed in midguts and salivary glands, were used to identify gel purified PoP midgut and salivary gland proteins from CLso-infected or CLso-free psyllids, analyzed by nano-liquid chromatography and tandem mass spectrometry (LC-MS/MS). Approximately 500 unique proteins were identified in the 'CLso-infected' compared to 'CLso-uninfected' organs. Identification of differentially abundant proteins and their corresponding differentially expressed transcripts in

response to *Liberibacter* infection of psyllids revealed that many were organ-specific. From among these, biologically interesting candidates were selected and used as bait for yeast-2-hybrid analysis of protein-protein interactions. Results indicated that transcripts involved in innate immunity, endocytosis, and cytoskeleton assembly are important to *Liberibacter* invasion of the psyllid host, and so could be involved directly or indirectly in psyllid-mediated CLAs transmission.

Developing a management plan for Asian citrus psyllid in retail nurseries: Evaluating uptake and retention of systemic insecticides in containerized citrus

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The broad objective of this study is to provide the California citrus industry with information on the conditions likely to affect the persistence of imidacloprid and other systemic neonicotinoids in potted citrus trees at retail stores. ACP adults and nymphs have been found on citrus trees for sale at retail outlets. The trees originated from production nurseries where they were treated with systemic imidacloprid before shipping to comply with CDFA regulations on the shipment of containerized citrus within quarantine. Many of the trees were present at the retail outlets for a period longer than 3 months, indicating that the trees had surpassed the 90-day certification period originally set by the CDFA. Leaf tissue samples collected from the infested trees and tested for imidacloprid residues confirmed that most trees had residues of imidacloprid below the 200 ppb threshold level required for protection against ACP. We have completed studies to evaluate the effects of watering amounts, potting media, and citrus variety on the uptake and persistence of systemic neonicotinoid insecticides applied to containerized citrus trees. There was a strong effect of variety on the overall titers of imidacloprid, with lemons having half the concentration measured in navel oranges. Excessive watering resulted in lower overall titers of imidacloprid in trees, although mean concentrations at all watering levels evaluated fell below desired thresholds after 12 weeks. The negative effects of overwatering were not improved by modifying the relative proportions of sand and organic components of the potting media.

Spectroradiometry for detection of HLB (*Candidatus Liberibacter asiaticus*) at field level: An alternative to visual detection methodology

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The Huanglongbing (*Candidatus Liberibacter asiaticus*) is a bacterial disease that nowadays have no cure. Molecular analyses (qPCR, PCR) are the most accurate methods for detection of HLB. The visual detection of the disease in the field can only be performed by trained technicians; this method is time-consuming, subjective, and expensive. Remote sensing is an alternative for detecting the disease in the field with the use of satellite images. The aim of this study was to apply the technique of Spectroradiometry for detection of plots infected with HLB and differentiate them from the healthy ones. Spectral signatures were obtained from 1,320 HLB-infected trees from different citrus groves (*Citrus aurantifolia*), and it was performed with a multi-spectral radiometer (485, 560, 660, 830, and 1650 nm). The Principal Component Analysis showed that around 660 nm and 830 nm were useful for the differentiation of plants infected with HLB from the healthy ones. Afterward, satellite image corrected (radiometric and by BDRF) of Landsat 8 (Path 28, Row 47, acquired 02/05/2013) were used in the ESAM algorithm using ENVI 4.7® software. The results of the spectroradiometrical analysis obtained and the field verification of the municipalities of Mugica and La Huacana, Michoacán (with official presence of HLB), showed 60% of correlation between predicted and observed data; each point evaluated corresponded to a pixel of 30 m x 30 m which presented one or more citrus trees infected by HLB. The first results of the study indicate a potential use for fast detection of citrus

infected trees in extensive citrus cultivates areas, as an economic alternative to the visual surveillance.

Functional and behavioral response of *Tamarix radiata* (Hymenoptera: Eulophidae) to different densities of its host, *Diaphorina citri* (Hemiptera: Psylloidea)

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It has been claimed that success of parasitoids as biological control agents can be predicted by response to increasing host densities, termed “functional response” as mediated by so-called “handling time” and “searching efficiency” first characterized by the Holling Disk Equation and later refined by Rogers (1972). How do these parameters relate to actual behavior exhibited by the female wasp in the presence hosts? A functional response was evaluated by holding pairs of 3-day-old *T. radiata* in 50 mL centrifuge tubes with access to an *M. paniculata* shoot infested with 10, 20, 30, 40, 50, or 60, 4th instar psyllid nymphs changed every 24 hours. Parasitism was highest with 10 hosts at 43% and least at 18.5% with 60 hosts. Superparasitism showed the opposite trend, being highest (39.4%) at the lowest host density. Results conformed to a Type II functional response with attack rate (a') estimated by the Rogers equation at 9.0 ± 1.3 cm²/hr and handling time (Th) of 50.4 ± 3.6 min/host. Behaviors of individual 3-day-old females in petri dishes with the same six host densities were recorded using Observer software for 30 minutes with much different results: handling time (3.1 ± 0.74 min/host), defined as probing + oviposition, and attack rate (333 ± 72.6 cm²/hr) calculated from the Rogers equation which equates encounters to the product of searching time, host density, and attack rate. The search continues for relationships between functional response, parasitoid behavior, and biological control success.

Early changes in host response to *C. Liberibacter asiaticus* measured by metabolomics

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Candidatus Liberibacter asiaticus (CLAs), a non-culturable, phloem-limited bacterium, is the presumed causative agent of the disease known as Huanglongbing ((HLB) a.k.a. Citrus Greening Disease) in the US. HLB is present worldwide and, since its initial discovery in 2005, has devastated the Florida citrus industry; it now threatens the California and Texas industries. Diseased trees suffer from discolored leaves and fruit, reduced fruit yield with poor quality, and premature death. There is no known cure for HLB, and though different citrus varieties exhibit varying tolerances for HLB, there are no resistant varieties. To understand how different citrus varieties respond to CLAs infection, leaf samples from Lisbon Lemon, Tango Mandarin, and Washington Navel Orange trees graft-inoculated with CLAs (originating from the initial find in Hacienda Heights, CA) were studied longitudinally with ¹H NMR metabolomics. Results were compared with that of qPCR, the current “gold standard” for CLAs detection. Changes in the overall metabolite profile of the infected plants were noted earlier than qPCR detection of CLAs, and suggest that metabolomics may be a more sensitive early detection test. Symptomatic leaves were significantly different from control leaves as well as asymptomatic leaves from infected trees, suggesting that different stages of infection may be detected. These biomarkers may be useful for early detection and staging of infection, helping to clarify CLAs-induced changes to host metabolism by providing insight into affected pathways. Furthermore, these results may be used to inform discovery of targets for treatment and methods of controlling the spread of HLB.

Multidisciplinary approaches for early detection of Huanglongbing

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Huanglongbing (HLB) is a devastating citrus disease caused by bacteria belonging to the genus *Candidatus* Liberibacter. In North America, the most common species infecting citrus is *C. Liberibacter asiaticus* (CLAs), which is a non-culturable, phloem-limited bacterium that can be transferred by grafting or psyllid feeding. The disease has devastating effects on fruit quality and ultimately causes premature tree death. With no known cure or resistant citrus variety, HLB severely threatens the global citrus industry. Gold-standard testing for this pathogen is detection of the bacterial genomic DNA using qPCR. However, qPCR relies on a minimum bacterial titer, and thus early detection of the disease is a matter of chance. Here, we explore other methods of early detection that measure the pathogen (secreted proteins and DNA) and host response to the pathogen (small RNAs, proteins, soluble metabolites, volatile organic compounds (VOCs), and optical reflectance spectroscopy) to compare with qPCR. In the first of two sets of experiments, graft inoculation of three varieties of citrus (Lisbon Lemon, Tango Mandarin, and Washington Navel trees) with *C. Liberibacter* isolated from the initial Hacienda Heights find have been longitudinally followed. Findings from each method have been converging to suggest that other methods may be more sensitive at detecting HLB prior to qPCR with high sensitivity. Moreover, several of the methods measuring response to infection may help to facilitate discovery of molecular targets to control CLAs infection and transmission.

Transmission of *Candidatus* Liberibacter asiaticus by the Asian citrus psyllid: A proteomic perspective

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The Asian citrus psyllid (ACP), *Diaphorina citri* is an economically important pest of citrus and a vector of *Candidatus* Liberibacter asiaticus (CLAs). CLAs is a phloem-limited, gram-negative, fastidious bacterium that is implicated in causing Huanglongbing, the most serious disease of citrus. The vector manipulation hypothesis predicts that CLAs manipulates ACP to promote its own spread; however, details of this molecular subterfuge are unknown. To understand ACP-CLAs interactions at the molecular level, we performed a quantitative proteomics analysis comparing healthy ACP to ACP infected with CLAs using nanoscale liquid chromatography coupled to high resolution mass spectrometry on an Orbitrap Fusion. Three biological replicates were used for each treatment and three different MS data acquisition strategies were used for each biological replicate, enabling proteome measurements over a wide dynamic range. Over 30,000 unique spectra were matched to the ACP and endosymbiont genomes with more than 3,500 proteins identified and quantified, including proteins from the different ACP bacterial endosymbionts. To the best of our knowledge, this comprehensive proteome analysis provides the first insights into the molecular events regulating CLAs infection of the ACP. Proteome changes are consistent with CLAs using a cadherin/catenin-mediated entry pathway used by pathogenic bacteria that infect animal epithelial cells via dynamic rearrangement of the plasma membrane and actin

cytoskeleton. ACP proteome changes show the insect is responding to bacterial effector proteins involved in immune system modulation. Changes were also observed in a number of metabolic enzymes and in proteins expressed by the defensive bacterial endosymbiont, *Candidatus Profftiella armatura* consistent with the hypothesis that CLAs infection causes metabolic disease in the insect which may promote the spread of the pathogen by vector manipulation. Functional assays are in progress to test the roles of these proteins in CLAs epithelial cell invasion, modulation of ACP metabolism, behavior, and endosymbiont interactions.

Subtle differences in the insecticidal response in field populations of the Asian citrus psyllid

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Insecticidal treatment to reduce populations of the Asian citrus psyllid (ACP) remains an important component in the management strategy to slow the spread of 'Candidatus' *Liberibacter asiaticus*, the causal agent of citrus greening (Huanglongbing, HLB). Protecting the utility of the available chemistries until a cure for HLB is available is of the utmost importance, and towards that goal, we conduct an annual survey to test the susceptibility of ACP to a panel of insecticides with different modes of action to monitor for developing resistance. In years 2009 through 2012, we began to see a drop in susceptibility to all major insecticide classes in a number of ACP populations across central and southern Florida. In the surveys conducted in 2013 and 2014, we found a reversion in the LD₅₀ response of all field populations to levels that were not significantly different from the reference laboratory strain. However, most population dose-response curves were significantly different from the laboratory strain and between one another, suggesting that there is divergence in insecticide response between populations. Whether these differences are within the natural variation for ACP populations or whether pressure from insecticides has resulted in the drift of metabolic and/or molecular profiles remains unknown and is being investigated. Regardless, these results suggest that certain populations of ACP may be primed for resistance development if insecticide use does not continue to be managed strategically and conservatively. The highlights of the major findings of this 2-year study with a comparison to previous years will be presented.

Systemic insecticides and reflective mulch for Asian citrus psyllid (*Diaphorina citri*) control in new citrus plantings

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Greening or Huanglongbing (HLB) is a devastating disease of citrus caused by *Candidatus Liberibacter asiaticus* and transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri*. HLB now occurs worldwide in most citrus growing regions except the Mediterranean and Australia. Management relies principally on insecticidal control of ACP which is insufficient, even for young trees which are most susceptible to the disease. We tested the ability of metalized polyethylene mulch to repel adult ACP, as well as effects on incidence of HLB, and early tree growth with and without insecticide treatments, as well as with and without foliar nutrition. The experimental design is a three-way factorial randomized complete split block design with four replications of four main plot treatments: (1) supplementary foliar nutrients only, (2) insecticides only, (3) nutrients plus insecticides, and (4) neither nutrients nor insecticides (control). Each main plot was split, with half being planted on metalized UV reflective mulch. Evaluations of all treatments include ACP populations on flush and sticky cards, trunk growth measurements, and leaf samples tested for the presence of HLB.

Protein-based detection systems in *Liberibacter asiaticus*

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Citrus Huanglongbing (HLB) disease affects citrus industry worldwide. The causal agent of HLB, a phloem limited alpha proteobacterium *Liberibacter asiaticus* (Las) transmitted by phloem sap feeding psyllid *Diaphorina citri*, is not yet culturable. Visible symptoms of

HLB-infected citrus trees such as asymmetric blotchy leaf mottle, stunted shoots, small off-taste fruits, and premature fruit drop start to appear in the advanced and late stages of the infection, for which little could be done to promote the survival of the trees as well as to implement better strategies to control the spread of the disease. Therefore, highly sensitive detection systems for HLB are necessary to rapidly identify the disease and, consequently, implement control methods in areas in which the bacterium has not yet been established. In this study, we have successfully purified eight *Liberibacter asiaticus* recombinant protein biomarkers that were over-represented in proteomics-based analysis using Proteomics-based Change Mediated Antigen Technology (PCMAT). Their correspondent polyclonal antibodies were successfully raised in rabbits. The antibody sensitivity against HLB-infected citrus materials is currently under investigation using enzyme-linked immunosorbent assay (ELISA). The result will be further confirmed by qPCR on the same infected citrus materials. If successful, this protein biomarkers-antibody system can be used as an alternative tool for HLB diagnostic and, thus, to better monitor the HLB distribution and control the disease.

FLS2 from *Solanum tuberosum* have interaction with FlaLas from *Candidatus Liberibacter asiaticus*

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The causal agent of Huanglongbing disease or citrus greening, 'Candidatus *Liberibacter asiaticus*' (las), is an uncultured, gram-negative, phloem-limited α -proteobacterium. Currently, there is no cure for this disease thoroughly, can only control the spread of this disease through removal and destruction of the infected trees. In the previous report, Las encodes a flagellin and hook-associated protein (Fla) of 452 amino acids that contains a conserved 22 amino acid domain (flg22Las) at positions 29 to 50 in the N-terminus. In this study, 108 genotypes of potatoes were tested for ROS activity using the flg22Las. Fortunately, the potato genotype TC72 and TC104 have the highly ROS activity; and the expression of related genes in the resistant pathway were also transiently up-regulated by the FlaLas via Agrobacterium-mediated transient expression, especially Flagellin-sensing 2 gene (*StFLS2*). Through the NCBI blast, there are two different FLS2 sequences in potato, named StFLS2a and StFLS2b, which have a difference in the number of Leucine-rich repeats and mutation in putative important amino acids. Through the further research, the up-regulated *StFLS2* is *StFLS2a* and the expression of *StFLS2b* has not changed in the FlaLas interaction with potato. The Yeast-two-Hybrid (Y2H) and Bimolecular fluorescence complementation (BiFC) approaches were taken to identify the interaction between FlaLas and StFLS2a. The result shows FlaLas has interaction with StFLS2a in the plant cell membrane, and in this process, the position 1 to 135 in the N-terminus of FlaLas is the important domain. Surprisingly, the *StFLS2a*-GFP and FlaLas-GFP is separately located the membrane and nuclear in the plant cell. We need further research to verify whether this interaction is donated to the cure for Huanglongbing in citrus.

Experimental psyllid traps

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Asian citrus psyllid (*Diaphorina citri* Kuwayama) is a vector for the pathogen *Candidatus Liberibacter asiaticus* that is decimating the Florida citrus industry. Most commonly, yellow sticky cards are used to monitor populations of *D. citri*. An alternative monitoring system that can avoid the sticky mess, preserve specimens for pathogen detection, reduce non-targets, and attract larger numbers of psyllids is desirable. A preliminary trap model has been designed that accomplishes the first three criteria, but the yield remains lower than that of the sticky trap. We are designing and testing alterations to the prototype using a 3D printer and 3D modeling software. A 3D printer allows us to model and print new ideas quickly without the need to search for and assemble components derived from various objects. The original prototype,

designed by Dr. Russell Mizell and our laboratory, consists of a hollow yellow cylinder with shaded holes at the top and a clear dome that allows light to shine into the cylinder and out the holes. The psyllids land on the yellow cylinder, climb upwards as is their natural inclination, go beneath the shade where they then see light through the holes, which they then enter. From within the trap, the holes appear dark and are avoided, trapping the psyllids inside. In making alterations to this design, we focus on shape, color, and light. We test the new designs against each other and against the original in release cages and in the field, making observations on the behavior of the psyllids in reaction to the designs. Some promising features include the use of battery powered LED lights, and ridges or stems to guide psyllids directly through the holes.

Antibiotic treatment of the Florida Citrus Arboretum for Huanglongbing

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The Florida Citrus Arboretum located in Winter Haven, Florida is a four and half acre collection of 212 cultivars of citrus and citrus relatives. The arboretum was established in 1975 to ensure that valuable citrus and citrus related germplasm are always available for study and use in Florida. Huanglongbing (HLB) was first detected in the arboretum in 2007 and the number of trees infected has steadily increased such that most of the arboretum is infected with HLB. In May 2015, the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI) initiated an experiment involving the use of Streptomycin sulfate and Penicillin-G potassium salt for potential mitigation of HLB in the arboretum trees. A total of six spray applications representing monthly applications (May-October 2014) of Streptomycin sulfate + adjuvant, Penicillin + adjuvant and adjuvant were made. Additionally, 10 trees were selected for passive injection of Streptomycin sulfate, 10 trees for Penicillin-G, and three untreated trees as controls. Passive injection and foliar sprays of both Streptomycin sulfate and Penicillin-G resulted in a reduction of *Candidatus Liberibacter asiaticus* (Las) populations but treatments via passive injection showed greater effect in reducing Las populations. Passive injection of Penicillin-G proved to be most effective.

Thermotherapy trials and the molecular mechanism behind the success of heat treatment for the control of citrus Huanglongbing

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Huanglongbing is associated with three species of *Candidatus Liberibacter* which have distinct variations in temperature sensitivity and tolerance. Las, the most prevalent and heat-tolerant species, can thrive at temperatures as high as 35°C. Our earlier work has shown that Las bacteria in potted HLB-affected citrus were significantly reduced and often eliminated when exposed to continuous temperatures of 40 to 42°C for a minimum of 48 hours. Feasibility and effectiveness of thermotherapy in the field was determined by placing portable greenhouses over citrus to achieve therapeutic temperatures through solarization. Most trees responded with vigorous new growth within weeks after treatment and Las titer was significantly reduced for more than a year. By two years, titer levels were similar to pretreatment numbers, but trees under good management continued to grow well. Unlike with potted trees, exposure to high heat in field conditions was not sufficient to eradicate the Las population. This may be attributed to reduced temperatures at night, rather than continuous high temperatures and failure to achieve therapeutic temperatures in the root zone. RNA-seq was used to monitor changes in gene expression of HLB-affected trees exposed to the thermotherapy, by collecting new flush leaves 3-4 weeks after treatment. Seventy-eight genes were differentially expressed before and after heat treatment. Thirty-one genes including four heat-shock related genes, two P450 genes, and one TIR-NBS-LRR gene, a peroxidase, one copper amine oxidase, and one CBL-interacting protein kinase, were up-regulated after thermotherapy. Forty-seven genes including one homogentisate phytyltransferase, two osmotin genes, and one WRKY transcription factor, were down regulated. These data

indicate that thermotherapy has marked effects on citrus gene expression in addition to affecting Las survival.

Construction of citrus gene coexpression networks from microarray data using random matrix theory

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After the sequencing of citrus genomes, gene function annotation is becoming a new challenge. Gene coexpression analysis can be employed for functional annotation using publicly available microarray data sets. In this study, the microarray probes were mapped to citrus genes based on *Citrus clementina* v1.0 genome assembly and annotation. 277 citrus microarrays were used to construct five coexpression networks, including one condition-independent and four condition-dependent (Citrus canker, Huanglongbing, leaves and fruits) networks. In total, these networks contain 33,939 edges among 4,934 nodes (genes), which accounts for 41.1% of the measurable genes of the microarray, or 20.1% genes in the *Citrus clementina* v1.0 genome. Then, these networks were partitioned into functional modules by the Markov Cluster Algorithm. 154 modules (38.8%) in these networks had some degree of Gene Ontology biological process enrichment, and 107 modules were detected with significantly enriched KEGG pathway terms. Finally, independent verification of these networks was done using RNA-seq data of 273 genes (5,586 edges). This study provides a comprehensive map of citrus gene coexpression networks, which should be quite useful for the citrus research community.

Evolving diversity and dynamics of ‘*Candidatus Liberibacter asiaticus*’ populations

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‘*Candidatus Liberibacter asiaticus*’ (Las) is the prevalent species of citrus Huanglongbing bacteria. We describe here the molecular characterization of Las bacterial populations and their association with insect transmission and disease development in host plants. By sequencing 35 Las isolates collected from different geographic origins, we found that most surveyed Las isolates encompassed at least two prophages/phages, but isolates from both Japan and California were missing a majority of the prophage regions. In addition, all but one of the 26 isolates from Florida clustered into two major phylogenetic groups. Variation within the chromosome was less than 1% overall but could be as high as 3% in the prophage regions. Since prophage/phages are known to carry pathogenicity-related genes, these dynamic components are key determinants that mediate the diversity and dynamics of Las populations. Based on their highly variable sequences, we identified nine Las prophages/phages variants. Types A, B, C, and D are the most abundant types in Las-infected plants, while only A, B, and C are abundant in Las-infected psyllids. The type D Las population does not appear to exist in the psyllids but is dominant in mealybugs. Interestingly, the type D-dominant Las population transmitted by the mealybugs does not cause disease; however, type D can be detected in infected citrus plants and is always associated with leaf blotchy mottle. Using graft transmission with severe and mild Las strains, we found the movement and titers of Las bacteria varied greatly in different genotypes of rootstocks and commercial varieties. Although significant differences were observed among 11 citrus genotypes, most Las infections were detectable in the shoots before the roots of inoculated plants, with both the presence and titers of Las being significantly higher in the shoots. Results also indicated Las phage activities were higher in the roots than in the shoots.

Genetic modification of citrus to create consumer friendly HLB tolerant plants

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To produce a genetically modified citrus tree acceptable to the consumer, juice processors, and packing houses, development of a consumer friendly plant is essential. This genetically modified citrus tree would either have genetic material derived exclusively from plants (all plant transgenics) or from sweet orange or closely related sexually

compatible species such as trifoliolate oranges and kumquats (all citrus intragenic plants). These plants will contain no DNA from viruses, bacteria, insects, or any other non-plant source. Use of this gene pool could lead to easier approval and consumer acceptance of a genetically modified product. We are exploring several strategies to create consumer friendly all plant and, eventually, all citrus DNA containing HLB tolerant citrus. We have evaluated a number of plant/citrus derived genetic elements (gene, promoter, terminator, selectable marker, etc.) necessary to create a consumer friendly transgenic/intragenic product. As an example, we have evaluated a single base pair mutant (A122V) of the *Citrus sinensis* acetolactate synthase gene as an herbicide resistance selectable marker gene. We have also developed a transformation system to generate reporter gene expression free citrus by coupling a visual anthocyanin producing transcriptional factor gene (derived from the blood orange) with a citrus derived embryo specific promoter to regenerate genetically modified plants that have the marker gene switched off. We are utilizing the cre-lox system to remove non-plant selectable marker genes following transformation. In addition, a large number of citrus derived promoters, genes, and terminators are being utilized to create an all citrus transformation vector, and have been incorporated into sweet oranges and mandarins by conventional *Agrobacterium* mediated transformation and our unique protoplast transformation methods.

Divergence of cultured *Liberibacter* strains with prolonged cultivation under laboratory conditions

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Liberibacter crescens is the only cultured member of the *Liberibacter* genus described. *L. crescens* has been used to test culture media for potential to support the growth of the *Liberibacter* pathogens such as *L. asiaticus* and *L. solanacearum* as well as in screening antimicrobial compounds. Though isolated from a diseased babaco papaya plant, attempts to re-infect known *Liberibacter* hosts with *L. crescens* are still in progress. Endosymbionts that are highly entangled with their host's metabolism have been known to undergo changes upon isolation that preclude reintroduction. To further examine the possibility of this mechanism occurring in the commonly used laboratory strain *L. crescens* BT-1, an ancestral strain from which BT-1 is derived was recently revived. This strain, *L. crescens* BT-0, exhibited growth characteristics that varied from the laboratory strain, calling for further investigation into potential regulatory changes that BT-1 has undergone during prolonged carriage in axenic medium. The genome sequence and methylation patterns have been determined from BT-0 and BT-1 growing under the same conditions using the PacBio platform. Initial analysis shows that a GANTC motif is heavily methylated in BT-0. It is suspected that a CcrM-like modification methylase, present in the other *Liberibacter*s and closely related to that in *Rhizobium*, is responsible for this behavior. Further investigation during in vitro growth using high-throughput Illumina transcriptome sequencing is in progress and is expected to give insights into *Liberibacter* metabolism and reveal potential adaptations that render BT-1 better suited to growth under laboratory conditions and potentially prevent the strain from re-colonizing its plant host as easily as the ancestral strain BT-0. A thorough investigation of these genomic and/or regulatory changes is expected to aid in the development of a *L. crescens*/plant system as well as identify key features needed for the growth of *L. asiaticus* in plants and artificial growth media.

Identification of semiochemicals in host and non-host plants associated with *Diaphorina citri* Kuwayama (Hemiptera, Liviidae) behaviour

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Diaphorina citri is a vector of Huanglongbing (HLB), the most severe disease affecting citrus production worldwide. The insect behavior can

be manipulated by semiochemicals and this can affect the potential of HLB dissemination. The aim of this work was to identify chemical volatiles from host and non-host plants associated with *D. citri* behaviour. Volatiles were collected from *Murraya paniculata*, *Citrus sinensis*, *Citrus reshni*, *Citrus limettoides*, *Poncirus trifoliata*, *Psidium guajava*, *Mangifera indica*, and *Anacardium occidentale*. A Pettersson olfactometer was used to determine behavior of *D. citri* females to the volatiles. Air was drawn from the olfactometer at the rate of 200 mL·min⁻¹. The chemical analyses were performed on a Hewlett-Packard 6890A GC with split-splitless injector and flame ionization detector using a HP-5 column. The residence time and number of entries of *D. citri* were subjected to paired *t* test. The chemical profile of volatiles obtained from *C. sinensis*, *M. paniculata*, and *A. occidentale* were analyzed by Generalized Linear Model and Deviance Analyses with gamma distribution and inverse as link function. *D. citri* spent more time on *M. paniculata* and *C. sinensis* arms compared to control arms (hexane). When *D. citri* was exposed to *A. occidentale* volatiles, they preferred control arms. As for the number of entries, significant differences were registered for *C. reshni* and *C. sinensis*. Regarding the chemical profile of *C. sinensis*, *A. occidentale*, and *M. paniculata*, the three species presented significantly qualitative and quantitative differences. Two compounds (terpenes) present in higher amounts in *A. occidentale* volatiles than in *C. sinensis* and *M. paniculata* might play a role in the host foraging behaviour. The identification of chemical compounds that elicit behavioral responses of *D. citri* is the first step for the development of a push-pull strategy for HLB vector control.

Low-cost, user-friendly solutions for VOC sampling of citrus for early detection of HLB

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In recent decades, Huanglongbing (HLB) has invaded citrus crops in the United States with devastating results. Researchers at UC Davis have established an HLB-specific volatile organic compound (VOC) signature for Valencia and Hamlin sweet orange varieties. Furthermore, they have established that VOC signatures are pathogen-specific and that the HLB VOCs can be discriminated in the presence of other diseases (like Citrus Tristeza Virus, CTV, and citrus canker, among others). In this same research, they also presented greenhouse study results indicating that VOC analysis provides health status information before PCR analysis with fewer false negatives. Early detection is key to managing this disease. One challenge to wide adoption of the technology is lowering the cost of performing the analysis and providing user-friendly solutions to collecting the VOC signatures. We will present different approaches that may be applied and early data that indicate how effective these approaches may be. Some of these approaches separate the task of collecting the VOC odor profile from the task of analyzing the profile. This separation of tasks provides solutions for detection in residential areas as well as for citrus groves. This also significantly lowers the weight and power burden on the survey crew.

Optimization of dsRNA knockdown-phenotype assays to evaluate RNAi efficacy of gene silencing in the potato psyllid

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The circulative, propagative transmission pathway of *Candidatus Liberibacter* in psyllid vectors appears to require multiplication in the gut, circulation in the hemolymph, entry into the salivary glands, and acquisition. This route presents a series of potentially hostile environments to which *Liberibacter* must respond, and survive in, to ensure psyllid-mediated transmission to the plant. *Ca. Liberibacter* transmission by Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama and potato psyllid (PoP) *Bactericera cockerelli* (Sulc.) exhibit different patterns of acquisition, in that adults and nymphal PoP can ingest, acquire, and transmit CLso, albeit with different latent periods. In contrast, the adult ACP transmits CLas efficiently only when ingestion occurs during nymphal stages (pre-adult). Although poorly characterized, bacterial 'effectors' that interact with psyllid proteins mediate interactions that enable *Ca. Liberibacter* to systemically invade ACP and circulate to the salivary glands. RNA interference (RNAi) is seen as a promising technology for management of insect pests and vectors of plant bacterial pathogens that are propagative in the vector.

Several factors can affect the efficacy of RNAi knockdown, including delivery method (direct or indirectly administered), dsRNA concentration, and presence of dsRNA- and siRNA-degrading enzymes. To this end, expression profiles were obtained for the midgut and the salivary glands, dissected from adult PoP and ACP either free of or harboring CLso or CLAs. Comparative analysis was used to select differentially expressed contigs associated with biologically relevant functions, as candidates for RNAi knockdowns. Adult or 3-4th instar psyllids harboring CLso from the first nymphal instar onward, or CLso-free psyllids, given an acquisition access period on CLso-infected tomato plants were treated with dsRNA using artificial membrane feeding, or topically. The efficacy of dsRNA candidate gene knockdown was quantified by qPCR for three replicates each. The goal is to develop and optimize laboratory assays for evaluating dsRNA knockdowns in conjunction with phenotypes of interest.

Genetic analysis of a nonpathogenic strain of *Ca. Liberibacter asiaticus*

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Huanglongbing (HLB) was discovered in Florida in 2005, and the causal agent, *Candidatus Liberibacter asiaticus* (Las) was widespread by 2007, but only now are dramatic yield losses apparent, indicating a very long incubation period. Most pathogenic strains of *Liberibacter*, and all Las in Florida examined to date carry two prophages, either of which may carry lysogenic conversion genes that increase the fitness and/or virulence of the bacterial cell in a host. In Las strain UF506, the two prophages (SC1 and SC2) both carry predicted peroxidase genes. Las gene SC2_gp095 conferred resistance to hydrogen peroxide to *L. crescens* (Lcr), and the enzyme appeared to be secreted, making this gene a likely virulence factor. Detoxification of host-derived hydrogen peroxide may enhance the ability of Las to withstand the initial burst of reactive oxygen species and may also impact hydrogen peroxide-mediated long distance signaling. Japanese Las strain B430 appears to be nonpathogenic in citrus but parasitic, with a reduced titer relative to other Las strains. B430 appears to be missing all six prophage genes examined, including the functional peroxidase. Japanese strain Ishi-1 appears to also lack the entire prophage region of Psy62 and UF506, but is pathogenic and may carry different virulent factors. It is unclear if it carries a peroxidase. The nucleotide sequences of B430 chromosomal genes flanking the expected prophage insertion site are identical to Las strain Psy62 and Ishi-1. A B430 ortholog of Psy62 CLIBASIA_01645, a chromosomally encoded C1 bacteriophage repressor, was found to exist as a mixture of three variants containing 12, 13, or 14 internal repeats in the same plant, two of which would encode a truncated (mutant) protein. The apparent lack of bacteriophage may ease selection pressure for a functional repressor, resulting in the observed mixture of variants.

Evaluating the biological control of the Asian citrus psyllid in the Lower Rio Grande Valley of Texas using the biological control agent, *Tamarixia radiata*

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Tamarixia radiata is a biological control agent of the Asian citrus psyllid that is being used as a tool to help reduce psyllid populations in urban environments of citrus growing areas in Texas. Methods have been developed to produce large numbers of these beneficial insects for field release. In FY14, the CPHST Mission Lab mass produced over 468,000 *Tamarixia radiata* for the biological control of ACP, bringing our cumulative total to over 1.2 million. Releases are made where plant tissue testing positive for HLB is being detected. In 2010, before we began our releases, we were detecting up to 43 immature psyllids per flush in residential citrus. Since our biological control releases began, we have seen the populations gradually decline. In 2014, we are only observing 20 immature psyllids per flush. This is a reduction of about 50% of the psyllid population.

Candidatus Liberibacter asiaticus progression in graft-inoculated oranges using DNA and protein detection methods

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Candidatus Liberibacter asiaticus (CLAs) is a fastidious phloem-limited bacteria associated with the development of Huanglongbing (HLB). Current strategies of HLB management provide little success. Understanding the mechanisms causing disease will contribute to detection methods and manipulation of host targets to enhance host resistance. Our objective is to study the infection process of CLAs in Navel and Mandarin after graft inoculation. Samples from graft-inoculated and mock-inoculated were taken from feeder roots and young and mature leaves. Detection of CLAs by PCR using CLAs specific primers were observed as early as 2 months post inoculation in feeder roots. Although CLAs was detected in both young and mature leaf tissues, feeder roots provided more consistent results over time. Using the sequenced CLAs genome, 27 potential secreted proteins called effectors were identified. Of the 27 candidate effector proteins, four have been shown to be consistently expressed in HLB-infected citrus. Stems from pruned branches have been utilized to detect the four effectors in the phloem. The stems were blotted onto a nitrocellulose membrane which was then probed with effector specific antibodies. Data will be presented on the expression patterns of each effector over time.

Metabolomic analysis of citrus Huanglongbing indicates contrasts in asymptomatic and symptomatic trees

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Huanglongbing (HLB) is one of the most severe diseases of citrus worldwide and is associated with yet uncultured *Candidatus Liberibacter* bacteria. HLB has caused large economic impacts for citrus producers such as São Paulo (Brazil) and Florida (USA). One informative approach to understand major characteristics of the involved plant-pathosystem is to study contrasts between diseased and non-diseased plants. Previous studies proved that gene expression is altered in plants under HLB pressure. We searched for metabolomic response of HLB-affected trees envisioning future use of this knowledge in the plant transformation system. We used five biological replicates, pairs of symptomatic and non-symptomatic trees next to each other, from a uniform 6-year-old commercial block of Valencia trees on Rangpur lime rootstock nearby Taquaritinga City, in the central area of São Paulo State. Leaves and root samples were subjected to NMR (Nuclear Magnetic Resonance) and chemometrics aiming to assess tissues in asymptomatic and symptomatic stages. Our study demonstrated that in symptomatic trees there is an increase of sucrose in leaves and decrease in roots, in addition to decrease of proline, proline betaine in roots, and malic acid in leaves. These results suggest important changes in fundamental metabolic processes in response to HLB. We observed a decrease in the content of proline and proline betaine in HLB-affected leaves. These metabolic alterations are not clear and may possibly be related to increase of proline catabolism and consequently to decrease of proline betaine biosynthesis, indicating an atypical plant-pathogen relationship, whose potential anti-oxidant defense system is being affected. We plan to explore these findings in future works involving evaluation of HLB in transgenic plants that overproduce proline, since proline is known to mitigate several stress damages. In addition, we also observed a direct relationship between the bacteria level and the metabolic profiles, even for asymptomatic plants.

RNA-Seq analysis of *Candidatus Liberibacter asiaticus* separated from infected trees

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Citrus greening (Huanglongbing; HLB) is a devastating disease of citrus trees with high economical costs to the worldwide citrus industry. In Japan, this disease is caused by phloem-limited fastidious bacterium, *Candidatus Liberibacter asiaticus* (Las) and is transmitted by grafting and by the sap-sucking psyllid *Diaphorina citri*. Here, we present the

significant genes expressed in Las cells during infection. After living Las cells were separated from the infected trees and concentrated using our new method, total RNAs from living Las cells were immediately purified and provided for RNA-seq. Rough lemon (*Citrus jambhiri* Lush) infected with Las strain Ishi-1 was used and replicated. By RNA-Seq analysis, we found that Las cells expressed genes related in transporters, lipopolysaccharide (LPS) synthesis, cell wall synthesis, and so on during infection. These genes may be involved in the pathogenicity of Las. Moreover, some of these gene products are predicted to be membrane proteins or extracellular proteins. Therefore, these might interact with the molecules of host cells for Las-citrus interaction as disease establishment or defense response.

Oral delivery of double-stranded RNAs shows mortality in the Asian citrus psyllid, *Diaphorina citri*

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This study aims to screen a series of double-stranded RNAs (dsRNAs) in order to identify optimal targets for RNA interference (RNAi) in *Diaphorina citri*. We first targeted the ATP synthase (ATPase) mRNA of *D. citri*. The ATPase coding region target sequence (542 bp) was used to design specific primers which included T7 RNA polymerase sequenced at their 5' termini. RNA from adult psyllids was extracted using the TRIzolTM (*Life Technologies*) and for the cDNA synthesis we used the SuperScript™ III Reverse Transcriptase (*Invitrogen*). After amplification of target region of ATPase, the amplicons were checked by sequencing using the BigDye® Terminator v3.1 Cycle Sequencing kit (*LifeTech*). The ATPase dsRNA was synthesized via *in vitro* transcription using the MEGAscript RNAi kit (*Ambion*) using PCR products from the target region of ATPase. For artificial diet bioassays, five concentrations (from 25 to 200 ng.µL⁻¹) of ATPase dsRNAs plus dsRNA for GFP (100 ng.µL⁻¹) were diluted in the artificial diet, containing 30% sucrose, yellow and green food dye. Groups of 30 teneral adult psyllids were used in each treatment and the psyllid mortality was scored daily for 5 days. After 5 days, we observed that all of the dsATPase concentrations increased the psyllid mortality. These results suggest that the oral delivery system will allow the efficient screening of psyllid candidate gene targets for RNA interference (RNAi) and dsATPase is an interesting target to be used to control *D. citri*. We are now screening more dsRNAs and assessing effects on mortality but also target mRNA knockdown.

Geospatial mapping platform: An approach for tracing the HLB and risk analysis

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The Geospatial Platform of HLB (GP-HLB) developed in the LaNGIF-CIACyT consists of a set of historic information databases, algorithms, and integrated communication systems. The GP-HLB executes data calculation, analysis and the output results are visualized through a geographical interface. ArcGis™, Mapserver™ are powerful software tools for publishing spatial data and interactive web applications that involve the use of maps. Some of its main features include the ability to query and display different types of formats like raster, vector, and database. The GP-HLB for epidemiological surveillance is very important, especially for monitoring process, to understand adaptation and dispersion process, and under the operational approach, to perform the epidemiological surveillance of Huanglongbing and its vector (*Diaphorina citri*). The GP-HLB used the following technologies: Silverlight™, API®, C#®, Blend 4.0®, JavaScript®, MySQL® database, Generation GeoDatabase with SHP files, and ArcGIS™ services. The platform integrates the spatial information, field management activities, biological and dispersion modeling, that allows determining the potential damage and identifying new ecological niches

potentially to be occupied by the bacteria and its vector, according to previous geographical and anthropic risk analysis.

CAPACIF: Training, application and collective heed of phytosanitary impacts. The case of HLB in Mexico

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The CAPACIF (training, application, and collective heed of phytosanitary impacts) is an entity created by UASLP, as a proposal for SAGARPA and The Campaign against HLB in Mexico. The principal aims of CAPACIF are generating and implementing plans for technical and scientific advice; it is focused on developing participatory methodologies that encourage ownership by the different actors of the citrus cropping system. This is possible because CAPACIF is formed by a team of social researchers that collaborate in an interdisciplinary manner with researchers of Plant Pathology and Geography. In the case of HLB disease, the CAPACIF team in 2011 made a diagnosis for the development of a protocol to strengthen control measures and mitigation of HLB through participatory methodologies developed in the Social Sciences. This diagnosis was made to assist in a holistic way for the control of pest and plant diseases. Subsequently, the proposal was joined to the strategies of the Campaign against HLB and it recruited Phytosanitary Technicians-Facilitators (PTF) to implement participatory nationally workshops in 2013, with the objective to sensitize the citrus producers, by promoting organized participation and appropriation of strategies, through guidelines of work designed for the operation of the Regional Control Areas (ARCO's) of the campaign against HLB. Currently, the CAPACIF team conducts by monitoring and mentoring of PTF at distance about methodologies and instruments that generate socio-economic and cultural information on HLB disease to generate social indicators and determine anthropic risks associated to the dispersion of HLB disease in Mexico.

Analysis of starch accumulation and phloem structure by confocal laser scanning microscopy in Mexican limes trees affected by *Candidatus Liberibacter asiaticus* (CLAs) after bio-products treatments

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Since 2009, Mexican citrus production has been affected by Huanglongbing disease (HLB) and no effective control has been reported. This study is a complementary work of a field test experiment in Colima, Mexico, in which three bio-products were evaluated on Mexican lime response against CLAs infection. After 8 months of applications, 15 foliar samples from representative trees showing a decrease in CLAs concentration measure by TaqMan® PCR were selected for the analysis. The tissue was fixed in FAA solution and embedded in Paraplast®. Sections of 12 µm were made on a microtome and stained with rhodamine B at 5% and calcofluor 1%, samples were observed by Confocal Laser Scanning Microscopy (LSM 710 NLO, Carl Zeiss). The images were converted to grey scale using Image J 1.48 software. Statistical analyses were done for image analysis results using JMP4 statistical program and two factor nested statistical model and Tukey test. Preliminary analysis showed a different response on starch accumulation and modification of the phloem structure, relating positively with CLAs titer in the affected trees. The data shows an improvement of vascular bundle capacity by decreasing the obstructions over the sieve plates with the bio-products tested. Dissimilarities in the size and area of starch grain accumulation were observed. Thus, in the F1 and F2, starch conglomerates were more evident when compared to F3 (Fitoplazmix™/Fitoxxi™) after 8 months of applications. In addition, the phloem structure seems to appear better organized and increases in size.

Drug repurposing: New chemicals and targets to combat Citrus Greening Disease

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One of the main obstacles in advancing the search for antimicrobials against ‘*Candidatus Liberibacter asiaticus*’ (CLAs) is the inability to culture this pathogen to perform classical growth inhibition tests. Consequently, we centered our research in the identification of chemicals that inactivate critical physiological pathways operating in this bacterium. To achieve our goal, the experimental design used in our laboratories consisted of a deep *in silico* multifactorial genomic analysis, followed by a conventional biophysical screening of selected targets. Using this approach, we identified new chemicals that targeted a transcriptional regulator belonging to the MarR-family (LdtR) in CLAs. The DNA binding sequence of LdtR was identified via DNase I footprinting. *In silico* analyses using this binding site indicated that LdtR modulates the expression of several genes involved in cell division and cell wall biosynthesis. A gene of particular interest within the regulon is *ldtP*, which is predicted to encode for an L,D-transpeptidase. This enzyme is involved in the modification of the bacterial cell wall using an alternative pathway and it is resistant to the activity of β -lactam antibiotics. A recombinant LdtP enzyme has been purified and used as a target to identify potential enzymatic inhibitors. Herein, we present the impact of the identified new antimicrobials on the regulatory activity of LdtR and on the enzymatic activity of LdtP. The compounds identified represent an important advance in the identification of chemicals to be used as bactericidal in planta. The results herein described provide important insights to understand CLAs regulatory networks and molecular foundations for the design of therapeutics for the treatment of this devastating disease.

Contributions from “Estación Experimental Agroindustrial Obispo Colombres” (EEOC – Tucumán – Argentina) to the National Prevention Program of HLB in Argentina

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Since HLB was first reported in Brazil (2004), the EEOC (along with enterprises involved in the production of citric fruits and phytosanitary organisms) implemented and strengthened different lines of work in four strategic areas. Provision of sanitized and certified vegetal material: EEOC is the only sanitation center in northwestern Argentina (NOA) and is in charge of providing sanitized and certified material (buds and seeds) to citrus nurseries. Observation and monitoring: a molecular diagnosis laboratory was set up for *Candidatus Liberibacter* spp. detection for both vegetal (citrus and *Murraya paniculata*) and insect samples. We visually inspected citrus trees from 44 places located in Northwest Argentina and collected leaf samples, displaying HLB-similar symptoms, and insect samples. From 2005 to present, 12,500 samples were analyzed by qPCR TaqMan, 50% corresponded to citrus leaf, 46% to insect, and the remaining 4% to ornamental plants. All samples were negative. To date, there is no evidence that *Candidatus Liberibacter* spp. are in the NOA; although, 3,000 sticky traps for *Diaphorina citri* were set across the citrus area in Tucumán province and checked periodically. Furthermore, citrus plantation and alternative hosts in the urban area were monitored too. This monitoring was conducted on a weekly basis, but was intensified in spring and summer. In April 2011, the abovementioned activities resulted in the detection of 11 *D. citri* specimens on *Murraya paniculata* plants from the urban area. This case has remained under effective control by the national and provincial phytosanitary authorities and has not led to any subsequent reports. As for the citrus area, over 600,000 shoots and approximately 25,000 colored sticky traps have been checked without detecting the insect vector. These results prove that Tucumán province is free from *D. citri*. In the north of NOA (Salta and Jujuy province), where the insect is present, the efficiency of 17 active ingredients was evaluated along with their correspondent fresh fruit residue analysis. We are currently evaluating the effectiveness of five entomopathogenic fungus strains.

An epidemiological perspective for the integrated management of Huanglongbing disease

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One of the principal challenges in managing rapidly spreading epidemics is to identify optimal control strategies. How, when, where, and which control methods should be used to manage disease effectively. Epidemiological modelling can help by providing a means to integrate the current status of knowledge and to provide a set of tools to compare the effectiveness of different control scenarios. The epidemiological approach is also well-suited, taking account of uncertainties in order to inform a risk-based management of disease. Drawing upon experience gained from modelling a range of emerging pests and pathogens including Huanglongbing (HLB) and Asian Citrus Psyllid (ACP), I propose to show how an ‘epidemiological toolbox’ can be used to predict disease and vector spread, to analyse the effectiveness of control and to compare ‘what-if’ scenarios for disease management. Successful control of disease requires us to match the scale of control with the inherent spatial and temporal scales of the epidemic. Identification and characterisation of the epidemic scales involve the formulation of mathematical models that capture the essential biological features of disease spread. The spatial distribution of hosts in the landscape is also important along with the effects of environmental variables and anthropomorphic activity, and the degree of stakeholder compliance. Using data for the spread of disease in Southern Gardens (FL) and data from psyllid traps in California, I propose to show how it is possible to extract signatures in the form of epidemiological parameters such as transmission rates and dispersal kernels for epidemic and vector spread.

Transcriptional response of tolerant rough lemon and susceptible sweet orange to Huanglongbing highlights differences in plant defense mechanisms

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We studied host transcriptional responses of rough lemon (RL) and sweet orange (SO) to infection in 7, 17, and 34 weeks after inoculation (WAI) with *Candidatus Liberibacter asiaticus* (CaLas) by using RNA-seq methodology. Plants were grafted with healthy or qPCR positive budwood. A completely randomized design with three biological replications was used. A Sub-Network Enrichment Analysis was used to construct protein-protein networks that provide a deep insight into the metabolism of affected plants. In brief, there were a greater number of differentially expressed genes in rough lemon than in sweet orange, particularly at the later stage of infection. Most of them were involved in defense mechanisms in RL, whereas in SO there were fewer and less up-regulated genes responding to the infection. A major defense component seen was in their hormonal response, specifically jasmonate, salicylate, and ethylene, which are well-known hormones involved in disease resistance. MYB and WRKY transcription factors were also highly expressed in RL 17 WAI and contributed to the activation of cell processes such as plant response, defense response, disease resistance, and hypersensitive response. Although the same set of transcription factors reached an equivalent expression level at 34 WAI, they still did not trigger in SO a response as strong as in RL. Additionally, all amino acid pathways at week 34 in rough lemon plants were affected by HLB in strong contrast to sweet orange. We confirm that distinct amino acid metabolic pathways constitute integral parts of the plant immune system. Our study suggests that the early and strong activation of the SA, JA, and ethylene hormone response to HLB infection may be one of the major reasons of rough lemon tolerance. A list of potential candidate HLB tolerance genes were identified in the study as well.

Alternative hosts for citrus diseases of quarantine concern in Florida: *Severinia buxifolia* (Poir.) Ten. and *Swinglea glutinosa* (Blanco) Merr.

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Alternative hosts usually play an important role in an epidemic disease, but they are often neglected in epidemiological studies, specifically when they cannot be recognized. Thus, the study of symptoms of a particular disease on all possible hosts is a significant aspect of any management program. Blotchy mottle, the most characteristic symptom

of the Huanglongbing (HLB) disease of citrus, has been identified on *Severinia buxifolia* in parks of Miami-Dade and Broward Counties in Florida. *Severinia buxifolia* has been reported to be attractive to the Asian citrus psyllid, infected with the bacterium *Candidatus Liberibacter asiaticus*, and a good donor of this pathogen. A recent study suggests *Severinia buxifolia* is probably a greater threat to HLB management compared with other alternative hosts, including the species *Murraya paniculata*. Citrus canker disease, caused by the bacterium *Xanthomonas citri* subsp. *citri*, is considered endemic in Florida. Lesions of the disease were found to naturally occur on a *Swinglea glutinosa* tree, the only species of the genus *Swinglea*, in a park in Broward County. Since its detection, the infection has been persistent and evident symptoms have been observed on leaves and stems. Typical lesions are corky, rounded, with a yellow halo and water soaked margin. The detection and identification of the citrus canker disease naturally occurring on this species is of more relevance considering that *Swinglea glutinosa* has been reported to be the only natural host of the Citrus Leprosis disease outside of the *Citrus* genus in Colombia.

Canine assisted early detection of HLB and integrated strategies for optimized control

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In Florida, an evolving strategy to offset HLB-induced declining production is to plant new groves, but protection of these new groves in the presence of widespread endemic HLB is challenging. With the ACP populations endemic in Texas, California, and Arizona, and HLB epidemic in Texas and still rapidly increasing, the major impetus has been to keep HLB from entering and establishing in commercial plantings in California and Arizona. To effectively combat HLB and minimize spread in all states and situations, early detection and early response are an absolute necessity. We've been training dogs for the detection of citrus canker for 15 years and achieved 99.7% accuracy in groves, packinghouses, and nurseries. We have transitioned this methodology to HLB, and now have trained two dogs to detect HLB-infected sweet orange, and grapefruit. Replicated randomized "field trials" consisting of trees in a gridded array with varying HLB-incidence, resulted in 100% accuracy (no false negatives or positives). Via USDA, APHIS, and MAC funding, we are currently training 20 additional dogs for early detection of HLB, to be deployed in new groves in FL, as well as for both commercial and residential early detection surveys in CA, AZ, and TX by mid-year 2015. We anticipate that canines will be able to detect 'Liberibacter asiaticus' (Las) in trees with subclinical infection, i.e., before symptom expression. We are currently exploring how far back into the asymptomatic state of infection that canines can detect unique Las-induced volatiles, and if subclinical infections can be detected even prior to trees becoming PCR positive. If so, this will give us a powerful tool for early detection that can be translated into immediate response (for instance tree removal) which will minimize disease spread and optimize planting longevity. Live canine demonstrations of HLB detection are planned for the IRCHLB conference audience.

Alternative method of thermotherapy application in citrus

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Thermotherapy has shown promise as a method to extend the life and production lifespan of citrus that is infected with HLB. However, the logistics of the current methods of application can be difficult, and the damage to the tree can be severe. Therefore, alternative methods of applying high heat to a tree, with no external power and a minimum of user input, were tested. Since these methods all use water to transmit heat (as opposed to directly heating the air around a tree), the term "hydrothermotherapy" (or HTT) is used to describe them. The motivation for this alternative thermotherapy approach was to develop a method that could be applied to a large number of trees simultaneously at low cost. Water for these systems is heated using solar energy, and then the hot water is transmitted to the tree via flexible black hosing, which is wrapped around the trunk to allow heat transfer directly into the tree without damage to the leaves. The first project was done to

determine the parameters needed for optimal treatment using the HTT systems. Trees were subjected to a set temperature, for a set period of time, to determine the heat tolerance of the tree. Once this was determined, trees were treated with various HTT arrangements, for the given period of time, and the effect on the HLB concentration within the tree was determined. Results indicate that using solar heated water can be an effective way to apply thermotherapy to citrus trees.

Psyllid management in the San Joaquin Valley of California

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Adult psyllid trap finds gradually increased in the San Joaquin Valley in Tulare and Kern Counties during 2014. The majority of recent finds have been in residential areas. California Citrus Mutual, California Citrus Pest and Disease Prevention Program liaisons, the Citrus Research Board, and the University of California personnel met during 2014 and began to develop Psyllid Management Areas that act as networks of communication among small units of 25-30 growers. At this time, the eradication approach of treating both commercial citrus and residential citrus with two insecticides in an 800 meter area around individual find sites will continue. When finds increase and 800 meter treatment areas begin to overlap, growers as directed by a task force will shift to voluntary, larger areas of treatment utilizing the Psyllid Management Area communication structure. University of California researchers continue studies on the efficacy of conventional and organic psyllid treatments, updating guidelines for growers and providing plans of action that minimize broad spectrum insecticides and utilize to the greatest extent possible treatments applied for other pests. Recent changes in quarantine structure in the San Joaquin Valley are likely to increase the spread of psyllid and eventually the spread of HLB.

Excess bicarbonate in soil and irrigation water increases fibrous root loss and decline of Huanglongbing-affected citrus trees in Florida

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An extensive survey of HLB-affected groves indicated that greater decline in fibrous root health and greater expression of HLB symptoms is observed where irrigation water is high in bicarbonates (>100 ppm) and/or soil pH >6.5. Affected groves employ micro-sprinkler irrigation that concentrates fibrous roots in the wetted zone and soils often have a history of excessive dolomite liming to manage high residual copper. Affected orchards have off-color foliage, thin canopies due to excessive leaf drop, twig dieback, and more severe HLB symptoms in leaves and fruit. HLB symptom expression of trees on different rootstocks is ranked Swingle citrumelo > Carrizo citrange > sour orange > Cleopatra mandarin which follows rootstock intolerance of bicarbonate. To identify the relationship between HLB decline and bicarbonate stress, fibrous roots of 8- to 15-yr-old Valencia orange trees on Swingle or Carrizo rootstock were sampled in 37 orchards with varying soil pH and irrigation water quality. Lower root density was correlated to irrigation water pH and soil pH >6.2. Fruit production over three seasons (2009-2012) during which HLB incidence was accelerating revealed that groves under high bicarbonate stress declined 20% in yield compared to groves under low bicarbonate stress with a 6% increase in production. Yield loss under bicarbonate stress was correlated with reduced fibrous root density compared to the non-stress condition.

Relationship between HLB-induced fruit drop, fibrous root loss and the interaction with *Phytophthora* spp.

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In Florida, incidence of Huanglongbing (HLB) caused by *Candidatus Liberibacter asiaticus* (Las) is approaching 100%. In the 2012-2013 season, HLB caused a record 20% fruit drop that increased to 30% in the 2013-2014 season. In 2014-15, the crop is projected to be reduced by another 15% which is the lowest since the tree-killing freezes in the 1960s. HLB-induced fruit drop is explained by early Las infection of structural and fibrous roots that causes root loss of 30-50% before

symptoms appear in the canopy, and later root loss of 70-80% as the tree canopy thins. Recent evaluation of root production on HLB-affected trees compared to presumed healthy trees confirms that root loss is due to reduced root longevity. This root loss is exacerbated by biotic and abiotic stresses in the rhizosphere. Increased susceptibility of Las-infected roots to *Phytophthora* spp. is evidenced by statewide populations that have fluctuated from unprecedented highs in the 2011 season to an unprecedented low in 2013 compared to 25 years of pre-HLB soil populations. *Phytophthora* propagules per soil volume and per root resurged in 2014 in response to a more than doubling of root density based on intensive (i.e., local repeated measures) and extensive (i.e., statewide survey) sampling compared to 2013. Substantially higher root density in Florida groves in spring and summer 2014 is predicted to result in less fruit drop than in the previous two seasons.

Soil-applied controlled release fertilizer (CRF) treatments impact the health and growth of HLB-infected trees—results from greenhouse and field experiments

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Evidence is accumulating that root system collapse is involved with HLB-induced tree decline, especially with commercial sweet orange and grapefruit trees on Swingle and Carrizo. Maintaining root health is imperative for keeping trees productive in an HLB endemic environment. In an effort to improve tree health by focusing on the roots, we have been experimenting with polymer coated nutrients (Florikan, Harrell's) and more recently TigerSul micronutrients in the field and greenhouse. In the greenhouse, Orange 15 (UFR-3) rootstock (Nova+Hirado Buntan pummelo x Cleopatra+Argentine trifoliolate orange) were side grafted with HLB-infected Valencia sweet orange. Treatments were established with 10 replications each. Control treatments received either 1) bi-annual Harrell's UF mix, or 2) bi-weekly liquid fertilizer. Experimental treatments received bi-annual treatments of the following: Harrell's UF mix supplemented with bi-annual treatments of 3x overdoses of individual polycoated essential minor elements (Florikan), TigerSul micronutrients (sulfate form of iron, zinc, and manganese, and the Schumann blend of all 3 products), or 2x overdoses of the individual polycoated essential macronutrients. The experiment was carried out for one year. Effects on tree health, tree growth, root mass, SPAD, leaf and root nutritional analysis, and leaf and root *Liberibacter* titers will be presented. There were clear differences among treatments. Several treatments significantly improved tree growth and health as compared with the controls, especially the 3x TigerSul manganese and 3x polycoated sodium borate treatments. Results suggest that trees in the HLB world have higher specific micronutrient requirements than what are currently being recommended. In the field, we have several experiments that involve controlled release, ground applied fertilizers; these include the St. Helena project near Dundee, where sweet orange trees on numerous rootstocks are being grown completely with CRF (Harrell's) and daily irrigation. The trees will be 7 years old in April, and they are now more than 80% infected with HLB, yet the large majority of the trees across all rootstocks have remained productive. Evolution of the CRF formulas and effects on tree health from St. Helena and other minor field CRF experiments will be discussed. It is clear that a constant supply of nutrients year-round is required to maintain productivity in the HLB world. Additional fine-tuning of fertilizer composition, type, and delivery method should lead to improved tree health and productivity. Improved ground nutrition will continue to play a key role in integrated approaches to controlling HLB.

Breeding rootstocks to prevent or mitigate HLB in commercial trees

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Genetic variability for HLB tolerance/resistance is being identified (grafted trees) in existing experimental rootstock germplasm planted throughout Florida, with both sweet orange and grapefruit scions. New rootstocks are being identified in these trials that show a reduced infection frequency, and less severe symptoms once infected, as compared to commercial rootstocks. Rootstocks showing promise include complex tetraploids, diploid citranges, and diploid pummelo x mandarin hybrids. Several of these rootstocks have been 'Fast Track'

released for large-scale commercial evaluation. Current focus is on the identification of rootstocks that can sustain or increase productivity under heavy HLB pressure. Data on these promising rootstocks will be presented. The fact that there is genetic variability in rootstock germplasm not pre-screened for HLB tolerance/resistance suggests that even greater progress can be made by focused selection, especially from crosses utilizing emerging HLB tolerant/resistant parents. Thus, we have adjusted our rootstock breeding/greenhouse screening program to focus on HLB by developing the 'Gauntlet' screening program described below. Following a preliminary calcareous soil/*Phytophthora* screen, selected individual hybrid rootstock candidates are transferred to citripots in commercial potting soil. Tops of these trees are propagated by rooted cuttings to produce seed trees on their own roots. The remaining individual liners are grafted with HLB-infected budsticks of Valencia sweet orange. The remaining rootstock top is then removed, forcing flush from the HLB-infected budstick. Trees are monitored for HLB symptoms, and healthy appearing trees are entered into a 'hot psyllid' house until psyllid feeding damage is observed on their leaves (usually for about 2 months), followed by field planting at a challenging field site (USDA Picos Farm, under DPI permit). Rootstocks capable of growing off healthy sweet orange trees are identified for further study. The oldest 'Gauntlet' trees have now been in the field for approximately 2 years, and 20 promising new rootstocks have been identified so far. Our goal is to develop rootstocks that will facilitate sustainable and profitable citriculture in an HLB-endemic Florida, and possibly eliminate the need for psyllid control.

Surprising results and implications of the Florida psyllid testing project

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Huanglongbing (HLB, citrus greening disease) is one of the most devastating citrus diseases in the world. In Florida, it is associated with a bacterium *Candidatus Liberibacter asiaticus* (Las) and transmitted by a psyllid, *Diaphorina citri* Kuwayama. We tested *D. citri* collected in many different venues over a period of 6 years for Las by molecular methods. Results surprised us. First, positive *D. citri* can be found long before symptoms develop on the plants at the site. Second, positive psyllids can ride on unprocessed fruit in trailers, even when there is no foliage. Third, about 10% of psyllid samples collected from plants for sale in Florida tested positive for Las. Finally, our data, and a related mathematical model, predict a form of transmission of Las that vastly increases the potential for spread of HLB. The mechanism now is known. The increase of infected vectors follows the growth of the insect population, independent of the incubation period in the plant. The implications of this new mechanism completely change our understanding of the epidemiology of HLB. It is possible to have positive *D. citri* throughout a grove before ever seeing a symptomatic plant. This mechanism has profound implications for disease spread, epidemiological research, early detection, long range dispersal, and grove management.

Production of transgenic citrus resistant to citrus canker and Huanglongbing diseases

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Huanglongbing (HLB or citrus greening disease) caused by *Candidatus Liberibacter asiaticus* (Las) is a great threat to the US citrus industry. There are no proven strategies to eliminate HLB disease and no cultivars identified with strong HLB resistance. Citrus canker is also an economically important disease associated with a bacterial pathogen (*Xanthomonas citri*). As part of the USDA citrus breeding program, we are trying to develop citrus resistant to both HLB and citrus canker through expression of antimicrobial peptides (AMPs). In grape,

enhanced *Xylella fastidiosa* resistance was reported from expression of a chimeral AMP-peptide (comprised of an AMP and a peptide with high bacterial membrane affinity) versus the AMP alone. Our study is being conducted with the AMP D4E1, a plant thionin, and a chimeric AMP composed of D4E1 linked to thionin. We generated transgenic Carrizo and Hamlin with each of these constructs through *Agrobacterium*-mediated transformation. So far, we have obtained many verified transformants of Carrizo and Hamlin with D4E1, thionin or chimeric AMP insertion. Gene expression was confirmed by reverse transcriptase PCR (RT-PCR) in some transgenic lines. Gene expression level was compared by RT-qPCR. Transgenic Carrizo lines containing these constructs were infiltrated with *X. citri* strain 3213. Several transgenic lines expressing the chimeric AMP or thionin alone showed marked canker resistance. Bacterial growth was inhibited in some transgenic plant lines expressing chimeric AMP and thionin but not those expressing D4E1 alone. These promising transgenic plants have been replicated and will be tested for HLB resistance.

The citrus Huanglongbing bacterium, '*Candidatus Liberibacter asiaticus*', encodes an effector targeting to citrus chloroplast

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'*Candidatus Liberibacter asiaticus*' (Las) is the agent causing destructive citrus Huanglongbing worldwide. A gene encoding a hypothetical protein (P235) was identified in the prophage region of the Las psy62 genome with a eukaryotic nuclear localization signal (NLS). P235 is a 123 amino-acid protein which was predicted to localize in plant nucleus by PSORT. In order to determine P235 cellular localization, *p235* and *p235* without NLS were cloned into the Gateway vector pGWB which allows the expression of GFP fusion protein. Transgenic Carrizo was obtained using *Agrobacterium*-mediated transformation. Surprisingly, we observed that both NLS+ P235 and NLS- P235 localized in the citrus chloroplast. Furthermore, putative *p235* sequence was constructed into a binary vector pBINPLUS/ARS and used to transform citrus. Over 40 independent transgenic lines were obtained. Gene integration was confirmed by PCR and gene expression was detected by RT-PCR. We observed that transgenic Carrizo lines expressing *p235* mimic HLB-like symptoms, with leaf chlorosis and plant growth retardation. Gene expression levels, determined by RT-qPCR amplification, correlated with HLB-like symptoms. Western blots and Co-Immunoprecipitation (Co-IP) are underway to detect P235 protein in transgenic plants and to pull down the P235 binding partner in citrus. In other pathosystems, bacterial effectors that target chloroplasts can suppress plant immunity. It is possible that repression or blockage of P235 function may reduce HLB development. This is the first report that '*Candidatus Liberibacter asiaticus*' encodes a protein that targets the chloroplast of plant cells and is a putative Las effector altering plant host physiology.

Regional fluctuation of *Diaphorina citri* in Central Veracruz, México

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Orange Jasmine (*Murraya paniculata*) is a host plant of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), the vector of the bacterial pathogen that causes Huanglongbing, considered the most serious citrus disease worldwide. It is important to know how psyllid populations fluctuate in citrus plants and urban areas, as these areas can be a source of infestation to nearby citrus plantations. Here, we studied the regional fluctuation of *Diaphorina citri* along a sampling route in the central area of the state of Veracruz, México, from September 2012 to August 2014. Yellow traps were placed in orange jasmine plants in five towns and on lemon plants in five plantations near the town. With the captures of adult psyllids, isodensity maps representing the spatial arrangement and temporal distribution of vector population were created. We observed that the vector is present throughout the year in all the sites and the highest abundances occurred in the months of March, May, and June

2013 and in the months of May and August 2014. Also placed was data logger to daily record the temperature in each of the sampling sites. With the data obtained, we can preliminarily say that there is a relationship between the months in which maximum temperatures and highest abundances of *D. citri* were registered.

Chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (Sweet Orange)

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Through utilizing the nutrient-rich phloem sap, sap feeding insects such as psyllids, leafhoppers, and aphids can transmit many phloem-restricted pathogens. On the other hand, multiplication of phloem-limited, uncultivated bacteria such as *Candidatus Liberibacter asiaticus* (CLAs) inside the phloem of citrus indicates that the sap contains all the essential nutrients needed for the pathogen growth. Genome sequencing studies revealed that CLAs can metabolize many sugars and amino acids found in the phloem sap. In addition, CLAs can act as energy parasites and scavenge ATP from its host through the use of an ATP/ADP translocase. Furthermore, reduction in some minerals such as Zn and P in CLAs-infected trees indicated that these minerals are required for the growth of CLAs. The presence of (*znuABC*) gene in CLAs genome also indicated that CLAs can import Zn from its host. The phloem sap composition of many plants has been studied; however, available data about citrus phloem sap is limited. In this study, we investigated the phloem sap composition of sweet orange. The phloem sap collected by EDTA or centrifugation method was derivatized with three different reagents and analyzed with GC-MS revealing 20 amino acids, 8 sugars, and 8 organic acids. Analysis of citrus phloem sap by inductive coupled plasma (ICP) showed that it was rich in potassium, calcium, phosphorus, magnesium, and sulfur. Trace amounts of iron, copper, zinc, and boron were also detected. The ATP concentration in citrus phloem was 24.0 ± 4.0 ppm. Analysis of citrus phloem sap high performance liquid chromatography (HPLC) showed that citrus phloem sap was rich in nucleotides.

Citrus seedlings from infected, Huanglongbing-symptomatic trees are not infected but express HLB-like symptoms: A possible model for understanding aspects of HLB pathogenesis

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Studies on seed transmission of '*Candidatus Liberibacter asiaticus*' (CLAs), the bacterium associated with Huanglongbing (HLB), described seedlings from infected trees which showed an abnormal growth phenotype but were free of CLAs. We germinated populations of seeds of 'Hamlin', 'Ridge Pineapple', and 'Valencia' sweet orange from infected trees and observed chlorotic and stunted seedlings which failed to grow substantially. Tests detected no CLAs DNA in these seedlings. These aberrant phenotypes were similar to symptoms expressed by infected trees, and persisted for 6 months or longer, at which point seedlings died or began to grow normally. Normal growth also was induced by grafting stunted seedling apices to healthy citrus seedlings. The absence of infection and their HLB-symptomatic phenotype suggest that extracellular factors produced by CLAs in the mother tree affected the normal development of these seedlings, possibly through the alteration of normal gene expression. The similarity of these seedlings and symptomatic foliage suggest these factors are involved in disease development in infected trees. We propose these seedlings are a model system for understanding the molecular basis of symptom development in HLB-affected citrus trees.

Psyllid and graft transmissions to *Murraya paniculata* seedlings indicate seedling populations segregate for resistance and susceptibility

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Huanglongbing is a bacterial disease of citrus associated with '*Ca. Liberibacter asiaticus*', (CLAs) which is transmitted by the Asian citrus psyllid (*Diaphorina citri*, Kuwayama) or by grafting. *Murraya paniculata* L. is a popular landscape ornamental no longer produced

because it is a preferred host for *D. citri*. To establish that *M. paniculata* is a host of CLAs, transmission experiments with *D. citri* and *M. paniculata* seedlings conducted in fall 2012 and summer 2013 yielded 1/16 and 11/80 infected seedlings, respectively. Grafting experiments done to transmit CLAs from citrus to *M. paniculata* showed a significant difference in the survival of budwood, with survival of 80% of budwood from non-infected trees and of only 17% of budwood from CLAs-infected trees. The psyllid transmission data suggest *M. paniculata* seedling populations are heterozygous for susceptibility to infection. These results are compatible with a model where resistance is a dominant trait controlled by two genes. The results from grafting experiments suggest this is an active resistance to infection and that resistant *M. paniculata* seedlings can be identified by grafting with budwood from infected citrus trees.

Infecting small citrus seedlings by grafting with intact HLB-symptomatic leaves: A small-scale model system for studying basic aspects of ‘*Ca. Liberibacter asiaticus*’ infection in citrus

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Vegetative grafting effectively transmits ‘*Candidatus Liberibacter asiaticus* (CLAs), the bacterium associated with the citrus disease Huanglongbing (HLB). It is common to infect citrus seedlings which typically are at least one year old and are thick enough to accommodate a side-graft as the inoculum. To produce infected plants which occupied less space, we grafted intact, HLB-symptomatic leaves to small citrus seedlings. Whole leaf grafts (WLG) succeeded with seedlings as young as 3 months, and with sweet orange, grapefruit, *Citrus macrophylla*, mandarin, citron, and citranges. Seedlings were inoculated by grafting the leaf to the top of a “decapitated” stump. One advantage of WLG inoculation is that new shoots are developmentally similar, so measured characteristics of the CLAs infection in different shoots can be compared directly. Colonization occurred first in tissues nearest the inoculum and progresses acropetally with sequential colonization of leaves; however, not all successive leaves were colonized. Most adventitious shoots which developed post-grafting expressed the classic foliar blotchy-mottle, whereas some shoots were stunted and chlorotic. Foliar blotchy mottle symptoms developed first and were most intense in tissues closest to the inoculum. At given time points, real-time PCR assays showed higher numbers of bacteria in symptomatic tissues closest to the inoculum and fewer in leaves further away.

Endosymbiotic control of the Asian Citrus Psyllid (ACP, *Diaphorina citri* (Hemiptera: Liviidae)): Diversity and ecology of *Wolbachia* in Florida ACP populations

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Due to their ability to influence host reproduction, endosymbiotic bacteria of the genus *Wolbachia* have been studied extensively across a broad range of species. Manipulation of *Wolbachia* spp. in some insect vectors has shown potential for mediating pathogen transmission. *Wolbachia* occurs naturally in the Asian citrus psyllid, which is the vector of *Ca. Liberibacter asiaticus*, the causal agent of citrus greening disease (Huanglongbing, HLB). We calculated the within-host density of *Wolbachia* in Florida *D. citri* populations using quantitative PCR for detection of the *Wolbachia* outer surface protein gene, *Wsp*. Gene quantities were normalized to the *D. citri* wingless gene (*Wg*) to estimate *Wolbachia* abundance in individual *D. citri*. Using this method, significant geographic differences in *Wolbachia* densities were detected among Florida *D. citri* populations, with higher infection levels occurring in male versus female hosts. Multi-locus sequence typing (MLST) of *Wolbachia* from geographically distinct ACP populations revealed the presence of several unique sequence types, with one predominant sequence type occurring in Florida psyllid populations. Although infection levels varied geographically, multiple infections of known strains were not detected. Temperature, life-stage, age, and gender contributed to variation in *Wolbachia* density among host insects. Preliminary evidence suggests that foreign *Wolbachia* strains may be artificially established in hemipterous insects. The ability of *Wolbachia* to influence host reproduction and pathogen transmission suggests that manipulation of psyllid *Wolbachia* infections may be a useful tool for

vector or pathogen management, as demonstrated in other vector-borne disease pathosystems.

Using the complexome analysis to explore the interactions between *Candidatus Liberibacter asiaticus* and Asian citrus psyllid

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In order to get a better understanding of the transmission mechanism of citrus Huanglongbing (HLB) by the insect vector, Asian citrus psyllid (ACP), we have been working on unraveling the protein-protein interactions (PPI) between ACP and the HLB associated bacterial agent, *Candidatus Liberibacter asiaticus* (CLAs) by means of proteomics. Complexome is the whole set of the protein-protein interactions in a particular cell or organism. During the transmission process, CLAs bacteria traverse inside the insect vector systemically and various PPIs and protein complexes (formed by protein constituents from both CLAs and ACP) must be involved; therefore, our aim is to find the proteins involved in the CLAs-ACP interactions by studying the differences between the complexome of CLAs-free and CLAs-infected ACP. We have established the Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) system in our lab to study the protein complexes (i.e. complexome) from CLAs-free and CLAs-infected ACP in their native status, and a further separation of the protein complexes in BN-PAGE by a second dimension of SDS-PAGE which helped us obtain more detailed information on all the subunits or constituents of the protein complexes at their denatured status. By comparing the differences between the 2D BN/SDS-PAGE gel images from CLAs-free and CLAs-infected ACP, we have successfully located several protein spot candidates for protein identification by mass spectrometry. Our next step is to use far-western blotting to show the specific protein-protein interactions between the identified protein(s) and CLAs, which will further validate our results from 2D BN/SDS-PAGE work.

Environmental fate of dsRNA in a citrus field environment

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RNA interference (RNAi) is a breakthrough technology that continues to demonstrate the potential for management of insect pests. To move the development of non-transgenic RNAi approaches to control insects in citrus, studies were established to evaluate the movement and persistence of dsRNA in the plants tissues, juice, and the soil environments post treatment. Five treated citrus trees (5-yr-old Valencia) and potted trees in glasshouse trials were either directly injected or topically applied to root zone. Concentrations ranged from 200 µg (micrograms) dsRNA in pots, to 200 mg on field root zones. Raw juice, 20 L, was spiked with dsRNA to a final concentration of 10 ng/µL. Samples at intervals up to 48 hours were collected. The dsRNA dynamic obtained showed that in potted plants the dsRNA was detected in leaves on the top of canopy (about 2 meters tall) 3 hour post treatment, and remained detectable up to 50 days post treatment. Field trees treated with dsRNA, had detection of dsRNA in old and new growth, as well as fruit peel, demonstrating the dsRNA moved to new growing tissues as they developed. Treatments of 100 mg dsRNA were detected out to 1.5 months and 200 mg dsRNA detected out to 3 months. Although the dsRNA was detected on the fruit peel, the persistence of dsRNA in fresh orange juice was relatively short, being degraded in raw juice after 24 hours. Preliminary results on soil analysis showed complete degradation of dsRNA in 3 days.

Validating the safety of RNAi products - Keeping honey bees safe

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The power of genomics has greatly advanced the emergence of new methods of pest/pathogen control strategies. Of these, RNA interference (RNAi) is being extensively studied worldwide as the next big breakthrough in insect pest management. The biggest advantages of

RNAi strategies is the ability to be highly specie specific (*target one insect*). Specificity is the foundation of RNAi, which depends upon genetic sequence, thus this knowledge provides researchers the ability to design dsRNA molecules to match only to the targeted mRNA in the insect, i.e., psyllid. Thus, the advantage of RNAi-based pest management is to not cause harm in non-target insects, such as pollinators, predators, and parasitoids. RNAi provides an environmentally sound pest management strategy. Thus, to validate the safety of RNAi-based products for use around honey bees and other beneficial insects, a new bioassay was developed to stringently evaluate direct and indirect genetic responses by honey bees to dsRNA's designed for RNAi which target the Asian citrus psyllid, ACP, *Diaphorina citri*. The RNAi Off-target Bioassay for Honey Bee Safety, was conducted in a double-blind test using three dsRNA constructs designed to the ACP (Dc-1,2,3) and with controls (GFP-dsRNA, and a sucrose blank). Upon emergence, nurse bees were fed a 10 ng dose of dsRNA in 60% sucrose solution, 20 μ L total volume. Bees were marked with non-toxic paints and returned to their original hive. Thirty nurse bees from each hive were fed the above dsRNA dose and then monitored for survival at 15 days. Surviving bees were analyzed for target mRNA expression levels as well as across a microarray panel of 21 bee immune transcripts for expression. No significant differences were observed for mortality or gene transcript expression between controls or honey bees fed ACP-designed dsRNA's.

Hemipteran cell cultures support *Wolbachia* {psyllids and leafhoppers}

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Insect cell cultures provide an important system for the study of insect viral pathogens, and microbes. Current interests are focused on manipulating *Wolbachia* species as a mechanism to suppress pathogens. An important part of this strategy is the ability to culture the *Wolbachia* bacterium. The leafhopper cell line, HvWH, (*Homalodisca vitripennis*) was established in 2009 and shown to support *Wolbachia* in culture for at least 4 years in continuous culture. Psyllid cell cultures have shown that microbes can be maintained in psyllid cell lines for several months. Use of these or other cell lines may therefore provide a system to experimentally attempt to alter a psyllid *Wolbachia*, or other endosymbiont for use as an agent to suppress CLAs and stop psyllid transmission, thus stopping the spread of HLB.

Systems biology approach to rapid solution development for agricultural problems - {psyllid-HLB}

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Cognitive computing – use of computers to identify complex interactions, to permit humans to make rapid, fact-based decisions – is changing the way researchers can approach problems. This paradigm shift in data visualization, interaction mapping, interpretation, and speed will increase the accuracy in researchers hypotheses, thereby decreasing the time and costs from problem to solution development. To this end, we produced a micro-CT Scan of Asian citrus psyllid anatomy, which permits digital sectioning of the insect in all planes of symmetry, covering the organ systems - alimentary tract, salivary glands, and reproductive. These systems are being linked to other descriptive data such as light, immunolabeling, and electron microscopy studies for added details. Further enhancements link the genomic data for these tissues, for known transcripts that are expressed in these tissues. The corresponding proteins and references will also pop up so that the search is more complete. An example for the Asian citrus psyllid can be observed at: http://www.youtube.com/watch?v=2tRXP_0vKryQ and the glassy-winged sharpshooter leafhopper at: http://www.youtube.com/watch?v=RcPeotDsBK8&list=UU7vEYx_PxWfi9LNT4CXeLvg. The enormous amounts of data produced each day exceeds the abilities of most researchers to effectively find and assimilate. Thus, combining the help of computer speed and interactive mapping with human intelligence, available information that was “invisible” or overwhelming, now becomes visible, organized, and associated to the other bits of

scientific information that makes the biological significance more obvious and readily useable to researchers. Current trends in computer science and biology are moving towards this type of “Systems Biology Approach” which is based upon ‘Cognitive Computing methods’. These systems will provide information in a format that will be easier to understand, while providing evidence for designing better products for medicines, materials, or solutions to complex agricultural problems, like HLB.

Progress on the development of methodology to measure and detect fruit that might contribute to HLB-associated off flavors in juice

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As Huanglongbing (HLB) has continued to spread across the state, there is a growing body of evidence to support that in some instances, there are some off-flavors that can be detected in juice coming from fruit of HLB-infected trees compared to juice coming from the fruit of healthy trees. The differences tend to be subtle but detectable in sensory evaluations by trained panelists, and in some instances by untrained panelists. Among the descriptors that have been used to describe HLB juice are bitter, sour, astringent, metallic, salty, umami, less orange aroma, less orange mouth feel, etc. Similarly, multiple studies have also identified several juice quality parameters, metabolites, or compounds that change with HLB infection; but, in all cases it has been changes in levels and not the presence or absence of a specific chemical. For instance, juice from HLB-infected fruit results in lower Brix, higher acid, and higher levels of limonin and nomilin. However, there is no one “smoking gun” parameter that can be measured that would indicate the potential for off flavors. In the present study, 14 different parameters were evaluated that encompassed sensory descriptors (sourness, metallic taste, saltiness, umami, spiciness, sweetness, and bitterness), basic juice chemistry (Brix, acid, ratio), secondary metabolites (limonin and nomilin), and harvest associated parameters (harvest date and fruit size). Samples were collected at random from fruit loads delivered to a commercial processing plant and were extracted using commercial equipment by the “USDA/State” lab that exists at commercial processing facilities. Sensory data were collected through the use of an electronic tongue. Basic juice chemistry data were collected using traditional wet chemistry techniques. Secondary metabolites were measured by HPLC and harvest date and fruit size were collected at the processing facility. Samples were collected from both Hamlin and Valencia varieties. Principal component analysis was used to reduce the number of variables considered for each of the varieties and the results were compared to a novel qPCR method to cross check the conclusions. Initial results indicated that a subset of parameters that include some sensory, basic juice chemistry, secondary metabolites, and harvest data can be used to identify fruit loads that have the potential to produce off flavors in Hamlin and to a lesser extent in Valencia varieties.

Have the HLB associated production losses in Florida bottomed out or are we in for harder times?

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Since its discovery in Florida in late 2005, citrus Huanglongbing (HLB), widely recognized as the most serious disease of citrus, has caused havoc in the Florida industry. Production costs have increased and production is down. Despite the apparent advances that growers and researchers have made in mitigating some of the symptoms of the disease, the statewide production continues to go down. All of the variables that go into the state forecast, tree number, fruit size, fruit per tree, and percent fruit drop are all trending in the wrong direction. This has led some growers to hold back on replanting in recent years which has further exacerbated the problem. The big question is how much lower will production go? Based on what we know, or in some cases based on our best guess, a model was created to estimate where we are in the HLB decline curve in the Florida industry. The model assumes that the industry focus will continue on the track of symptom mitigation

instead of inoculum removal and management. Based on some conservative assumptions used in the model, the significant losses that have occurred the last several years should have been predicted. Going forward with the same assumptions, it appears that the production decline is near its predicted lowest significant drop and future losses will track the rate of replanting. That is to say, if tree attrition is greater than the rate of replanting, production will continue to decline. If the replanting rate is equal to or greater than the attrition rate, then the production will hold or increase slightly over time.

Asian citrus psyllid-derived protein suppresses *Ca. Liberibacter asiaticus* holin promoter activity

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Two circular prophage genomes (SC1 and SC2) have previously been described integrated in *Ca. Liberibacter asiaticus* (Las) genome; similar prophages are found in all described Florida Las strains. The SC1 lytic cycle, marked by upregulation of several late genes, including a functional holin (SC1_gp110), is activated when Las is *in planta*, but not when infecting the Asian citrus psyllid (*Diaphorina citri* Kuwayama) host. Expression of the holin in *E. coli* is lethal. The SC1_gp110 promoter was cloned into the wide host range vector pUFR071, structurally replacing the native *lacZ* promoter to allow the holin promoter to drive the β -glucuronidase (*uidA*) reporter gene in pLF057. The GUS reporter was also cloned downstream of the native *lacZ* promoter in pUFR071 to yield pLF058 as a positive control. Both plasmids were separately transformed into *L. crescens* BT-1. Although BT-1 cultures transformed with pLF058 showed GUS activity using X-Gluc, as expected from the constitutive *lacZ* promoter, BT-1 transformed with pLF057 exhibited much higher GUS activity, indicating that the holin promoter was strongly active in BT-1. Upon treatment of BT-1/pLF057 (Abs600 = 0.6, 500 μ l) with 50 μ l of aqueous psyllid extract (25 psyllids/ml), GUS activity was inhibited in a dose-dependent manner. The inhibition of GUS activity by insect extracts in Lcr cells was heat labile and abolished by proteinase K treatment, suggesting that psyllids produce a protein inhibitor of the phage holin. The inhibitor was precipitated with acetone, and size fractionation demonstrated an inhibitor in the size range of 10-50 kDa. This reporter system may be developed into a high-throughput chemical screen for treatments that may interfere with psyllid or plant regulation of the phage lytic system.

Functional validation of SC2 prophage encoded peroxidase (SC2_gp095) gene in *Candidatus Liberibacter asiaticus*

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All pathogenic *Ca. Liberibacter asiaticus* (Las) strains examined to date carry two nearly identical prophages, named SC1 and SC2. In strain UF506, prophage SC2 was observed to replicate as an excision plasmid. Replicative SC1 forms are discernible in citrus, and at higher copy number in the artificial host periwinkle (*Catharanthus roseus*). Given that the highly reduced Las genome encodes no known defense against host generated ROS, the putative phage-related ROS scavenging functions annotated as peroxidase (SC2_gp095) and glutathione peroxidase (SC2_gp100) may represent important "lysogenic conversion" genes whose expression may increase bacterial fitness and delay symptom development in the host plant. Both SC2_gp095 and SC2_gp100 were expressed at significantly higher levels in periwinkle than in citrus or insects. SC2_gp095 alone, and in tandem with SC2_gp100 were separately cloned in a wide-host-range (repW) shuttle vector pUFR071 (under control of the *lacZ* promoter), and transformed into *E. coli* and into *L. crescens* (Lcr), a culturable proxy for Las. The transformed Lcr cells showed enhanced *in vitro* resistance to H₂O₂, 23% higher enzymatic activity and faster growth rates in culture as compared to Lcr cells transformed with only pUFR071. Moderate enzymatic activity was also evident in transformed Lcr culture supernatants, but not *E. coli* supernatants, confirming a predicted non-classical secretion potential for SC2_gp095, and suggesting such secretion from Las. Experiments are underway to further characterize the role of SC2_gp095 *in planta*. We hypothesize that Las peroxidases: 1) mitigate the direct antibacterial effect of reactive oxygen species (ROS) on Las cells, and 2)

disrupt systemic cell-to-cell self-propagation of ROS (H₂O₂) -mediated signaling in the host plant. The latter idea may explain the surprisingly long incubation period before symptoms appear. Las peroxidase(s) may be secreted effector(s) that function to suppress host symptoms, a tactic used by most biotrophic plant pathogens.

Modifying citrus genome using Cas9/sgRNA

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Citrus is one of the most economically important and extensively grown fruit tree crops worldwide. Citrus production in most citrus producing countries, e.g., US, Brazil, and China, is facing an unprecedented challenge caused by Huanglongbing (HLB). Currently, no effective HLB management is available. Development of HLB resistant or tolerant citrus will provide a long-term, effective, and sustainable solution to HLB. Traditional plant breeding is unlikely to lead to HLB resistant or tolerant plants due to the lack of resistant varieties. Targeted genome engineering is expected to contribute significantly to future varietal improvement. Genome editing technologies using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9/single guide RNA (sgRNA) have already been successfully used to genetically modify plants. Here, we reported our progress in modifying citrus genome using Cas9/sgRNA technology. We first developed a novel tool, *Xanthomonas citri* subsp. *citri* (Xcc)-facilitated agroinfiltration, for enhancing transient protein expression in sweet orange leaves. Our results showed Xcc-facilitated agroinfiltration could be used on other citrus varieties including Duncan grapefruit, Valencia sweet orange, Key lime, Carrizo citrange, Sour orange, and Meiwa kumquat. We then employed Xcc-facilitated agroinfiltration to deliver Cas9, along with a synthetic sgRNA targeting the *CsPDS* gene, into sweet orange. DNA sequencing confirmed that the *CsPDS* gene was mutated at the target site in treated sweet orange leaves. The mutation rate using the Cas9/sgRNA system was approximately 3.2 to 3.9%. Off-target mutagenesis was not detected for *CsPDS*-related DNA sequences in our study.

Citrus Huanglongbing stimulates root growth while causing overall root loss

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Huanglongbing (HLB) is a devastating disease of citrus, caused by phloem limited *Candidatus Liberibacter asiaticus* (Las). Early symptoms of HLB include fibrous root loss and blotchy mottle, followed by yield declines, leaf drop, and dieback. Early root loss was previously described as a 30-50% reduction in root density evident before foliar symptoms develop, is dependent on local bacterial infection, and occurs before canopy phloem plugging-induced carbohydrate starvation. Continued sampling of Hamlin/Swingle and Valencia/Swingle trees in different stages of HLB development has revealed that root loss occurs in two stages and has been confirmed for at least four other rootstocks. The second phase of root loss (70-80%) begins at the early stages of canopy thinning from leaf drop. During this phase, carbohydrate supply to the roots is severely limited by canopy phloem-plugging. Four root cages were buried under each of 40 trees of different HLB ratings and replaced every 2 months to measure new root growth. Surprisingly, in both phases of root loss, prior to severe tree decline, root growth was stimulated compared to presumed healthy trees. When looking only at infected trees, root growth in the cages was positively correlated to the presence of Las in these new roots. This suggests that root density reductions result from the shortened lifespan of fibrous roots and that induction of root growth is unlikely to mitigate HLB-associated root loss.

Data-driven mathematical models for HLB: Testing interventions in a virtual world

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Designing effective and cost-conscious intervention strategies for HLB that are useful for different types of growers in multiple locations is a

major goal of HLB research programs. It requires synthesizing biological data across different temporal and spatial scales, and from both laboratory and field studies, while taking into account economic constraints. This is often very difficult using traditional empirical approaches alone. Adding bespoke mathematical models to the toolbox of techniques has the potential to facilitate synthesis of already collected data to improve our understanding of what drives observed patterns of transmission and provide feedback on data collection and experimental efforts. Further, models can provide insights into the cost effectiveness of intervention strategies faster, more cheaply, and with less risk than almost any other approach. They can also be used to test a much broader range of controls, including ones that are still in development, and compare their likely efficacy with current interventions. We present a simple, interactive model of the spread of HLB in a grove over time that incorporates laboratory data on the effects of temperature on the ACP life-cycle to show the possibilities of using mathematical models for understanding transmission and planning interventions. We discuss how models can be used to quantify uncertainty of components to guide experimental priorities, what data and information are needed for parameterization and testing, and how results can be used in practice.

Huanglongbing reduces the effectiveness of *Phytophthora* fungicide control

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Both field surveys and greenhouse experiments show that Huanglongbing (HLB) increases the susceptibility of the citrus root system to the damaging root pathogen *Phytophthora nicotianae*. This increased *P. nicotianae* infection suggests that root system damage may occur more quickly in the presence of both pathogens. Chemical control of *P. nicotianae* may slow root system decline and subsequent yield decline in HLB-affected trees in *Phytophthora*-infested groves. To test the value of *P. nicotianae* control in slowing tree decline in the presence of both pathogens, 100 *P. nicotianae*-infested nursery trees were inoculated or mock-inoculated with HLB-affected budwood. After HLB inoculation, trees were treated bimonthly with Ridomil Gold SL (mefenoxam) or Aliette WDG (Aluminum tris O-ethyl phosphonate) compared to untreated controls. HLB status, *P. nicotianae* infection and propagule counts, and visible symptoms were assessed bimonthly. After 12 months, the trees were harvested and biomass of roots and shoots was measured. As expected, Ridomil and Aliette significantly reduced *P. nicotianae* root infection and increased fibrous root mass compared to untreated controls for HLB(-) trees. Neither Ridomil nor Aliette significantly reduced *P. nicotianae* root infection or increased fibrous root density for HLB(+) trees, although both measures quantitatively improved with Ridomil performing slightly better than Aliette. These data suggest that HLB reduces the effectiveness of fungicide control of *Phytophthora* root rot in addition to increased susceptibility to *P. nicotianae*.

Comparative metabolomics of Citrus phloem sap to explore the nutrition needs of *Candidatus Liberibacter asiaticus*

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Citrus greening disease or Huanglongbing (HLB) is putatively caused by *Candidatus Liberibacter asiaticus* (CLAs) which is vectored by the Asian citrus psyllid *Diaphorina citri* (ACP), a phloem sap feeding insect. Little is known about the composition of citrus phloem sap and why CLAs transmission from ACP to citrus trees is so successful within this medium. Plant phloem sap is an aqueous solution of mostly sugars produced during photosynthesis, as well as other organic and inorganic compounds. Phloem sap samples were collected from HLB-tolerant varieties such as *Citrus latipes* and HLB-susceptible varieties such as *Citrus sinensis* 'Valencia'. Samples were derivatized using a two-step trimethylsilylation (TMS) for GC-MS which is very sensitive and specific to organic compounds, especially organic acids and sugars. Using this method, 60-90 compounds including amino acids and amines, organic acids, and carbohydrates were detected and quantified in citrus and other phloem sap samples to establish their compositions. It was found that while the overall composition was similar between sensitive and tolerant citrus varieties, the metabolite profile was unique for each

variety, with some trends that correlated with tolerance to HLB. Glucose concentration was higher than sucrose in 'Valencia' and *Poncirus trifoliata*, but sucrose was higher than glucose in all other varieties. Only 'Valencia' phloem sap contained raffinose in significant amounts. *C. latipes*, had the most L-proline and the highest concentration of amino acids in the varieties examined. Carbohydrates were highest in two grapefruit varieties, lemon, and lime. Organic acids were lowest in 'Valencia' and *P. trifoliata* and highest in *Citrus macrophylla*. Sugar alcohols are known to support other gram-negative bacteria, and were found in abundance in citrus phloem sap, suggesting they may be required for CLAs proliferation. Overall, the comparison of phloem saps between susceptible and tolerant varieties of citrus revealed some differences that could be important for understanding the nutritional requirements for culturing the CLAs bacterium.

Rapid deployment of best practices from global collaboration on zebra chip disease in potatoes when applied to HLB – A case study

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Zebra Chip (ZC) disease of potato represents a quality defect that is caused by the bacteria, *Candidatus Liberibacter solanacearum* (*Lso*), which is transmitted by the potato psyllid, *Bactericera cockerelli*. Between 2004 and 2012, a concerted effort between industry, academia, and USDA-ARS scientists led to a multi-faceted approach to expeditiously understand the causation of the disease. A global network of scientists, growers, and producers worked together to rapidly identify and implement practical solutions. While ZC still presents challenges, significant progress in understanding and managing the disease was made and losses due to ZC have been mitigated. Similarities between ZC and HLB go beyond sharing a similar strain of *Liberibacter* and a psyllid vector. The importance of leveraging collaborative research and quickly finding practical strategies to effectively combat HLB in citrus is a global concern. Committed individuals, representing different stakeholders (i.e., growers, universities, government groups, processors, and other interested parties) will be the best chance to find a viable solution(s) to protect and reboot the citrus industry. Based on the ZC case study, the rapid deployment of promising technical solutions to combat HLB disease in the various citrus-producing countries would significantly benefit the preservation of this important food source. The formation of an HLB Solutions Technology Transfer team comprised of interested and dedicated volunteers from growers' groups, academia, industry, and government agencies is being proposed to support this critical global initiative by establishing an infrastructure for exchange of this information.

Candidatus Liberibacter caribbeanus, a new citrus associated *Liberibacter* from Colombia, South America

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We report a new species, *Candidatus Liberibacter caribbeanus* (CLca), from both the Asian citrus psyllid and *Citrus sinensis* from Cordoba in the northeastern region Colombia, South America. Additional psyllids carrying CLca were found from Barranquilla in the northern part of Colombia close to the Caribbean Sea. Quantitative real-time PCR assays using primers and probes routinely used for *Ca. L. asiaticus* were capable of detecting CLca. About 5000 psyllids and 100 plant samples were tested for the presence of *Liberibacter*. We conducted conventional PCR and Sanger sequencing of the 16S rDNA region, followed by phylogenetic analysis, 16S metagenome analysis of the psyllid DNA, and PAC-BIO sequencing of the bacterium from psyllid. A new method, digital PCR, was utilized for sensitive detection of CLca. Confirmatory tests using multiple genomic regions of both CLca and CLas were conducted. CLca is about 92–96% similar to previously reported *Liberibacter*s based on 16S rDNA sequence analysis. At this time, it is not clear if CLca can be cultured and if it causes HLB-like symptoms in citrus. The titer of the bacterium reaches very high levels in the psyllid. Further research to determine the pathogenicity of the organism and its role in citrus HLB is in progress.

Effect of temperature and the infection with *Candidatus Liberibacter asiaticus* on the energy metabolism of Asian citrus psyllid, *Diaphorina citri*

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Asian citrus psyllid, *Diaphorina citri*, is a tropical and subtropical pest that invaded many new regions around the world and threatened citrus industry as a vector for Huanglongbing (HLB) disease. ACP mechanism of temperature tolerance is poorly understood. We investigated adult survival, cellular energy balance, gene expressions, and changes in nucleotides and sugar-nucleotides under the effect of different temperature regimes. The optimum degree for survival was 25°C. Low temperatures such as 0°C and 5°C caused 50% mortality after 2 and 4 days, respectively, while at high temperature (40°C and 45°C), one day was sufficient to achieve more than 95% mortality. Correlation between ATP quantities and ATPase enzymes' activities was significantly negative. Twenty-four nucleotides and sugar-nucleotides were identified and quantified using HPLC in ACP adults exposed to low, high, and optimum temperatures. Its pattern profile was clearly distinguished in response to each treatment. AMP, IMP, GMP, GTP was decreased in response to low and high temperature. The ratios between AMP:ATP and ADP:ATP were significantly decreased and positively correlated with adults' survival, whereas, the adenylate energy charge was increased in response to low and high temperatures. Gene expression of Hsp70, V-type proton ATPase catalytic subunit A and ATP synthase α subunit confirmed these results. We also investigated the effect of the infection with CLAs on ACP energy metabolism. We noticed accumulation of ATP and low ATPase activities in infected psyllid. The ratios between AMP:ATP and ADP:ATP were significantly decreased while the adenylate energy charge was increased. CLAs cells acquire ATP and other nucleotides from surrounding media using ATP translocase. This finding suggests that CLAs manipulates its vector for its benefit. Exploring energy metabolic regulation, nucleotide content in context of ACP adult survival might help in understanding the physiological basis of how ACP in newly invaded regions respond to environmental stress including weather and infection with CLAs. These findings might lead to development of genetic tools, as RNA interference for nucleotides pathways, and innovative strategies for ACP management to limit the spread of the HLB disease.

LuxR solo quorum sensing mediates plant virulence and insect transmission of *Candidatus Liberibacter asiaticus*

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The genome of *Candidatus Liberibacter asiaticus* (CLAs) reveals the presence of *luxR*, which encodes the LuxR protein. This protein is one of the two components typical of bacterial "quorum sensing" or cell-to-cell communication systems. Interestingly, the genome lacks the second component; *luxI*, which produces Acyl-Homoserine Lactone (AHL), suggesting that CLAs has a solo LuxR system. We have confirmed the functionality of the CLAs solo *luxR* by constructing a *luxR* gene promoter fused with a GFP reporter. This has resulted in a functional CLAs *luxR::GFP* monitor strain of *E. coli* similar to that reported by Kock et al. (2005. Microbiology. 151:3589-3602). This *E. coli* strain produces fluorescence if the *luxR* promoter binds to AHLs or to a eukaryotic signal. Several AHLs, including N-butanoyl homoserine lactone, N-hexanoyl homoserine lactone, N-3-oxo-hexanoyl homoserine lactone, N-3-oxo-octanoyl homoserine lactone, and N-3-octanoyl homoserine lactone, as well as, extractions from insects and from citrus plants, have been shown to activate CLAs *luxR*. The plant-derived extracts are likely to be structurally unrelated AHL mimics. As a response to infection by CLAs, citrus may increase the production of its AHL mimic(s), which would bind to LuxR and possibly limit CLAs bacterial growth by triggering cell aggregation and consequently limit bacterial movement *in planta*. The insect extract has a structurally related AHL, which may be produced by endosymbiotic bacteria and bind to CLAs LuxR. As a result of this binding, CLAs may form biofilm on the surface of the gut of Asian citrus psyllids (ACP), *Diaphorina citri*. Currently, we are investigating the effect of many compounds known to be signals for bacterial LuxR, especially those found in citrus phloem sap, on the activity of CLAs LuxR. These compounds include,

but are not restricted, to Indole-3-acetic acid (IAA), indole, γ -amino butyric acid (GABA), salicylic acid (SA), Riboflavin and Lumichrome. We expressed CLAs LuxR in citrus using the CTV-based vector system. LuxR expressing citrus showed equally distributed severe symptoms when infected with CLAs. Rearing infected ACP on uninfected LuxR plants resulted in a diminishing CLAs population. Interfering with CLAs cell-to-cell signaling may lead to a new avenue of control strategies.

Development of efficient trapping citrus to attract Asian citrus psyllid and increase sustainability of citrus groves

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Citrus Huanglongbing (HLB) is the most destructive disease in citrus. HLB has caused a significant economic impact on citrus production in Florida, the second largest orange juice producer in the world. An available option to citrus growers is to uproot infected trees and/or to use enhanced nutritional supplements that mask symptoms and postpone the underlying problem. The latter strategy does not eradicate the bacterium, *Candidatus Liberibacter asiaticus* (CLAs), which causes HLB. Judicious use of the pesticides against Asian citrus psyllid (*Diaphorina citri*) combined with use of biotechnological approaches, including RNAi, may be useful to control psyllid populations. Towards this end, we modified citrus plants (through RNAi) to become more attractive to psyllids. These plants may have application as perimeter trap plants along grove edges or interspersed with other plants within a grove. Additionally, these citrus plants should be kept regularly pruned so as to not allow flowering to prevent possible gene flow to the commercial population of trees. We observed that psyllids are attracted to yellow and mixed yellow areas and thus to the chlorotic and semi-chlorotic regions in plants. We have developed citrus plants with silenced endogenous genes, Phytoene desaturase (PDS) that interferes with the carotenoid biosynthetic pathway and δ amino levulinic acid dehydratase (δ -ALAd) that interferes with the chlorophyll biosynthetic pathway using silencing potential of the citrus tristeza vector. Citrus trees that have silenced PDS and δ -ALAd exhibited generalized chlorotic phenotypes because of interference with their pigments' biosynthesis and attracted more psyllids than normal plants. Developing citrus plants as trap plants that attract psyllids may contribute to protecting and increasing sustainability of new citrus plantings by employing the trap crop technique.

Molecular basis of Citrus Greening and related diseases gleaned from genome analyses of hosts and pathogens

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While significant efforts are spent on remedies against Citrus Greening, its exact molecular mechanisms remain poorly understood. In order to successfully eradicate this plague in the long-term, we need to gain knowledge about these mechanisms, learn which molecules of the pathogens are causing the infection, which molecules of the host are the targets, and devise strategies to specifically interfere at molecular level. Comparative analysis of multiple genomes offers a bird's eye view of a pathogenicity landscape and will generate hypotheses about molecular players that are most likely to be involved. We performed comparative analysis of available genomes of *Liberibacter*, citrus and psyllid with the emphasis on the differences between pathogenic and non-pathogenic *Liberibacter* species. The results on protein structure and function prediction are presented as a comprehensive web-site with web-pages dedicated to each protein. Based on our analysis, we formulated hypotheses about proteins that are more likely to be causing HLB and vulnerabilities in citrus targeted by these virulence factors. Some of the important players involve the *Liberibacter* prophage, plant-like secreted factors, and steroid biosynthesis enzymes.

Determining the effects of citrus genotypes on CLAs transmission via root grafting

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Citrus greening disease (Huanglongbing, HLB) caused by the bacterium, *Candidatus Liberibacter asiaticus* (CLAs) vectored by the Asian Citrus

Psyllid has adversely affected the citrus industry in Florida. This project seeks to investigate if root grafting is a viable transmission pathway for CLAs between trees. Previous studies have demonstrated that the intensity of HLB symptom development on the aerial parts is affected by scion/rootstock combination. Thus, research on the alternate routes of CLAs inoculation and the influence of citrus genotypes in disease spread needs further investigation. Therefore, the goals of this project are to determine: (a) if genotype susceptibility to HLB influences root grafting inoculation of CLAs and (b) if removal of aboveground parts affects CLAs transmission via root grafting. Commercially important citrus genotypes (Ray Ruby grapefruit and Valencia orange) on five various rootstocks were planted in 3-gal pots in January 2013. Pots containing three trees each (two bud-inoculated and one healthy) were randomly assigned as follows: (1) Trees left intact; (2) Inoculated trees clipped after 9 months; (3) same as 2, trunks treated with glyphosate; (4) Inoculated trees clipped after 12 months; (5) same as 4, trunks treated with glyphosate; and (6) a non-inoculated control. Each treatment has nine replicates, except for control with only three. Data on growth morphology, leaf chlorophyll content, and photosynthetic response are collected on a monthly basis. Visual assessment of symptom development is performed prior to clipping followed by leaf sampling for PCR. We expect that root-grafting is a viable mode of CLAs transmission even after the removal of aerial parts of the diseased tree. This project will provide information on the response of commercially important citrus genotypes to HLB symptom development and will help grove managers in decision making while adopting methods to control the spread of HLB in Florida.

Surveys to determine HLB presence in Asian citrus psyllids and citrus samples from Barbados

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In June 2012, the presence of *Candidatus* *Liberibacter asiaticus* (CLAs) in 115 Asian citrus psyllid samples, collected from Barbados citrus trees, was confirmed by real-time and conventional PCR assays and by the sequence of its partial 16S rRNA and ribosomal protein genes. During March-April 2014, 145 HLB-symptomatic leaves of different citrus cultivars (predominantly lime) and sweet lime, from different regions of Barbados were collected, DNA was extracted in Barbados, and DNA extracts were shipped to TAMUK Citrus Center to test for the presence of CLAs. CLAs was not determined from any of these samples; but 20 of them produced high borderline threshold cycle (Ct) values in real-time PCR assays. These DNA extracts were found to be of poor quality, based on the amplification of an internal control plant cytochrome oxidase gene in qPCR assays. More surveys will be conducted to collect HLB-symptomatic leaves and test them for the presence of CLAs.

Incidence, spread, and current situation of Huanglongbing in Texas

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Huanglongbing (HLB) was confirmed for the first time in Texas in January 2012 through visual inspection of typical disease symptoms, quantitative real-time PCR, conventional PCR, and graft-inoculations of 'Valencia' sweet orange healthy seedlings with *Candidatus* *Liberibacter asiaticus* (CLAs)-infected budwood. Prior to this first detection, a comprehensive survey and testing of psyllid samples in 2011 revealed an extremely low percentage (0.03%) of CLAs-positive psyllids with threshold cycle (Ct) values <32 indicating an approximately one-year lapse between positive HLB detection in psyllids and trees in Texas under field conditions. Analysis of psyllid survey samples over a 2-year period revealed an increase in incidence of CLAs-positive psyllids from 0.03% (n=) in 2011 to 0.33% (n = 11075) in 2013. Similarly, the percentage of CLAs-positive leaf tissue increased from 0.61% (n = 7680) in 2012 to 1.03% (n = 8320) in 2013. A positive and significant relationship was obtained between the yearly percentage of CLAs-positive leaf tissue samples (Y) and the percentage of CLAs-positive

psyllids (X) ($Y = -0.0302 + 3.21X$ with adjusted $-R^2 = 0.99$), for the period of 2009-2013. The results suggest that an increase of 3.21% CLAs-positive psyllids could result in ~1% increase in CLAs positive leaf tissue samples, thus affirming psyllids as better indicators of early HLB detection under field conditions.

Detection of Huanglongbing-associated bacteria using diverse DNA extraction methods and RCR primers for roots of 'Valencia' sweet orange on sour orange rootstock

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Fibrous root samples from Huanglongbing symptomatic (S) and asymptomatic (AS) 'Valencia' sweet orange trees (*Citrus sinensis*) grafted onto sour orange (*C. aurantium*) rootstock were analyzed using different DNA extraction procedures and primers to detect *Candidatus* *Liberibacter asiaticus* (CLAs). DNA extraction kits including Qiagen DNeasy, Mo Bio PowerSoil and PowerPlant generated root DNA suitable for polymerase chain reaction (PCR) diagnostic assays. Quantitative PCR (qPCR) using HLBspr primers and probe on root DNA extracts from a total of 206 samples from 60 AS trees showed unreliable results for the presence of *Ca. L. asiaticus*. Except for one sample, all the remaining samples gave negative qPCR result using LJ900fpr primers and probe. Further analysis of roots samples from S and AS trees indicate that HLBspr is not suitable for CLAs diagnosis in root samples. None of the 206 leaf samples from the same AS trees showed positive qPCR result with HLBspr. Conventional PCR (cPCR) using OI1/OI2c primers on 12 randomly selected root samples produced non-specific amplification products which were easily distinguished from products specific to *Ca. L. asiaticus*; however, the same root samples did not produce any amplification product in cPCR using A2/J5 primers. Any of the above mentioned cPCR or qPCR assays could accurately detect *Ca. L. asiaticus* in S leaf samples of *Ca. L. asiaticus*-infected trees. Root samples from 10 (5 S and 5 AS) additional trees were analyzed by cPCR using A2/J5 and Las606/LSS primers and qPCR using LJ900fpr and CQULA (CQULA04F/CQULA04R, and TaqMan probe CQULAP10) clearly showed that all these primers are efficient in detecting *Ca. L. asiaticus* in root samples. The frequency and consistency of *Ca. L. asiaticus* detection was improved when root samples were collected near the tree trunk compared to the roots collected farther. *Ca. L. americanus* was not detected in either root or leaf samples.

In planta virus-based expression of artificial microRNAs targeting the potato psyllid *Bactericera cockerelli*

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Silencing genes by RNA interference (RNAi) has potential to be a useful tool for controlling pests. Previously, we showed that recombinant *Tobacco mosaic virus* (TMV) containing insect sequence inserts can efficiently infect and generate small interfering RNAs (siRNAs) in a variety of plant species, and more importantly, delivery of interfering sequences via recombinant TMV induces target-specific RNAi effects in insects feeding on these plants, even yielding decreased progeny number as compared to plants infected by recombinant TMV containing GFP. Here, we cloned artificial microRNAs (amiRNAs) targeting the ATPase gene of *Bactericera cockerelli* into agroinfection-compatible TMV and *Tomato mottle virus* (ToMoV-A, TAV) expression vectors, and a virus-free 35S promoter driven binary vector. RNAs were extracted from the *Nicotiana benthamiana* tissues infiltrated with agro-amiR-TAV, agro-amiR-TMV, agro-amiR, and infected with amiR-TMV *in vitro* transcripts, and analyzed by Northern blots. The results showed that the amiRNAs were successfully produced in the infiltrated *N. benthamiana* and *N. tabacum* plants. Two of the amiRNA clones with each vector were used to prepare small RNA cDNA libraries, and these were then utilized for small RNA deep sequencing by using the Hi-seq platform. The results of deep sequencing showed that DNA viral-vector (TAV) gives the highest and very specific accumulation of amiRNA compared to RNA viral-vector (TMV) and non-viral vector. In addition to plant analysis, we used the filtrated tissues for psyllid feeding tests. We also used *in vitro* transcripts of amiRNAs for membrane feeding tests for *B. cockerelli*. In both feeding experiments, psyllids were analyzed by

reverse transcription (RT)-real time PCR. Our goal is to understand how viral vectors affect the quality, quantity, and profiles of amiRNAs in plants and find the best way to deliver amiRNAs for potential applications. By designing 21 nt sequences to have a perfect match to a target sequence, the amiRNA could potentially avoid off-target effects and be a better strategy for practical applications.

Identification of essential genes of the Huanglongbing (HLB) model bacterium *Liberibacter crescens* BT-1

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Liberibacter crescens BT-1 is a gram-negative, rod-shaped, α -proteobacterium isolated from mountain papaya. It is a close relative of the causal citrus HLB agents *Liberibacter asiaticus*, *L. africanus*, and *L. americanus*. Citrus greening is one of the devastating diseases with high economical costs to the worldwide citrus industry. The inability to culture any of the HLB causal agents limits our understanding of the mechanism of infection and delays the development of proper treatments for citrus greening. *L. crescens* BT-1 is the first cultured member of the *Liberibacter* genus and can serve as a model to study methods to kill *Liberibacter* pathogens. Here, mutagenesis is used to identify essential genes in *L. crescens* BT-1 that can serve as antimicrobial targets for the closely related pathogens as has been done in other systems. Analysis of the essential genes, protein-coding sequences necessary for the survival of *L. crescens* BT-1, has not been previously attempted due to the limitations of available genetic tools for the organism. In order to gain insight on the virulence, metabolism, and culturability of the genus *Liberibacter*, a mini-Tn5 transposon derivative system consisting of a gene specifying resistance to kanamycin, flanked by a 19-base-pair terminal repeat sequence of Tn5, was used for the genome-wide mutagenesis of *L. crescens* BT-1 and created an insertion mutant library. By analyzing the location of insertions using massively-parallel sequencing technology (transposon directed insertion sequencing), the genes essential for *L. crescens* BT-1 survival under *in vitro* conditions are determined. The information obtained may reveal unique aspects of the physiology and metabolism of *L. crescens* BT-1, aiding in the discovery and the development of therapies against the disease.

Asian citrus psyllids respond to degradation products of common citrus plant volatiles

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We used electrophysiological techniques to record antennal signals and feeding behavior of the Asian citrus psyllid, *Diaphorina citri*, to gain insight into insect-plant interactions that may be of value in the fight to control *Candidatus Liberibacter asiaticus*, the bacterium responsible for Huanglongbing disease of citrus. We were unable to demonstrate antennal responses to most of the volatile organic compounds produced by citrus leaves using either head space volatiles or purchased pure compounds. However, large and consistent antennal responses to degradation products of common citrus volatiles were obtained under laboratory conditions. Glass cartridges loaded with β -ocimene or citral became stimulatory only after 3 to 9 days at room temperature. Gas chromatograph-coupled mass spectrometry (GC-MS) demonstrated that both compounds degraded completely over 3 to 9 days to acetaldehyde, acetone, acetic acid, formic acid, and other compounds. GC-coupled electroantennographic detection (GC-EAD) identified two peaks that elicited consistent and large antennal responses: acetic and formic acids. Both acids were highly stimulatory to *D. citri* antennae and responses were correlated with log dose. Probing behavior of *D. citri* was studied by incorporating blends of citrus volatile compounds in varying proportions and amounts into an emulsified wax substrate (SPLATTM, ISCA Technologies, Inc). More probes were observed on SPLAT containing blends of acetic and formic acids compared with either acid separately or other compounds. Our study suggests that *D. citri* may orient to formic and acetic acid present in the citrus tree canopy. These observations of antennally active compounds, both constitutive and arising as degradation products from constitutive plant volatiles, may contribute to development of attractants and/or repellants for this important species.

Optimizing intra- and inter-orchard sampling for early detection of citrus Huanglongbing

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In Brazil, Huanglongbing (HLB) was reported only in São Paulo, Paraná, and Minas Gerais, but the vector is widespread in the country. It is of the utmost importance to develop and improve sampling procedures in order to maximize the probability of early detection in regions where the disease is still unreported. Our objectives were: (i) to find an efficient spatial sampling scheme to detect the disease as soon as possible, (ii) to know how many times one should scout a given orchard in order to find 100% of symptomatic plants, and (iii) to optimize the sampling frequency and intensity in order to maximize the detection probability before the disease reaches a given incidence. For the first two objectives, an excess of 650 observed field maps were used as a baseline. Objective (i) was accomplished by simulating 21 intra-orchard sampling schemes, including patterns in X, W, Double W, Diagonals, in clusters, per planting rows and edge focused patterns. In each simulation, the plants were selected according to the sampling pattern and 'evaluated' considering the reported efficiency in Brazil (47.6%). In all cases, a monomolecular function was fitted describing the relationship between HLB incidence and probability of detection. The best sampling procedures were always those which included the evaluation of plants on the orchard's edges ("Edge", "Double Edge", "Florida", or "Mexico") or systematically assessing a fraction of the planting rows. As the results consider only the detection probability, issues such as efficiency per assessed plant, sampling costs per plant and total sampling time should be further evaluated. Objective (ii) was accomplished by simulated scouting of 100% of the plants in an orchard, assuming a given detection efficiency. As the detection efficiency increases, a steep decrease in required re-inspections occurs, stabilizing after 50% of detection efficiency. Considering the reported detection efficiency in Brazil, an average of three re-inspections are needed to find 100% of the symptomatic plants. Objective (iii) was accomplished by developing a spatially explicit compartmental model; simulating HLB epidemics in a Brazilian citrus landscape; and simulating different sampling schemes, frequencies, and intensities. Results expressed as the probability of detecting the disease before the regional incidence reaches a given value allowed the development of scenarios to be used by policy makers.

Modeling the impact of RNAi treatments on the spread of HLB among asymptomatic trees

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Based on recent experimental results indicating that newly infected young flush can become infectious to nymphs and adult psyllids within 10-15 days, we incorporated this short time to infectiousness into an agent-based microsimulation model of HLB transmission. A major implication of our results is that waiting to implement control measures until trees are symptomatic is a strategic decision that can lead to unnecessary rouging. A high priority needs to be given to monitoring and ongoing surveillance of groves so that invasion by psyllids is detected when, or soon after, this process is initiated. Then intensive control of the psyllid population during the asymptomatic phase of transmission can profoundly enhance the useful fruit producing life of citrus trees. Improved strategies for psyllid and nymph surveillance are critically needed. One potential control mechanism is RNAi constructs, which currently is being investigated. Citrus trees are infected with a genetically modified strain of CTV that produces extra double stranded RNA sequences specific to psyllid genes. When these RNAs are ingested by psyllids, they inhibit production of proteins vital to psyllid development. An RNAi construct may act by reducing the survival or

reproduction of the psyllids, as well as inhibiting the acquisition of *Ca. Las* by the psyllids. We have extended our original model to incorporate the effects of various RNAi constructs. Our model predicts that RNAi constructs can significantly reduce the psyllid population and slow the spread of *Ca. Las* infection.

Improvement of the use of yellow stick card for monitoring adults of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae)

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Increasing the effectiveness of *D. citri* monitoring is of great importance to improve the control of this insect. Thus, this study aimed to evaluate the effect of periodic training of inspectors in identifying *D. citri* on yellow sticky cards (YSC) and to compare the effectiveness and costs of reading the YSC in the field versus in the office. The effect of periodic training was determined by comparing the performance of nine inspectors who had been trained a year before and immediately after a new training session. Each inspector assessed 20 YSC that had been exposed in the field for 7 days. For comparing YSC reading performance in the field and in the office, eight inspectors assessed the presence of *D. citri* on 90 YSC. In both experiments, one psyllid was placed on some YSC at a marked position. The assessment was first conducted in the field and a week later in the office using the same YSC. For this study, the eight inspectors were analyzed as a single group and as two groups of four inspectors based on the highest and lowest performances in the field. Periodic training of inspectors improved the efficiency of *D. citri* detection on YSC by 28%. Overall, reading the YSC in the office increased the detection of psyllids by 15.8% compared to the field. The efficiency in detecting the insect on YSC in the office increased by 18.4 and 10.2% for inspectors with lowest and highest performances in the field, respectively. Furthermore, the assessment in the office reduced the difference of effectiveness in detecting *D. citri* between the worst and best inspectors from 12.5 to 3.8 percentage points. The assessment of YSC in the office reduced inspection costs at 60%.

Validation of early detection technologies using droplet digital polymerase chain reaction amplification

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The official regulatory techniques for Huanglongbing (HLB) diagnosis are quantitative polymerase chain amplification (qPCR) or conventional PCR analysis of DNA from the HLB causing *Liberibacter*. However, the concentration of *Liberibacter* DNA has to be high enough to allow for confirmation by conventional PCR followed by cloning and sequencing. By the time titers are high enough in plants for this, the infection has already spread to adjacent trees—especially if Asian Citrus Psyllid (ACP) are present. This scenario is completely inadequate if the California citrus industry is to keep HLB out of groves. Several early detection techniques are being evaluated by the California Citrus Research Board. These include both direct measurement of elicitor proteins produced by the bacterium and the detection of small interfering (si) and messenger (m) RNAs, proteins, metabolites, and volatile organic compounds produced by the citrus tree in response to infection. Many of these approaches measure systemic responses. This holds a huge advantage over any PCR technique which is significantly limited by sampling since *Liberibacter* are not uniformly distributed in an infected tree. Nevertheless, a PCR protocol is urgently needed to validate these early detection methods that would provide acceptable evidence of their efficacy to federal and state regulatory agencies. Droplet digital polymerase chain reaction (ddPCR) amplification is such a protocol. The technique utilizes the same qPCR primers and probes and amplified products can be isolated, cloned, and sequenced from ddPCR as with conventional PCR. We have evaluated 87 transect/risk-based survey site samples from around the Hacienda Heights positive with ddPCR that have been evaluated by several of the early detection protocols. Correlation of ddPCR results with those of these methodologies will

determine which protocol(s) show the most promise for detecting HLB early enough in the infection process to make tree removal, and therefore elimination of inoculum, feasible.

Development of novel control strategy to citrus HLB by targeting critical traits of *Candidatus Liberibacter asiaticus*

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Huanglongbing (HLB) in Florida is caused by *Candidatus Liberibacter asiaticus* (Las), a phloem-limited fastidious α -proteobacterium, which is transmitted by Asian citrus psyllids (ACP, *Diaphorina citri*). HLB is causing an unprecedented crisis for citrus industry in Florida and poses a severe threat to citrus production in California, Texas, and Arizona. Currently, no effective HLB management is available. We aim to control HLB by targeting critical traits of Las to break down its infection cycle. Interestingly, Las contains the General Secretory Pathway (Sec), which is important for the viability and secretion of putative Sec dependent effectors (SDEs) of Las. SecA, an ATPase, is vital for the function of the Sec pathway and a good target to develop antimicrobials. We have identified multiple SecA inhibitors with high antibacterial activity to *Liberibacter*s and their relatives. We will represent our recent progress in controlling HLB using SecA inhibitors and other antimicrobial compounds. In addition, our study indicates Las contains a functional salicylic acid (SA) hydroxylase which breaks down SA and its derivatives. SA and its derivatives play a central role in plant defenses, e.g., systemic acquired resistance (SAR), and are exogenously applied on plants as SAR inducers to control plant diseases. Here, we will present our recent progress in controlling HLB by nullifying SA hydroxylase of Las. Finally, breaking down the interactions of virulence factors and their targets *in planta* has been suggested to be one strategy to generate disease resistant plants. We will present our recent progress in identifying SDEs and their putative targets. We aim to generate HLB resistant or tolerant plants by disrupting the interaction between SDEs and their targets in citrus.

Ten-year screening and confirmatory PCR tests of citrus Huanglongbing - Performance, challenges, and improvements

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Conventional PCR (cPCR) and real-time PCR (qPCR) methods had been developed and systematically validated for detection of citrus Huanglongbing (HLB) before the disease was detected and confirmed in Miami, Florida, in 2005. Since then, the two rapid and sensitive multiplex qPCR assays (HLBaspr/COXfpr and HLBampr/COXfpr) have been used to screen field samples for HLB as part of an on-going survey conducted by state, industry, and/or USDA-APHIS regional laboratories. The two screening qPCR assays can detect all three known species of HLB bacteria: '*Ca. L. asiaticus*' (Las), '*Ca. L. africanus*', and '*Ca. L. americanus*'. Since 2005, over 200,000 samples of HLB host plants and Asian citrus psyllids collected mainly in Florida, Texas, South Carolina, Louisiana, Georgia, and California have been tested using the screening qPCR assays. Confirmatory tests which include three multiplex qPCR (HLBaspr/COXfpr, HLBafpr/COXfpr, and HLBampr/COXfpr), three cPCR assays (OI1/OI2c, A2/J5, and GB1/GB3), and sequence analysis of the cPCR amplicons are done exclusively at USDA CPHST Beltsville laboratory on samples that test suspect positive in the screening qPCR assays. The first regulatory detections of HLB-Las by the states using the screening assays were confirmed by the federal confirmatory assays in new quarantine areas. We combined the two screening qPCR assays and validated them as a single assay (HLBasmpr/COXfpr). The performance of the new combined assay remained unchanged; however, the cost was reduced by half. We have supplemented our confirmatory assays by developing and validating two additional qPCR assays (CPRfpr/COXfpr and HSPfpr/COXfpr) that target two different conserved genes of Las. Although the current qPCR assay is at least 100-fold more sensitive than cPCR, which in 1996 proved useful for pre-symptomatic detection of the disease, in the future we will evaluate

several emerging technologies and/or platforms such as digital PCR and digital sequencing for advanced HLB diagnosis.

Identification of traits involved in rhizosphere competence of beneficial bacteria isolated from HLB-infected citrus trees

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Huanglongbing (HLB), associated with *Candidatus Liberibacter asiaticus* (Las), is the most devastating disease of citrus and threatening the citrus industry in Florida. Early root infection has been suggested to play a central role in HLB disease development and of significance to tree health. Therefore, introduction of beneficial bacteria to roots to promote root health might be an alternative approach to management of HLB. Our recent investigations showed that three beneficial Bacillus and closely relative isolates were able to promote citrus plant development with stronger root systems and delay the development of HLB symptoms and Las populations in greenhouse assays. Here, we characterized the rhizosphere competence of these three isolates in both greenhouse and natural environment. Using culture-dependent and -independent approaches, bacterial populations of these isolates on roots of citrus and in rhizospheric soil were determined following soil inoculation. The bacterial populations on the roots of citrus and in soil one month after inoculation were approximately 5.0×10^4 CFU/g (dry weight) and 5.0×10^3 CFU/g (dry weight), respectively, for the three isolates. The assays revealed a similarity in rhizosphere competence with survival rates ranging from approximately 0.05% to 0.5% for the three isolates. Further analysis revealed that the rhizosphere competence of these isolates may be associated with various phenotypic traits, including substrate utilization, nitrogen dissimilation, siderophore mediated iron acquisition, stress tolerance, copper resistance, and the production of antimicrobial substances.

Genetic analysis of Huanglongbing pathogen from sweet orange “Hongjiangcheng” in Guangxi

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“Hongjiangcheng” is one of the major citrus varieties planted in southern Guangxi. It has been a great loss for Huanglongbing. So, it is very important to know the diversity of “hongjiangcheng” *Candidatus Liberibacter asiaticus* for the preventing and control of “hongjiangcheng” Huanglongbing in the future. In this paper, suspicious samples with Huanglongbing were collected from “hongjiangcheng” from different places in Guangxi. Nested-PCR was used to detect the samples with primers fD1/fD2 and OI1/OI2c. Some positive samples were selected to conduct genetic analysis. Total 67 clear loci were amplified from 30 samples collected in 9 counties of Guangxi with 35 pair SSR primers. The phylogenetic tree showed: the *Ca. liberibacter asiaticus* from southern Guangxi Fangcheng, Nanning and Fusui were clustered together; Longan, Daxin, and Jingxi were grouped in another place. The pathogen in Bama of central Guangxi and Rongshui of western Guangxi were probably from Fangcheng, Nanning and Fusui group. Longzhou’s pathogen, also located in southern Guangxi, has the greatest difference with others. So, the diversity was obvious for the *Ca. liberibacter asiaticus* from “hongjiangcheng”, and the pathogenic genetic differences were associated with geographical areas.

Preference and colonization of *Diaphorina citri* in frequently established backyard citrus in the Peninsula of Yucatán, México

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In México, citrus growers and the government face HLB through a regional management, focused in reducing *Diaphorina citri* populations in assigned regional areas (ARCOs). In Yucatan, a significant percentage of citrus is located in urban areas. So, ARCOs frequently fall over urban backyards, with presence of citrus uncommon in commercial orchards and coexisting with *Murraya* plants. In addition, *Murraya* sprout continuously, which could affect the *Diaphorina* timing control and performance of regional management. This research determined the *Diaphorina* adult feeding, egg-laying and development preferences in

eight citrus species common in backyard orchards. The experiment consisted in inducing simultaneous shooting in 10 plants of the following species: *C. sinensis*, *C. aurantium*, *C. paradisi* (‘Star Ruby’ and ‘Rio Red’), *C. aurantifolia*, *C. latifolia*, *C. limetta*, *C. limetioides*, *C. reticulata*, and *Murraya*. Once sprouts grew up to 3 cm, 2200 newly-emerged *Diaphorina* adults were released (1:1 sex ratio). The adult preferences were measured in the first hour, and 24 hours after being released. After 10 days, four shoots per plant were collected, and individual number and nymphal stage were determined. The experimental design was completely randomized in a factorial arrange (3 x 10). Experiment was replicated in March, July, and October. Collected data were submitted to analysis of variance from a series of experiments; the means were compared by Tukey tests ($p > 0.05$) with the program SAS 9.2. Experiments were different among them, since average temperature was different in every onset. *Diaphorina* adults preferred *C. aurantifolia* to feed on, while *C. aurantium* was the least preferred ($p < 0.05$). However, *Murraya* resulted in a higher egg laying preference; also nymphal development was higher. The grapefruit cv. Star Ruby was the citrus with least presence of eggs and nymphs. The obtained results prove useful to determine the best trap setting location for monitoring of *Diaphorina* in urban areas.

A model system for analyzing expression of *Ca. Liberibacter asiaticus* gene expression

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The pathogen *Ca. Liberibacter asiaticus* (CLAs) is the inferred cause of citrus greening, a threat to US citrus growth. CLAs cannot be grown in pure culture, which precludes usual approaches for functional genomic analysis. We aim to develop a synthetic biology system for assaying major CLAs regulatory proteins. This could facilitate high-throughput *in vivo* screening of molecules to reveal novel lead bactericide compounds active on CLAs. The closest bacterial relative to *Liberibacter* is the benign nitrogen-fixing symbiont, *Sinorhizobium meliloti*. The core RNA polymerase (RNAP) of the two organisms is very similar: we anticipate that all CLAs transcription factors will function in association with *S. meliloti* RNA polymerase. We propose to use the model *S. meliloti* system to provide “read-out” of function for the CLAs RpoH σ factor and a set of transcription factor proteins, then use these in a high-throughput screen to seek antibiotics that are specific to *Liberibacter*. We constructed a unique *S. meliloti* mutant that lacks all secondary RNAP sigma factors, which will be an ideal recipient to express CLAs genes. A synthetic *La rpoH* gene was designed with optimized codon usage and ribosome binding site for expression in *Sinorhizobium meliloti*. The complete DNA for the gene was synthesized using Invitrogen’s GeneArt Gene synthesis, and this synthetic DNA was cloned into variants of the expression plasmid, pSRK-Tc; optimal cloning was achieved using versions with alternate resistance markers (Km, Gm). The corresponding *S. meliloti rpoH* gene was cloned into the same vectors and will be used as a control for sigma factor activity. The constructs are being sequenced to assure fidelity of the cloning.

Seasonal variation in ‘*Candidatus Liberibacter asiaticus*’ titers in new flushes from citrus trees growing in locations with distinct climates

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The Brazilian citrus belt extends from south to north and northwest São Paulo State (SPS) plus the Triângulo Mineiro (TM) region of Minas Gerais State. Significant variation occurs between the climates of each region and seasonally within the regions. Historically, summer is hotter and the winter drier in the northern region of SPS. HLB is present in all regions but less prevalent in the extreme southern and northern regions of SPS. To determine potential association between local climate and ‘*Ca. Liberibacter asiaticus*’ (Las) titers in new flush growths, qPCR was used for 1.5 years to periodically assess pathogen titers in graft-inoculated trees under screened houses (SH) in 4 SPS locations (south, central, north, and northwest), or in naturally-infected mature caged trees in farms in Analândia, central SPS, and in Frutal and Comendador Gomes, within the TM region. Dataloggers were used to record local

ambient air temperatures hourly. In the lab, five adult *D. citri* were caged for 48 hours on each of the 25 to 50 flushes sampled from each location and evaluation date. Flushes and insects were then individually processed for qPCR analysis. During evaluations, air temperatures varied from 4.8° to 40.1°C inside the SH and from 4.2°C to 48.5°C inside cages. Average Las titers and Las acquisition rates were lower in flushes from caged (1.6 ± 0.4 log Las cells/g tissue; $9.7 \pm 5.5\%$) than from SH (2.8 ± 0.2 log; $23.2 \pm 4.4\%$) trees, and lower in Comendador Gomes (1.1 ± 0.4 log; $4.9 \pm 3.2\%$) than Frutal (1.5 ± 0.4 log; $8.1 \pm 3.8\%$) or Analândia (1.9 ± 0.3 log; $15.5 \pm 8.8\%$). Multiple regressions showed high association ($F = 25.0$, $R^2 = 0.85$, $p = 0.0027$) between Las titers in caged trees and climate parameters registered in the last 7 days before sampling, namely, the number of degree-hours below 20°C or above 30°C and the amount of rain. High positive association ($R^2 = 0.89$) was also observed for Las titers from caged trees and rate of Las acquisition by *D. citri*.

A census-travel predictive model for introduction sites of HLB to inform and optimize detection surveys

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Globalization has increased long-distance human-mediated pathways for invasive disease introduction. Detection of *initial introductions* of exotic pathogens/pests is challenging because they occur in very low incidence. Optimal probability of eradication/mitigation depends on early detection prior to spread. The earlier the detection, the more likely the pathogen can be eliminated or the epidemic slowed, lessening impact over multiple years. To find point introductions across a broad geographic landscape of mixed agricultural/residential areas requires substantial manpower and fiscal resources. Point introductions often go undetected for prolonged periods until incidence exceeds the lower threshold of sampling sensitivity. The Census/Travel model utilizes probable pathways, parses regions into smaller areas (census tract/zip code), and predicts the most likely locations in a given geographic area for introduction. The model's geospatial method uses US census and international travel data combined with a pathosystem's epidemiological characteristics, i.e., latency; detection sensitivity, reliability of confirmation, reproductive rate, environmental suitability, dispersal rate, ease of control, etc. Combining existing foreign population habitat and international pathway data, the model generates a risk index map to identify locations with the highest introduction potential. The risk map is linked to a survey optimizer that calculates the number of samples to be taken in a given area based on risk, and estimates manpower and fiscal requirements. It also ranks foreign countries by their relative contribution to risk of disease introduction. Risk maps were generated for HLB and other pathogens/pests. For prior disease introductions in Florida with identified distributions, the model performed well and validated known points of introduction. The census/travel model is being integrated into existing risk-based model platforms to optimize early detection surveys in California, Texas, Arizona, in Florida to optimize disease intervention/control. The model is independent of pathosystem, and can be extended to all States to predict introductions of human, animal, or plant diseases/pests.

CHMA design and construction for Central Valley in California

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Citrus Health Management Areas (CHMAs) facilitate the coordinated control of ACP populations, the clean-up of abandoned groves, and the removal of infected trees. Growers cooperating within a CHMA have been effective in suppressing ACP populations and slowing the spread of HLB in FL, indicating CHMAs are a viable management strategy. It has been confirmed that ACP populations are decreasing where coordinated spray efforts have been implemented in commercial groves in FL. However, the boundaries of CHMAs in FL are not optimized in size, but constructed primarily on arbitrary boundaries with an attempt to combine resources and garner neighbor participation. There are other various relevant factors, such as urban population size, abandoned groves' acreage, ratio between commercial citrus and residential area,

will have a significant contribution to CHMA performance. Improving from CHMA design deficiency in FL, we intend to construct CHMA boundaries for Central Valley based on estimated HLB/ACP risk level under 1-mile² grid (also call STR, Section-Township-Range of public land survey system) resolution. Where mixed landscapes exist, an optimal mix of residential and commercial landscape is considered so that regional disease management decisions can be implemented more effectively. Through cluster analysis and spatial statistics, we have developed maps that organize plantings based on the spatial pattern and dynamics of ACP populations and HLB risk. K-means clustering method is preferred to construct CHMA as homogenous as possible for risk. The CHMA size and locations (e.g., total citrus acreage, grower number) are also optimized for cost-effective management. Through thousands of simulations and optimization, 28 CHMAs are currently proposed for California Central Valley, which explains more than 55% of the total risk variance. For super large CHMAs, sub-CHMAs can be further constructed with approximate balanced between acreage and ownership. A similar methodology can be applied for CHMA construction in other citrus producing areas or states (i.e., Southern CA, TX, and AZ).

Modeling ACP spread within mixed residential and commercial landscape

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Citrus Huanglongbing (HLB), spread by the psyllid vector, is a devastating disease threatening nearly every citrus producing area worldwide with the exception of Australia and Mediterranean countries. The recent finds of ACP in California central valley emphasize the urgency for regulatory intervention and disease control, as this would pose a major threat to the viability of the citrus industry. Increasing ACP incidence and risk are considered inevitable for the Central Valley. A spatial explicit ACP simulation model is developed with attempt to understand the dynamic of ACP population changes, in particular, how ACP spread in a mixture of residential and commercial citrus landscapes in the Central Valley. A 16 mile² area of Porterville, CA is used as the baseline for ACP spread simulation. The mathematical model considers the following parameters for ACP progression: psyllid life span and mortality, net reproduction rate in natural environment, psyllid dispersal distance, citrus host type and density, new flush production, and effect of different spray schemes. An initial ACP population was introduced in one/multiple locations before the simulation. We then modeled factors influencing ACP population variation and interaction with new flush availability in relation to temperature (e.g., seasonal effect) for a period of 3 years using a daily time step. ACP spread occurs more frequently and faster within commercial citrus clusters, but comparatively slower for low density or well separated residential areas. This study also evaluates the behavior and distribution of ACP by subjected to different spray strategy scenarios. A comparison between simulation outputs confirms that the synchronize rate for coordinated spray plays an important role in slowing ACP epidemic development. Besides justifying the benefit of large-scale disease management, the outcome of this simulation model can also quantify the influence of input epidemiological parameters on ACP development, and can assist management decision-making by running specified scenario-based analyses.

Demographic and socioeconomic influences on citrus species preference: Influence on residential tree populations and potential influence on ACP population and HLB epidemics

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Powerful computational tools have enabled us to learn/extract patterns of residential host plant distribution. To a large extent, residential citrus biodiversity/choice is influenced by both the physical environment and preferences of household for specific host types. An understanding of social preferences for dooryard citrus tree is critical to residential citrus host density mapping and modeling. Dooryard citrus preferences are heterogeneous and far from random, where certain factors may be able

to explain part of the variation in preference. In addition to local climatic and environmental factors, we postulate that a range of demographic and socioeconomic characteristics can also affect the residential preferences for citrus types. Based on residential HLB/ACP survey from 2013/01-2014/05, about 102,404 properties with dooryard citrus in seven counties (focusing on Los Angeles) of southern California was visited. Dooryard citrus tree distribution and associated ACP population counts were observed for each property during the survey. Principle component analysis was used to characterize the variation of dooryard citrus distribution. Subsequently, we evaluated the relationship between demographic and socioeconomic factors (obtained from 2010 US census database) and citrus tree preference using generalized linear model regression where statistically appropriate, and evaluated the fit of the relationship to determine the degree to which variable has significant association with preferences. Lastly, we summarized the spatio-temporal pattern of ACP finds (including nymphs) in each citrus host type, and quantified the effect of citrus host on ACP tolerance. Specifically, lemon and lime dooryard trees are most preferential for ACP, followed closely by orange and mandarin trees. Grapefruit was the least preferred by ACP (3-fold difference compared to lemon and lime) of all citrus hosts studied. Determination of dooryard citrus host preference rating is a necessary step towards a comprehensive understanding of the ACP spread in residential areas and its influence on HLB epidemics.

Assessment of Citrus Health Management Areas and their performance in Florida

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Huanglongbing (HLB) is considered the most devastating disease of citrus worldwide, and the Asian Citrus Psyllid (ACP) is the vector responsible for HLB spread and epidemics. ACP was first found in South Florida in 1998 and rapidly spread across all citrus producing areas in the State. Citrus Health Management Areas (CHMAs) have been created to coordinate area-wide spray efforts among neighboring growers to reduce ACP populations and slow the spread of HLB in Florida. Currently, 48 CHMAs have been established covering more than 480,000 acres of commercial citrus. ACP populations are monitored for approximately 20% of total citrus (e.g., 4,500 ~ 5,200 citrus blocks) on a 3-week interval via a Multi-Pest Survey (MPS) program. The structured MPS program was established in August 2011 and since its inception there has been more than a 60% reduction in ACP counts during peak season in the monitored area. Spatially interpolated ACP population density assessments from the MPS are used to estimate ACP density for all non-surveyed locations during each cycle, which is then summarized at a 2 km grid resolution for optimal visualization. This allows growers to monitor sudden ACP density changes at a fine spatial resolution. By investigating ACP changes through all cycles, a new rating system has been designed for benchmarking and analysis of performance characteristics of all individual CHMAs. Also, various relevant factors, such as urban population size, abandoned grove acreage, ratio between commercial citrus and residential area, are analyzed to quantify their influence on CHMA performance. Utilizing these CHMA performance measures, CHMA leaders can tailor their management strategies for improved control. In addition, analytical results can be utilized to further optimizing CHMA size, location, and design, and assist future MPS inspections by redesigning sampling protocols to target survey efforts in areas with high ACP variability.

Host plant influence on Asian Citrus Psyllid titer and transmission efficiency of *Candidatus Liberibacter*: A proteomic analysis

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Interactions between plant hosts and insect vectors are well studied at the molecular level from the perspective of the plant response. However, almost nothing is known regarding the impact of host-insect interaction on the insect vector and the vector's ability to transmit plant pathogens.

Recent work from the Cilia lab showed that aphids reared on turnip were less efficient at transmitting potato leafroll virus compared to aphids reared on *Physalis*. The difference in transmission efficiency was correlated to differences in the expression of digestive proteases within the insect gut. Previously, the Hall lab showed that titers of the bacteria *Candidatus Liberibacter asiaticus* (CLAs) in Asian citrus psyllids (ACP), the vectors of CLAs associated with citrus greening disease, are lower when reared from *Murraya paniculata* than in psyllids reared on infected *Citrus sinensis*. To understand the biochemical basis of the difference in transmission efficiencies in the ACP, two sibling colonies of healthy ACP originating from the same population were simultaneously reared on healthy *C. sinensis* and healthy *M. paniculata* for three generations. Protein profiles of these sibling synchronized ACP colonies were analyzed via 2-D difference gel electrophoresis (DIGE) and high-resolution mass spectrometry and quantified. Data analysis is in progress. These complementary approaches will reveal differences in protein amounts as well as changes in post-translational modifications between insects adapted to the different host plants to gain insight into differences in their vectoring capacity. Our future plans include measuring proteins of interest in CLAs-infected insects using targeted mass spectrometry, localizing these proteins to specific insect tissues using fluorescent *in situ* hybridization, and testing for their function using RNA interference.

Vibration lures for monitoring and mating disruption of psyllids in citrus tree canopies

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Methods have been developed to attract male Asian citrus psyllids with microcontroller devices operating vibration sensors that detect male mating calls and piezoelectric buzzers that send out female-mimic vibrations in reply. The devices are in early stages of field testing to monitor and/or trap psyllids walking and feeding in citrus tree canopies in experimental orchards at the University of Florida. Preliminary studies indicate that the male call detection algorithm used by the microcontroller works well when wind and other background noise is low, but has difficulty in detecting calls when wind levels exceed 5-10 miles per hour. Frequency filtering and correlation analyses are being tested as potential ways to increase the efficiency of call detection in low to moderate levels of background noise. Efforts also are in progress to decrease the power usage so that the traps can remain in the field longer between battery replacements, to improve discrimination of psyllid calls from background noise, and to store signals on SD memory cards for later recall and analysis. In addition, studies are in progress to develop methods of interfering with psyllid mating communication.

New orchards of Mexican lime (*Citrus aurantifolia*) in a scenario of high incidence of Huanglongbing

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In the state of Colima, Mexico, the presence of Huanglongbing (HLB) was detected in the orchards of Mexican lime (*Citrus aurantifolia*) in April 2010. HLB is currently present in all citrus areas of the state with an incidence of nearly 100%. The planting densities used in orchards of Mexican lime are low with an average of 200 trees per hectare. Also, HLB is causing a yield reduction and it is required to improve the crop management in order to maintain the profitability of the plantations. Under this scenario with HLB, the question is whether it is appropriate to establish new orchards of Mexican lime? To answer this question, two experiments were established in April 2012 for testing high planting densities and new lime production systems. One experiment includes the Mexican lime varieties 'Lise' and 'Colimex', and also the planting densities with 313, 417, 556, 667, 800, 833, and 1,250 trees per hectare. The second experiment includes planting on a ridge, plastic mulching, and drip irrigation, with three different planting densities (800, 833, and 1,000 trees per hectare). The variables recorded were growth and fruit production. The results obtained 12 months after planting show that 100% of the trees have symptoms of HLB. However, trees also show a

growth that almost resembles that for healthy plants of these Mexican lime varieties in the state of Colima. The lime trees have started the production stage. The higher yields were 15 tons per hectare in 2014 and the highest planting densities yielded 20 tons per hectare. Nowadays, some treatments with planting densities using ridge, mulching, and drip irrigation show good performance and they can be an option for the establishment of new plantations of Mexican lime in the presence of HLB.

Three-year results of the area-wide management program for the Asian Citrus Psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) in southern Sonora, Mexico

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Since its detection in southern Mexico in 2002, the Asian Citrus Psyllid (ACP) *Diaphorina citri* Kuwayama, has dispersed rapidly to all citrus production areas in México. This insect vectors the most destructive citrus disease known as Huanglongbing or HLB, which is now present in 250 municipalities of 16 citrus production states in Mexico. Most damaged plants are found in commercial groves of Colima, Nayarit, Jalisco, Michoacan, and Sinaloa. In Sonora, ACP has been present since 2006, but HLB has not been detected up to now. A proactive action plan was implemented in the winter of 2010 in order to reduce the ACP population and risk of HLB introduction to Sonora. Actions include ACP and HLB monitoring, two area-wide insecticide applications; one in the dormant stage (February-March) another in the fall (September-October). During the growing season, if monitoring indicates that an orchard is over the regional ACP population mean, it is considered as hot spot and recommended for control. Monitoring and control of ACP is also performed on rural and urban areas. Introduction of citrus or ACP host plants from other states to Sonora is prohibited and revision points are established for this action. We have a great participation of growers which have decided to support the strategic plan by a self-initiative of warranting the cost of one application for those that do not spray during the area-wide period of control. Information is provided to housewives, gardeners, and general public through bulletins, pamphlets, newspapers, and radio and television programs. Three-year cumulative data obtained by tap sampling of all orchards in this region were statistically analyzed, and results indicate a significant reduction of ACP populations since 2010; the lower slope values through the years reflect the impact of the management actions on ACP populations.

Color morph and infection with *Candidatus Liberibacter asiaticus* influence flight capability and dispersal of the Asian citrus psyllid

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In the context of a vector-borne pathogen system, the spread of disease depends on movement of vectors across a landscape. We examined the flight capability of the Asian citrus psyllid (*Diphorina citri* Kuwayama, Hemiptera: Liviidae) with a laboratory flight mill. *D. citri* is the vector of the bacterial pathogen *Candidatus Liberibacter asiaticus* (CLAs) potentially causing Huanglongbing or citrus greening disease. Initially, we compared the flight capability of *D. citri* depending on their sex and morphotype (color of abdomen). Two general morphotypes exist within this species: green/blue and gray/brown. A total of 84 psyllids were tested and we observed that approximately 32% of psyllids from the green/blue morphotype tested exhibited long durations of flight (>60 s); whereas, less than 5% of psyllids from the gray/brown morphotype performed such long duration flights. There was no difference in flight performance between the two sexes within both the gray/brown and green/blue morphotypes. We measured pronotum width and wing length. However, neither pronotum, nor wing size, were associated with flight capability of *D. citri*. Subsequently, we tested if psyllids infected with the plant pathogen, CLAs, were more prone to flight than uninfected psyllids. A total of 66 psyllids were tested and we observed that 58% of the green/blue psyllids infected with CLAs performed long durations of flight, while only 25% of uninfected psyllids performed such long duration flights. Finally, we performed a third experiment where psyllids were allowed to settle on plants for 3 days, and subsequently allowed to

disperse to a newly introduced plant during the next 4 days. In this case, we observed that CLAs-infected psyllids were more prone to disperse than uninfected psyllids. Greater propensity for dispersal by psyllids infected with CLAs than uninfected counterparts may indicate host-phenotype manipulation by the pathogen. By increasing dispersal of its vector, the pathogen may be manipulating vector behavior to increase its own proliferation.

Absence of windbreaks and solid set plantings increase density of Asian citrus psyllid in citrus

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Densities of an herbivorous pest might be impacted by landscape and grove architecture. We present two field experiments where the densities of the Asian citrus psyllid were compared depending: (1) on the presence or absence of windbreak and (2) if the groves consisted of a solid set of plantings or a grove with a mixture of mature and reset trees. (1) Psyllid abundance was measured on the edges of five groves. The factor investigated was the presence or absence of a windbreak. For the five groves, we observed significantly fewer psyllids on the edges of groves with windbreaks as compared to those without windbreaks. We found no significant difference in the number of natural enemies between the edges with or without windbreaks, suggesting that windbreaks do not affect densities of psyllid natural enemies. (2) During two consecutive years, we compared the densities of psyllids on reset trees planted in a solid set plantings (all trees planted within the last 2 years) versus the densities of psyllids on resets (trees planted within the last 2 years to replace mature trees) present within mature groves. This was conducted in four groves and among three citrus varieties. More psyllids were found in the solid set plantings as compared to the resets within mature groves.

HLB on a small scale - What can we learn from a “model” system?

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Here we demonstrate that exposing very small grafted citrus trees to free-ranging ACP and CLAs inoculum in the greenhouse can mimic results seen in the field. Two experiments to quantify the incidence of CLAs infection and HLB-symptom severity during exposure to free-ranging ACP and CLAs-infected inoculum plants were conducted. The first experiment included a diverse group of 16 citrus types. Genotypes included in the experiment were selected based on commercial significance in Florida, purported resistance to HLB (positive or negative), or both. During 45 weeks in the greenhouse, the incidence of CLAs infection increased from none detected (100% of initial samples) to 70% after 45 weeks. Frequency distributions of CLAs titers (Log copy number) after 45 weeks in the greenhouse and for 20,000 samples collected by field scouts were very similar. Among the 16 genotypes, we were able to detect statistically significant differences in the “severity” of CLAs infection. The objective of our second experiment was to determine the effect of duration of exposure to ACP on CLAs infection and HLB symptom severity. Three hundred CLAs negative ‘Valencia’ sweet orange grafted onto US 812 were placed into an ACP inclusionary greenhouse. At 2-month intervals, groups of 60 plants were removed from the inclusionary greenhouse, treated with insecticide, and then placed into an ACP exclusionary greenhouse. Proportions of CLAs positive plants at the time of transfer were 0.02, 0.12, 0.18, 0.30, and 0.95 following exposure to ACP for 2, 4, 6, 8, or 10 months, respectively. At 11 months after initiation of the experiment, proportions of HLB-symptomatic plants were 0.08, 0.10, 0.20, 0.25, and 0.37 for plants that had been exposed to ACP for 2, 4, 6, 8, or 10 months, respectively. Our results indicate that meaningful results can be obtained using this model system to study HLB.

The development of a babaco papaya – *Liberibacter crescens* interaction as a model system for citrus greening disease

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Progress on our understanding of citrus greening disease has been hampered by our inability to culture the pathogen, *Liberibacter asiaticus* (Las). The closest cultured relative of Las, *Liberibacter crescens*, was recently characterized and found to be very similar genetically and physiologically to Las. It is still unknown why *L. crescens* is culturable and Las is not. It is also unknown whether *L. crescens* causes disease on any plant host including the plant from which it was isolated, babaco papaya hybrid (*Vasconcellea* × *heilbornii*). Our objective is to infect babaco papaya with *L. crescens* BT-1 to determine whether this bacterium causes HLB-like symptoms on this host. If *L. crescens* causes HLB-like symptoms, the babaco papaya – *L. crescens* interaction would serve as a reasonable model system since both the host and the bacterium are genetically tractable with relatively small genomes. In addition, mutations in every non-essential gene of *L. crescens* are now available to explore this interaction. If *L. crescens* does not produce HLB-like symptoms yet is able to colonize the babaco host, we aim to identify genetic differences between Las and *L. crescens* that may contribute to the pathogenicity of Las. Experiments to infect babaco papaya with *L. crescens* will be described that include pressure infiltration and transmission through papaya leafhopper and dodder. The relative merits and success of each approach will be described as well as the amount of symptomatology and infection observed with each.

Embedding epidemiology and technology in their socio-economic context to assist with strategic planning of HLB management

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The continuing impact of HLB on citrus production globally in infected regions, and the potential impact in as yet uninfected ones, has highlighted the need for epidemiologists to consider social factors when predicting the risks of new infections and potential responses when infection is confirmed in new areas. We discuss several issues where socio-economic considerations are already important, or are likely to become so, in current efforts to manage HLB in US citrus production and efforts to develop novel methods of disease management. Before the arrival of an exotic, invasive disease such as HLB, an emphasis is given to risk assessment, prediction of initial sites of entry, and effective surveillance. In each of these activities, traditional epidemiological analyses have much to offer; but, in studying the spread of HLB and other high profile plant diseases, it has become apparent that social factors such as transport between immigrant populations and their traditional population centers, overall volume of human traffic into a region, among others, can be indicators of high risk of disease arrival and have useful predictive value for locating early infections. Similar social factors, and a range of indicators of economic activity (both in the legitimate and gray economies) and transport intensity, all help to define the risk of disease spread within an area; essentially by accounting for the capacity of human activity to vector the disease. When combined with traditional epidemiological analyses, inclusion of these factors in models of HLB risk accounts for a high proportion of the explainable variation and allows the design of efficient survey plans for the disease. After arrival of HLB/ACP in a citrus production region, the need is obvious for technologies and practices which suppress the vector population and circumvent or mitigate the impacts of the disease on production. Experiences in the US suggest two particular groups of socio-economic factors are (or are likely to be) important in determining the success of these efforts. Within the first group are factors which determine the capacity for growers to cooperate in area-wide vector population management. We discuss lessons which can be learned from the experience of establishing Citrus Health Management Areas (CHMAs) in Florida in response to the rapid spread of HLB, and ongoing efforts in California to establish a comparable system for coordinating vector suppression in advance of HLB being found in commercial citrus. The second group of factors determines grower and public attitudes to genetic modification. Various GM approaches to

improve management of ACP or mitigate the effects of HLB on citrus production are being investigated. If the sustainability of citrus production is dependent on use of genetic modification to overcome the threat posed by HLB, the social acceptability of such technology, both to growers and to the public in domestic and international markets, will be vital in determining the potential for use of the technology. We discuss the prospects for use of GM solutions in light of evidence from other crops and the known epidemiology of HLB.

Effect of internal and external inoculum control practices on HLB epidemic progress in a commercial citrus grove

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Huanglongbing (HLB) is difficult to control because the primary infections by infective immigrant Asian citrus psyllid (ACP) are not totally prevented by rigorous vector control and removal of HLB-symptomatic trees only within the grove. This study was conducted in a 400-ha sweet orange grove planted in 2007 in northeastern São Paulo state, Brazil, surrounded by coffee farms. Even with six annual insecticide sprays, the disease incidence increased from 0.2% in 2011 to 1.0% in 2012, and 1.1% in the first 5 months of 2013. Changes in internal HLB management after that time included increased training of personnel for ACP monitoring, repositioning of yellow sticky traps at the grove edges, and reducing spray volume to allow for 10-day spray frequency. External to the grove, inoculum assessment and actions on neighboring properties with citrus and *Murraya* trees were initiated. Twenty-six non-commercial sites with 1,261 HLB-affected trees were discovered within a 10-km radius from the grove. Seven hundred and sixty-one trees were eradicated after negotiation, including replacing the citrus with other fruit trees, monthly supply of orange or other services provided by the grove owner. ACP monitoring and insecticide sprays were started with the neighbors' permission where tree eradication was not permitted. *Tamarixia radiata* was released during vegetative flushes in sites far from the grove or in residential areas. The result of this inoculum management program was initially observed in the second semester of 2013, when removal of HLB-symptomatic trees was 46% lower than during the same period in 2012. As of July 2014, the disease incidence was 0.4% compared to 1.3% in 2013 and 0.5% in 2012. This study confirms the importance of non-commercial HLB-infected trees without ACP control in promoting HLB epidemics in commercial citrus groves and how efficacious removal of neighboring trees or at least controlling the ACP on those trees is for prevention of new primary infections in citrus commercial groves.

Early detection of Huanglongbing using mass spectrometry-based proteomics and transcriptomics

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Citrus trees infected with Huanglongbing (HLB), associated with infection by *Candidatus Liberibacter asiaticus* (CLAs), typically have no visible symptoms for the first months of infection, making it difficult to distinguish between healthy and infected citrus trees during this time. Previous attempts at early detection used real-time, quantitative PCR for amplification and detection of CLAs 16s rDNA, but the success of this approach depends on the presence of a sufficiently high titer of CLAs in the host tissue sampled. Measuring systemic changes in plant proteins in response to HLB offers an opportunity for early detection of HLB infection that does not rely on localized detection of the pathogen. Using tandem mass tag (TMT) labeling and high resolution mass spectrometry, we constructed proteomic profiles of HLB-positive Lisbon Lemon over the first several months of infection. At the UC Davis Contained Research Facility, Lisbon Lemon trees were grafted with HLB-positive or healthy tissue, and leaf samples were collected at time of grafting and every 2 weeks thereafter. Extracted protein samples were multiplexed using TMT labeling for high-throughput analysis, and then analyzed by high-resolution mass spectrometry. Proteins differentially expressed

between control and infected samples were identified at each time point. A drastic change in protein response between control and infected lemon trees was measured at week 10 post-inoculation, although changes could be detected as early as 2 weeks post-inoculation. Samples from the same set of trees were analyzed by RNAseq to generate an annotated database of expressed genes and predicted proteins to facilitate analysis of proteomic data. These complementary methods will provide insight into the correlation of transcripts and protein changes over time. Ultimately, HLB early detection data generated using the TMT strategy will be useful for data mining and machine learning, which may further improve early detection efforts in field collected samples.

Evaluation of three releases densities levels of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) for the control *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)

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Asian Citrus Psyllid (ACP), *Diaphorina citri*, is one of the most important pests in citrus production worldwide because it is a vector of a bacterium associated with Huanglongbing (HLB) or citrus greening disease. ACP was found in California in 2008 in San Diego County, and in 2012, HLB was found in Southern California in Los Angeles County; after this initial detection, no other have been detected. Control the ACP population is one of the most important strategies to prevent disease spread. The strategy to control ACP includes chemical and biorational insecticides and biological control. *Tamarixia radiata* is an effective ectoparasitoid of the ACP nymphs imported from Punjab Pakistan. Since 2012, more than 750,000 *Tamarixia* have been released throughout Southern California – mostly in residential areas. The implementation of biocontrol in commercial citrus is just getting going and it is not clear how viable a strategy it will be. *Tamarixia radiata* females preferentially parasitize fourth and fifth instars of ACP. In addition, host feeding on younger nymphs has been observed. We evaluated ACP parasitism and establishment of *T. radiata* in a commercial Navel field in Southern California using three levels of release intensity. An orchard in Mentone, CA was chosen, divided into three blocks, and one of the tree levels of infestation assigned. *Tamarixia* was released every week for 6 weeks in a ratio of 1:3:9 for low, medium, and high. Every 2 weeks, we monitored ACP populations and parasitism rates in each of the three blocks. *Tamarixia* parasitism was determined in the laboratory from 20 terminals. The ACP pressure was low and only first and second instar nymphs were detected for low and medium release treatment and very low levels observed in high. No fourth and fifth instar nymphs or parasitism was observed in any of the treatments.

Biological control of the Asian Citrus Psyllid in California

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In 2013, the California Department of Food and Agriculture took over lead responsibilities for producing and releasing *Tamarixia radiata* in all locations of California where ACP is present and eradication is no longer being attempted. More than one million *T. radiata* have been released in the seven southernmost counties of California; an area of 40,000 square miles. Eighty percent of releases are carried out in a grid pattern, each release site three miles apart, in urban areas. The remaining 20% of releases are made in organic, unmanaged, and abandoned citrus groves. Releases of a second biological control agent *Diaphorencyrtus aligharensis* started in 2014. This presentation describes facilities, production methods, and release strategies used by the state-operated ACP biological control program in California.

Projecting the potential climate suitability of HLB using a presence-only model (MaxEnt)

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The species distribution model MaxEnt was used to predict the global and local potential distribution of HLB. This model needs the present

distribution of a ‘species’ and long-term climate data to make predictions for new areas. The current global distribution of HLB was gleaned from online databases, literature review, and personal communications with experts. The long-term climate data which included 19 bio-climatic variables were sourced from the CliMond website. Preliminary results showed that the model successfully predicted Florida as highly suitable for HLB establishment, although we did not use HLB occurrence in Florida. This finding is in agreement with the rapid spread and current distribution of HLB in this region. The model also predicted that limited areas in California are climatically favorable for HLB establishment, but the probability of establishment was predicted to be much lower compared to Florida. To increase the confidence regarding the model projection, a Principle Component Analysis (PCA) was performed to investigate the climate similarity of regions with proven HLB presence with the California climate. PCA analysis showed that the climate in areas around Los Angeles overlapped with the climate of regions where HLB is currently present. On a global scale, HLB predictions from MaxEnt combined with expert knowledge could be informative for countries such as Australia, New Zealand, and European countries, where HLB has not been reported thus far.

Effect of infection with CLAs and/or infestation with Asian citrus psyllid on phytohormonal balances in citrus

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Huanglongbing (HLB), is a destructive disease of citrus causing great losses in citrus industries worldwide. HLB is caused by *Candidatus Liberibacter asiaticus* (CLAs) which is transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri*. The infection with CLAs and/or the infestation with ACP affect the citrus phytohormonal balance. We investigated the effect of infection with CLAs and/or infestation by ACP on citrus phytohormonal profile. Studied phytohormones groups include: (1) auxins (AUs), i.e., indole-3-acetic acid (IAA), indole-3-carboxylic acid (ICA), indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), and their methyl esters; (2) salicylates (SAs), i.e., salicylic acid (SA), benzoic acid (BA), cinnamic acid (CA) and their methyl esters; (3) jasmonates (JAs), i.e., *trans*-jasmonic acid (*t*JA), *cis*-jasmonates, i.e., jasmonic acid (*z*JA) and their methyl esters; (4) abscisic acid (ABA); (5) Cytokinins (CKs), i.e., *trans*-Zeatin (*t*Z) and *trans*-Zeatin riboside (*t*ZR); (6) Gibberellins (GAs), i.e., Gibberellic Acid (GA3), Gibberellin A4 (GA4), and Gibberellin A7 (GA7); In the current study, we reported differential responses to the infection with the bacteria and the infestation with the vector. We identified a large number of hormones that increased or decreased as a result to the infection with the bacteria and/or the insect. A better understanding on how citrus phytohormonal profile responds to the attack with CLAs and/or ACP, will lead to development new strategies to help control the disease.

Novel tetracyclines designed specifically for activity against the causative agent of HLB

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Oxytetracycline has been used extensively for over 60 years in agriculture for both plants and in animal health. The tetracyclines as a chemically diverse family of compounds have multiple activities across many organisms. Within this large family of compounds, a select subgroup has discreet and potent activity against α -proteobacteria, including CLAs, while demonstrating inactivity against human bacterial pathogens labeling them as “non-antibiotic tetracyclines” a designation accepted by the FDA. Our efforts in tetracycline semi-synthesis have produced three separate series of compounds found effective against the surrogate strain *Liberibacter crescens* and with increased potency, while in a study of over 35 derivatives distinct structure-versus-activity parameters have emerged guiding the further design and synthesis of compounds active against *Liberibacter* species. One series of positional derivatives was three orders of magnitude more active than oxytetracycline, while the others were at least two orders of magnitude

more active in this surrogate strain. The most potent compounds derived from the screening were examined in further studies of trans-bark uptake in young citrus trees, showing that proper formulation demonstrated transport throughout the plant and into the canopy. Further studies in HLB-infected citrus trees by bark or foliar application decreased PCR-based bacterial levels after one month of treatment while shoots collected from HLB-infected Valencia orange shoots showed significant repression of L10 and 16S mRNA levels. These preliminary results demonstrate that chemically-modified tetracycline derivatives are active specifically against HLB, lowering infection burden in whole plants, and are considerably more active than oxytetracycline. The case for novel and inexpensive compounds specifically designed for citrus plants harboring α -proteobacteria infections will be presented along with its potential to replace oxytetracycline for agricultural use.

Simulation of the impact of Huanglongbing in the production of citrus grove under disease management scenarios, disease incidences, and grove ages at the beginning of the epidemic

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Besides the availability of financial and technological resources, and citrus growers' awareness about the damages that mismanagement of the Huanglongbing (HLB) can cause to their and neighbors' groves, the decision of applying the Asian citrus psyllid (ACP) control and symptomatic trees eradication by citrus growers depends on an economic analysis of the benefits and costs of any measure adoption in the short (5 years), medium (10 years), and long-term (20 years). Although basic information about the temporal progress of HLB and damage in plants of different age, scion/rootstock combination, and under different HLB management still scarce, a macro of Excel (Microsoft) was developed to estimate and quantify the future impact of HLB in groves adopting different kinds of disease management. In this study, that macro was adapted to simulate the impact on production of citrus groves under different scenarios of HLB management (scenarios: A – without HLB management; B – without eradication of symptomatic plants and with ACP control; and C – with eradication of diseased plants and ACP control), grove ages at the beginning of the epidemic (2 to 10 years old), and initial incidences of HLB-symptomatic trees (0.01% to 10%). Considering that the assumptions and models were corrected, for high expected yield of healthy groves (average >1,600 90-lb boxes/ha in 15 years) and moderate annual rate of HLB incidence in scenarios with ACP control ($rG = 0.25$ by Gompertz model), it can be concluded that groves younger than 6 years old at the time of HLB detection will have higher expected productivity in any term and initial disease incidence only if managed with eradication and reset of symptomatic plants and ACP control. Older groves with lower initial disease incidence may have a medium-term productive survival ($\geq 1,000$ 90-lb boxes/ha), only controlling ACP enough to not allow the secondary spread of the disease. However, the impact on fruit quality of symptomatic trees kept in the field was not considered in this study.

Small RNA deep sequencing and transcriptome profile analysis to identify viruses in worldwide populations of *Diaphorina citri*

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The Asian citrus psyllid, *Diaphorina citri* Kuwayama, transmits *Candidatus* Liberibacter asiaticus, causal agent of the deadly bacterial disease called Huanglongbing or citrus greening. *D. citri* and *Ca. L. asiaticus* are well established in Florida, and have been reported from other states including Louisiana, Texas, and California; they pose a real threat to the entire US citrus industry. In order to discover known and unknown *D. citri* viruses which might be useful for virus-based biological control strategies, we constructed small RNA cDNA and RNA-seq libraries and used next generation Illumina-based sequencing for selected, worldwide *D. citri* populations from Taiwan, China, Brazil, and the US. Through deep sequencing analysis and *de novo* contig assembly, larger contigs were obtained. BLASTX and tBLASTx searches against the viral database available in GenBank suggested distinct viruses shared protein sequence similarity with Reoviruses, and with Picorna-like viruses from the genus *Iflavirus*. Subsequent RT-PCR and Sanger sequencing confirmed the presence of these viruses in some

but not all populations of *D. citri* investigated in this study. Here, we are reporting the first Picorna-like virus discovered in *D. citri*. However, our analysis suggested that, although encoded proteins show low (30-35%) but significant similarity to viruses belonging to the genus *Iflavirus*, the *D. citri* virus is not an *Iflavirus* and appears to be representatives of a new genus. We were able to build 70% of the genome of this new virus through bioinformatics analysis. The genome sequences were completed by filling up the gaps using RT-PCRs and specific designed primers. Both 5' and 3' UTR full-sequences were obtained using RACE strategies. Our intent is to engineer this virus and assess its effects on *D. citri*.

Proteomics analysis reveals novel host molecular mechanisms that could be associated with heat-induced resistance to '*Ca. Liberibacter asiaticus*' in lemon plants

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Sustainable long-term measures to combat HLB via breeding or genetic engineering methods are hampered by the fact that no true genetic resistance has been found in citrus germplasm. All cultivated citrus species or citrus relatives are susceptible to the disease. However, the degree of HLB susceptibility or tolerance varies among citrus species. There have been suggestions toward the identification of innate '*Ca. Liberibacter asiaticus*' (Las) resistance-associated molecular mechanisms in citrus plants for application in breeding or genetic engineering crop development programs. Furthermore, a recent study showed that a continuous heat treatment of 40°C to 42°C for a minimum of 48 hours was sufficient to significantly reduce Las titer or eliminate Las entirely in HLB-affected citrus seedlings. Plant exposure to one form of stress has been shown to serendipitously induce resistance to other forms of stress. In this study, we conducted proteomics analysis of heat-treated HLB-affected lemon plants, detected proteins that were markedly up-regulated only in plants that were simultaneously exposed to heat and Las. This suggests that heat treatment induces proteins in Las-infected citrus plants that could play an active role in the suppression of Las growth. Hence, this research demonstrates that: (1) the application of a proteomics approach to elucidate the molecular mechanisms involved in heat-induced Las-resistance in citrus plants and (2) the use of the information from proteomics analysis to develop genetically-modified Las-resistant citrus plants by altering host gene expression to mimic heat-induced conditions.

Nationwide survey and strain identification of Huanglongbing (HLB) disease of citrus in the Philippines

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A nationwide survey was conducted to come up with a comprehensive data on the extent of HLB incidence and strains, and its insect vector on Philippine citrus. A total of 120 citrus farms in the various citrus growing areas in the country were surveyed. Typical symptoms of HLB were found in varying degrees of severity in 57% of the farms surveyed. The typical HLB symptoms assessed include sectorial yellowing, mottling, small leaves with zinc deficiency symptoms, interveinal chlorosis, vein corking, lopsided fruit, inverted fruit coloration, and aborted seeds. Disease samples were collected and confirmed through starch Iodine test and Polymerase Chain Reaction (PCR). There was a good agreement between the visual assessment of symptoms and identification by iodine starch test and PCR. Besides HLB, the occurrence of Asian Citrus Psylla (ACP) was also noted through visual search and stem tap technique. ACP was noted in 12% of the farms surveyed. In some farms, however, no ACP was found although HLB symptoms were observed and disease spread is evident due to the presence of newly infected trees within the orchards. Disease samples were also graft-inoculated onto differential hosts to determine the HLB strains present in Philippine citrus. Based on disease index on differential hosts, Strain I and II are the predominant strains of citrus HLB found in the Philippines.

Salicylic acid mediated defenses in Huanglongbing pathosystem

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Citrus *Huanglongbing* disease is associated with three species of fastidious, phloem-limited, gram-negative α -proteobacteria. Two of them occurs in Brazil—*Candidatus Liberibacter asiaticus* (*CaLas*) and *Candidatus Liberibacter americanus* (*CaLam*). Despite the several studies that have been carried out, there is not an ultimate model which explains the mechanisms of evolution of the HLB. It is known that salicylic acid (SA) is the main plant hormone which triggers defense responses against biotrophic pathogens and that callose accumulation may be one important event regulated by this hormone. In this study, we performed a transcriptional time-course analysis of SA genes between two contrasting citrus genotypes—*Poncirus trifoliata* (tolerant) and *Citrus sinensis* (susceptible)—exposed to *CaLas* or to *CaLam*. Plants were inoculated with infected budwoods by grafting. Total RNA was isolated from leaf tissues harvested 7 and 30 days after inoculation and in symptom stage. We carried out real-time PCR for detecting gene transcripts and used the GenEx software for gene expression analysis. We also evaluated callose deposition in petioles of symptomatic leaves by means of aniline blue staining. Compared to other treatments, it was observed that *C. sinensis* infected with *CaLas* had more significant alterations in the profile of gene expression. We saw that genes upstream SA pathway such as NDR1 and PAD4 showed a slight upward regulation in sweet orange during late stage of infection. A SA methyltransferase BSMT1 was also upregulated. In contrast, genes downstream SA pathway showed a slight downward regulation. Callose accumulation was observed in citrus and trifoliata sieve tube elements (STE); however, collapsed STEs—which were observed only in citrus petioles—might have a more prominent implication in the symptomatology of highly susceptible plants. This results suggests that systemic defense responses mediated by SA are not fully activated during citrus-Liberibacters interaction.

Developing exchange/recombinase founder lines to introduce HLB resistance genes into Citrus

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We have designed an innovative system to deploy a novel pair of recombinase enzymes, namely *Bxb1* and *CinH*, for performing precise genetic engineering of citrus. They control the integration and the excision of sequences based on the presence and orientation of specific recognition target DNA sites allowing the precise integration of transgenes, with the simultaneous removal of marker genes and/or any other unneeded sequences. Their unidirectional activity ensures that the integration and excision events produced are non-reversible and stable, generating citrus lines with reliably and uniformly expressed introduced traits. The binary pTAG vector contains the double enhanced 35S promoter constitutively expressing the fusion gene *codA:nptII*, and a *DsRed* visual reporter gene expressed by GmUbi3 promoter, which are flanked by fused recognition sites, with the integration recognition sites (*attP* - for *Bxb1*) on the 5' side and the excision recognition sites (*Res* - for *CinH*) on the 3' side. The translationally fused *codA:nptII* selectable marker gene, provides both positive selection (via the antibiotic kanamycin in the medium, for which *nptII* provides resistance) and negative selection (via 5-FC, which is made toxic by *codA*). Single copy insertion lines containing TAG have been generated and will be tested for re-transformation with use of the negative selection to select against lines where the marker has not been properly excised. Epicotyl explants from Carrizo and Hamlin were transformed using *Agrobacterium tumefaciens* strain EHA105 carrying pTAG. A total of 335 candidate founder lines were characterized by Southern blot analysis to identify lines with a single copy of the recombinase platform in the genome. These founder lines will be utilized in developing transgenic plants with priority traits such as HLB resistance.

Production of mandarin + *Citrus latipes* somatic hybrid citrus rootstocks with potential for improved tolerance/resistance to citrus greening

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Citrus greening or Huanglongbing (HLB) is one of the most destructive diseases of citrus worldwide and now is threatening the survival of the citrus industry in Florida. The causal agent of HLB in Florida is *Candidatus Liberibacter asiaticus*. The search for alternative citrus greening resistant rootstocks that also withstand the other constraints is now considered an urgent priority for a sustainable citrus industry. The production of somatic hybrids allows accumulating all the dominant traits of both parents, irrespective of their heterozygosity level, and has proven to be an important tool for rootstock improvement. We have developed a new embryogenic callus line and suspension culture of the seedy and polyembryonic ‘W. Murcott’. *Citrus latipes* (papeda) is monoembryonic and has been purported to exhibit tolerance to HLB in other countries. Somatic hybridization of ‘W. Murcott’ mandarin hybrid with *Citrus latipes* was conducted by protoplast fusion of embryogenic suspension culture-derived protoplasts isolated from ‘W. Murcott’ mandarin with leaf protoplasts of *C. latipes*. Analysis of ploidy level by flow cytometry confirmed the regeneration of 26 new interesting tetraploid somatic hybrids from this combination. Furthermore, the morphology of the new putative somatic hybrids was intermediate between the parents, exhibiting the larger winged petiole and elongated leaf characteristics of *C. latipes*. Molecular markers analysis of the new somatic hybrids is underway to determine the allotetraploid and autotetraploid hybrids. The new somatic hybrids will be propagated to evaluate their horticultural performance and ability to mitigate or prevent HLB in grafted commercial scions. These potential somatic hybrid rootstocks should also have potential to control tree size due to polyploidy, and should be amenable to standard seed propagation via nucellar seed production. These hybrids will add new germplasm to the citrus rootstock breeding program addressing the HLB problem.

Oils and plant extracts to control the Asian citrus psyllid (*Diaphorina citri*) in Mexican lime in Mexico

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The Asian citrus psyllid (ACP) was detected 10 years ago affecting Mexican lime (*Citrus aurantifolia*) in the citrus growing area of Colima, Mexico, and 6 years later Huanglongbing (HLB) disease was found. At the present, HLB and its vector are the most important problems on this citrus specie. The main management strategy against HLB in Mexican lime orchards is based on agronomic practices and *D. citri* control. ACP control is based on the use of synthetic insecticides. The search for sustainable alternatives is a priority in the management of the vector. In this study, we report the effect of different oils and plant extracts against ACP. Six assays were carried out in trees heavily infested with the insect. One foliar application of each treatment was done on tagged shoots of 5–7 days old. Temperature during application of the treatments was about 30–32°C. Alive nymphs were evaluated before and after the application. Paraffinic oils (PureSpray Foliar 22E®: weight percent 98.3%, Anasef T®: 80%, and Saf-T-Side®: 80%), and petroleum oil (Citrolina: 67%) at dosages of 1.0%, showed a good control of APC. At 7 days after the application, the biological efficacy with all the oils was over 90%. Also, different cooking oils (corn, soybean, safflower, olive, and canola) at 1.0% reduced ACP population. The control efficacy was from 77 to 90%. Only the cooking oils caused a slight toxicity on the foliage of Mexican lime, which was manifested as slight browning leaves and defoliation. Seeds extracts of *Jatropha curcas* L., and *Azadirachta indica* (Juss) had an ACP nymph reduction of about 92.0%. Plant extracts of garlic (*Allium sativum* L.), onion (*Allium cepa* L.) and soursop (*Annona muricata* L.) showed a poor to regular control. The results indicate that the paraffinic oils and *J. curcas* and *A. indica* extracts can be included in programs for ACP control in Mexican lime.

Field cage production system for *Tamarixia radiata* in California

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Asian citrus psyllid (ACP) is well established in much of southern California. The area-wide application of pesticide treatments for ACP and host plant removal of HLB-infected trees is the current strategy for managing the threat of disease establishment in commercial citrus orchards. In ACP infested urban areas distant from commercial citrus production areas, area-wide applications of pesticides may not be feasible because of the size of the area needing treatment. It is in these areas, releases of specialized biological control agents that attack ACP nymphs are well suited as an additional treatment strategy. The goal of this project is to adapt and develop methods for a field insectary production system of *Tamarixia radiata* under California conditions. Adapted from work by USDA scientists in Texas, in this system, mature citrus trees are caged to confine ACP movement, pruned to induce fresh flush and ACP and *T. radiata* are introduced and managed for their production. Plant foliage with ACP mummies is harvested, brought to the laboratory, held for wasp emergence where they are collected and shipped for field releases. Field cages have been set up for testing suitability of various Citrus species, including lime, lemons, grapefruit, oranges and ornamental Rutaceae species curry leaf and orange jasmine. *Tamarixia radiata* have been successfully reared on all tested Citrus varieties. Environmental conditions prevailing during June through October are suitable for *Tamarixia* production under California conditions. A total of 118,642 wasps were produced from 19 cages in 2013. Similarly, 125,415 wasps have been produced from 20 cages in 2014. As more cages await parasitoid harvest, total wasp production for this year is expected to exceed 200,000. Establishment of *Tamarixia radiata* following field release and redistribution is expected to suppress ACP population eventually slowing HLB spread should the disease make its appearance in California.

The behavioral response of *Diaphorina citri* to ultraviolet light

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The Asian citrus psyllid (ACP) is responsible for transmitting greening or Huanglongbing (HLB) to citrus. It is considered the most debilitating disease of citrus worldwide. Currently, citrus growers rely on insecticides to control ACP. ACP is a dominantly visual insect and, as such, it is important to understand its visual behavior. Previous studies have shown that young trees planted on a bed covered with metalized polyethylene mulch reduced ACP populations and, consequently, incidence of HLB. However, the actual mechanism whereby the metalized mulch protects trees from ACP is not known. One hypothesis is that radiation of ultraviolet light from the ground confuses the approaching psyllid and disrupts flight. To test this hypothesis, we developed laboratory experiments to evaluate ACP response to UV light. We tested both light emitting diodes (LEDs) and monochromatic colored visual targets produced with narrow band pass filters. ACP were attracted to UV more strongly than to blue and red, but less strongly to yellow and green. UV LEDs emitting a wavelength of 375 nm were found to be the most attractive to ACP amongst a range of UV LEDs (355, 365, 375, 385, and 405 nm). There was no difference between male and female ACP in terms of their attraction towards UV. We hypothesize that metalized mulch, which reflects UV, could be disorienting to the ACP as UV light is in nature primarily found in the sky. These studies improve our understanding of ACP visual behavior and provide the basis for future studies.

Epidemic modelling and surveillance design for early warning of an HLB epidemic

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Early warning surveillance is crucial in areas that are not yet infected with Las or that have been newly planted with healthy trees. The success of HLB control in these areas relies on the detection of disease and instigation of mitigation procedures as early as possible in the epidemic. Control measures that are instigated too late may not be effective and will incur far greater control costs and disease-induced yield losses than epidemics that are caught early. Surveying and scouting is however expensive and surveillance programs necessarily cover large geographic areas, stretching fiscal and manpower resources. Understanding how to target resources across vast spatial areas is not trivial given the complex spatial distribution of plantings, vector dispersal patterns, interactions between residential and commercial citrus areas and unknown points of disease entry. Using state-of-the-art epidemic simulation methods coupled with geographic information systems (GIS), we incorporate this information and use it to simulate realistic spread patterns. By combining the epidemic models with stochastic optimisation algorithms, we are able to identify optimal 'smart-surveillance' programs that maximize the probability to achieve early warning based on the predictions of spread. We identify a number of general rule-of-thumbs to help inform optimal surveillance design. In particular, we illustrate that the optimal spatial distribution of sampling resources at the landscape scale depends on the sensitivity and specificity of sampling at the scale of individual trees and plantations (e.g., diagnostic tests and visual symptom scouting). Thus, as new sampling, diagnostic, and detection technologies become available, the methods we are developing can help identify deployment strategies that get the most out of available technologies. The method can be tailored to specific areas and regions, each of which have unique environments and face different situations in terms of, for example, vector densities and inoculum pressures.

Attractants for ACP trapping technology: Challenges, status, and opportunities

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Psyllid host searching behavior is complex and sophisticated. It can be influenced by host species, growth stage, and physiological condition, psyllid gender and mating status, behavioral plasticity, usurpation by phytopathogens of host aromas, and psyllid-induced emission of foliar volatiles. ACP relies on visual cues to locate its host plants, but evidence suggests that olfactory cues mediate visual response. Scent lures could potentially enhance psyllid response to the bright yellow and green background colors of the sticky traps used in ACP monitoring programs. However, because host foliage aromas are complex, dynamic, and species-specific, developing an effective scent lure is challenging. Here we explore some aspects of lure composition and deployment which may increase trap yield: (1) Scent lure efficacy may be influenced by setting. In residential areas, a generalized scent lure comprised of volatiles common to several host species may work well, while a scent lure used in groves might need to mimic the scent of the grove trees to be effective. Alternatively, a lure that mimics 'super hosts', such as orange jasmine, may work well in a variety of situations. (2) Volatiles that signal the presence of flush may be important components in scent lures. Flushing shoots are essential for ACP reproduction. Signature odors might include cuticular hydrocarbons, ammonia, methanol, and carbon dioxide, all of which are emitted during leaf expansion. These compounds may synergize ACP response to terpenes emitted by foliar scent glands. (3) Sesquiterpenes may be important signal compounds. These terpenes are prevalent in the aromas of 'super hosts' but most are commercially unobtainable; little is known about their effect on psyllid attraction. A better understanding of these factors could lead to the development of scent lures that are consistently effective in a variety of application situations. Better monitoring tools would especially be useful where ACP population densities are low or for tracking psyllid movement patterns.

Expression of the LasAI effector of 'Candidatus Liberibacter asiaticus' induced proliferation of root hair and trichome in transformed plants

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'*Candidatus Liberibacter asiaticus*' (Las) is the globally prevalent species of HLB bacteria. Like other bacterial pathogens, Las encodes different effectors that can alter the host's response to the pathogen. Two such effector proteins, LasAI and LasAII show characteristics of the leucine-rich repeat (LRR) family of proteins, and are mitochondria-targeting autotransporters. In this study, we have generated several different constructs from LasAI containing the three different domains (the N-terminal sequence, the C-terminal sequence, and the repeat sequence). Using *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana*, we have observed that both the full length LasAI and the repeat domain specifically localized to the mitochondria, while the N-terminal domain targeted the nucleus and the C-terminal localized to other organelles. The ability of the different LasAI domains to target the different organelles may explain why an increase in the repeat numbers of LasAI correlated with a more aggressive Las bacterium. Interestingly, when LasAI was expressed in plants, we observed a dramatic increase in the number of root hairs in transgenic Arabidopsis, while the number of trichomes were greatly increased on the leaves of *N. benthamiana* with transient expression of LasAI. Based on these results, we have developed a novel *in vitro* screening system that can evaluate small molecules against Las effectors in less than 2 weeks.

Nutritional sprays to manage Huanglongbing (HLB) in the field: Can they mitigate orange juice HLB-induced off-flavor?

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Citrus groves receiving nutritional sprays to manage HLB were compared with groves in the same areas receiving conventional fertilization treatments. Fruit were harvested from healthy and HLB-affected trees. In 2009–2012, HLB fruit were sorted into symptomatic (small, green, lopsided – HLBs) and asymptomatic (normal looking fruit – HLBA), while later (2012–2014), no distinction was made between symptomatic and asymptomatic HLB fruit. Fruit were washed and juiced using JBT extractors and finishers, then pasteurized following industry standards. Sensory tests were performed with trained (descriptive analyses) and untrained (difference-from-control tests) panelists. In 2009–2010, differences between juice from healthy and HLBA fruit were minimal, and only perceived by trained panelists. Juice from HLBs was off-flavored, bitter, sour, with some metallic, tingling, and umami taste. These descriptors were mostly used for early season Hamlin, while the effect of HLB on Valencia juice flavor was minimal. Nutritional treatments had no consistent effects on juice flavor over the 5-year duration of the study. Difference-from-control tests showed improvements due to nutritional treatments on Hamlin in January 2012, December 2012, and January 2013, i.e., panelists could not detect differences between juice from healthy and HLB fruit. Trained panelists found more differences between juice from healthy and HLB fruit than between juice from different nutritional treatments, except for one Hamlin nutritional juice in December 2012. Blends made with 75:25, 50:50, and 25:75 ratio of healthy and HLB Hamlin juice were also tested. Trained panelists classified the 75:25 blend with healthy juice, and 25:75 with HLB juice, confirming previous blending studies, while the flavor of the 50:50 blend varied with harvest. In conclusion, no management practice in the field was able to alleviate the effect of HLB on juice flavor. However, much of the off-flavor depended on the season and levels of sugars, acids, and other components in the juice.

Residual toxicity of various organic and conventional pesticides to Asian citrus psyllid and its parasitoid *Tamarixia radiata*

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The Asian citrus psyllid (ACP) was detected in California in October 2008 and has invaded most of the citrus grown in southern California. Huanglongbing (HLB) was detected in Los Angeles County in 2012 and poses a significant threat to California's ~\$2 billion citrus industry. Establishment of ACP and HLB can cause huge economic loss and it is

important to slow the spread of the HLB pathogen. At present, this can best be achieved by controlling the size and spread of psyllid population through integrated management strategies. A series of lab trials were conducted to evaluate the residual efficacy of various conventional and organic chemicals for ACP control and for selection of chemicals that were least toxic to natural enemies like *Tamarixia radiata*. The most persistent organic insecticide tested was Entrust + oil and to a lesser extent Trilogy. Among four pyrethroids, Danitol was most effective and persistent, while Mustang was weaker when tested with adult ACP. However, when toxicity was tested with adult *Tamarixia*, both the organic and conventional pesticides were not as toxic as they were to ACP adults. In a separate study, a field trial was conducted to test the efficacy of organic insecticides against ACP nymphs. Among the materials tested, Entrust + oil provided better control in comparison to two other products. Future plans include further trials under lab and field conditions to evaluate products for ACP control that are least toxic to natural enemies.

Development and reproduction of the Asian citrus psyllid on *Zanthoxylum fagara* Rutaceous relative of citrus

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The Asian citrus psyllid *Diaphorina citri* is the principal vector of the bacteria *Candidatus Liberibacter* spp. causal agent of Huanglongbing (HLB) or citrus greening disease. Both vector and disease are now well established in Florida and also reported throughout the Americas and Asia. The host range of *D. citri* is limited to citrus and some rutaceous relatives. Use of additional host plants by *D. citri* could accelerate the spread of HLB. Adults and a small nymphal colony of *D. citri* was observed on wild lime *Zanthoxylum fagara* during field surveys in Florida. Experiments were conducted to evaluate survival and development of *D. citri* on *Z. fagara* and the hybrid rootstock 'Swingle' citrumelo. Adult longevity of 56.7 ± 3.1 days and 76.2 ± 1.5 days was observed on *Z. fagara* and 'Swingle' citrumelo, respectively. However, reproduction and nymphal survival was not successful on *Z. fagara*. Considering very short acquisition and transmission times for HLB pathogens by *D. citri*, adult longevity on *Z. fagara* appears to be sufficient to acquire and transmit the causal pathogens. However, probability of acquisition is low due to unsuccessful survival of nymphs, which are considered more efficient at acquisition of HLB causal pathogens than adults. Nevertheless, adaptation to hosts presently inappropriate for reproduction could increase over time. There is need to determine if HLB pathogens can be transmitted to and/or acquired from *Z. fagara* and other potential rutaceous hosts. Field monitoring of citrus and *Zanthoxylum* spp. is warranted in order to maintain effective plans for vector and disease management.

Organic insecticides and parasitoids for integrated management of Asian citrus psyllid, *Diaphorina citri*

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The Asian citrus psyllid (ACP) vectors pathogens that cause Huanglongbing (HLB) or citrus greening disease. Management of ACP is critical in all habitats including organic citrus for area-wide suppression of HLB. Mature citrus trees in Florida go through dormancy in winter producing little or no new growth required for ACP to develop and reproduce. Targeting overwintering ACP adults with sprays of broad-spectrum insecticides has been shown to provide significant reduction into growing season and opportunity for biological control and selective insecticides. This tactic is adopted area-wide in Florida and Texas and also used in California. However, organic farmers struggle with management of ACP and HLB because they cannot use synthetic insecticides. Consequently, we have initiated development of ACP control programs testing use of organic insecticides and release of *Tamarixia radiata* also suitable for conventional citrus and urban habitats. We evaluated dormant winter sprays of an organic insecticide, Pyganic (natural pyrethrum) applied in November, December, and January compared to a single spray of the synthetic pyrethroid Danitol

(fenpropathrin) in January against ACP in Valencia and Hamlin oranges. Pyganic was applied either alone, with horticultural mineral oil (HMO), or soybean oil (HSO) at 2% volume by volume. Significant effects of Pyganic plus either oil lasted for 1–2 weeks after November and December applications. Pyganic + HMO and Danitol maintained ACP at or below 0.1 adults per tap sample for 4–5 weeks, and significant suppression lasted 2 months after the January application. Monthly applications of Pyganic with HMO appear to be a reasonable option for organic growers to suppress overwintering ACP. Organic programs evaluating promising insecticides alone or altered with oils or other surfactants are being evaluated parallel to a conventional program. *Tamarixia radiata* are also being released for integration in these programs. Findings and implications for ACP management will be discussed.

Tolerance to citrus Huanglongbing in Australian citrus relatives, *Microcitrus* and *Eremocitrus*

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Tolerance, or resistance, to citrus Huanglongbing will be important as a long-term solution for this disease. In a field trial conducted with over 1000 plants belonging to different genera in the sub-family Aurantioideae, we observed field tolerance in many Australian citrus relatives. To confirm these results in a controlled greenhouse setting, we have exposed 140 plants to *Candidatus Liberibacter asiaticus* via psyllid feeding under no-choice conditions for a period of 2 weeks. We have maintained these seedlings under greenhouse conditions and conducted detailed analysis on the presence of the pathogen and level of tolerance observed in various plants after exposure to the pathogen. We have analyzed the samples using qPCR and droplet digital PCR (ddPCR) to estimate the bacterial titer in accessions that were either susceptible, tolerant, or resistant in field studies. To confirm the presence or absence of the pathogen in plants with questionable qPCR results, ddPCR analysis was conducted targeting multiple genomic regions of *Candidatus Liberibacter asiaticus*. Since these experiments are conducted using open pollinated seeds where the pollen parent is not known, we have also conducted about 2000 pollinations using *Microcitrus*, *Eremocitrus*, pummelo, and mandarin as both seed and pollen parents. The targeted crosses were also conducted with known HLB tolerant cultivars of mandarins like Temple tangor and Fallglo mandarin.

Sensitive quantification of *Candidatus Liberibacter asiaticus* in HLB-infected citrus

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Citrus greening, also known as Huanglongbing (HLB), is a devastating disease on Citrus plants and is caused by the yet unculturable bacterial *Candidatus liberibacter* spp. This gram-negative bacterium is limited to vascular tissue and mainly accumulates in the symplasm of autotrophic organs. In addition, the accurate quantification of viable bacteria is necessary to assess the status of potentially-infected trees as well as to test any possible control strategies. A number of methods have been broadly described to quantify total bacteria content; which includes the detection of both live and dead bacteria as well as the different bacterial pathovars. In the present work, an improved detection method, based on PCR is described, which employs new designed primers. Real-time PCR was also employed to test the sensitivity of the method, which was able to detect 10 copies per genome in tree-infected trees. In addition, Droplet Digital PCR (ddPCR) was also developed to detect two copies per plant genome, which is the highest sensitivity reached in current protocols. The accuracy of the tested techniques will allow assessing the feasibility of possible controlling methods of this devastating disease.

Merging a crowdsourcing challenge with molecular research to find an RNAi-based biological control strategy for the insect vector of Citrus Greening Disease

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Researchers have been exploring more effective ways to control the Asian Citrus Psyllid (*Diaphorina citri* Kuwayama, ACP), the only known insect vector of the phloem-limited bacterium “*Candidatus Liberibacter* sp.” responsible for Citrus Greening or Huanglongbing (HLB) disease. Research results are reported on the use of a crowdsourcing challenge-based strategy to identify gene targets in the ACP that can function as interdiction points to control the insect using an oral uptake RNA interference (RNAi) strategy. This challenge was sponsored and developed by the Florida citrus industry (Florida Citrus Research and Development Foundation) and posted on the web through Innocentive, Inc. Interested participants (Solvers) were allowed to submit suggested targets with justifications for orally administered dsRNAs that would be hypothesized to induce mortality by RNAi. Of the sequences submitted, a committee of experts selected 43 for an oral uptake bioassay performed using an artificial diet screen to identify those that induced mortality. Subsequently, a citrus phloem-limited virus vector was used to engineer citrus to produce candidate dsRNAs in the phloem. When ACP fed on these plants, there was much greater ACP mortality than was observed by artificial diet delivery, in some cases producing 100% adult ACP mortality in detached-leaf bioassays. RNA Seq analysis shows that resulting ACP transcriptome changes were attributable to genes functionally linked to the gene targeted by RNAi.

Analysis of circulative transmission of *Liberibacter* in the Asian Citrus Psyllid using Protein Interaction Reporter (PIR) technology

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New methods are needed to control the spread of the citrus greening pathogen, *Candidatus Liberibacter asiaticus* (CLAs), by the Asian Citrus Psyllid (ACP). This collaborative project funded by the California Citrus Research Board is focused on discovery of protein-protein interactions involved in Huanglongbing transmission. During transmission of CLAs within the ACP, interactions between the vector and host regulate how the pathogen moves through the insect, replicates, and is transmitted into a plant. In this study, Protein Interaction Reporter (PIR) technology has been used to characterize protein interactions between CLAs and ACP. The PIR strategy was developed by the Bruce Lab and uses novel cross-linker molecules which covalently link interacting proteins. Mass spectrometric analysis of PIR cross-linked peptides provides information on the relative proximity and orientation of surface exposed lysine residues in cross-linked protein complexes. The Bruce and Cilia labs have previously successfully applied PIR technology to study interactions with an aphid-transmitted virus. Percoll density gradient centrifugation has been applied to purify enriched populations of CLAs cells from infected ACP. Using this method, intact CLAs cells retaining interactions with ACP tissues are recovered. Infected psyllids were homogenized in a non-denaturing buffer, and the concentrated lysate was applied to a Percoll gradient. Centrifugation resulted in two distinct bands in the Percoll gradient—these fractions were recovered, the cells were washed, and qPCR was used to quantitate the amount of CLAs cells present. The PIR cross-linker was used to capture protein interactions in the enriched CLAs sample, and the cross-linked peptides were subjected to mass spectrometry analysis. Given the challenges of working with this non-culturable pathogen, this Percoll method for CLAs enrichment has a range of potential applications, including the use of purified CLAs cells as immunogen for antibody production.

Can the molecular characterization of the citrus tristeza virus resistance gene locus shed light on *Poncirus* resistance to Huanglongbing?

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Huanglongbing (HLB) is the most serious recent threat to the US citrus industry. Several studies have indicated that *Poncirus* is resistant to HLB, but the *Poncirus* genes for HLB resistance are to be identified and characterized. The *Ctv* locus in *Poncirus* confers durable broad-spectrum resistance to citrus tristeza virus (CTV), a virus that is phloem-limited like CLAs, has been identified and sequenced. We compared the genomic organization of the *Ctv* region to the corresponding regions (*ctv* regions) from three susceptible citrus species and analyzed the genes, particularly the resistance genes (*R*-genes) within the locus, in an attempt to gain perspective of the to-be-identified HLB resistance gene(s) in *Poncirus*. Copy number and localization of *R*-genes at the *Ctv* and *ctv* regions vary considerably from species to species; *R*-genes have been on faster paces of sequence change than surrounding genes. Dramatic structural rearrangements have occurred, resulting in an expansion of ~60 kb at the *Ctv* region over the *ctv* region. While the center of the *Ctv* region is packed with a rich and diverse group of retrotransposable elements (REs), the *ctv* regions are free of REs. Pondering these phenomena at *Ctv*, it is intriguing to speculate the likely structure and evolution of the *Poncirus* genomic regions harboring HLB resistance genes.

Comprehensive meta-analysis, gene co-expression, and miRNA nested network analysis of genes differentially expressed in citrus-HLB interactions

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Huanglongbing (HLB) is the most devastating disease of citrus. A number of transcriptome studies have been conducted and reported to characterize the gene expression dynamics in citrus-HLB interactions. These studies indicated that many citrus genes/pathways were modulated by HLB. The present study was conducted to gain comprehensive insight about the underlying molecular mechanisms in citrus-HLB interactions. Twenty-two publicly available citrus gene expression datasets, including 18 from HLB-susceptible (S) and four from HLB-resistant (R) citrus selections, were retrieved; and previously identified, differentially expressed genes (DEGs) were analyzed using the LIMMA and the RankProd methods. Out of a combined list of 7,412 DEGs, we identified the most significant 65 common genes and 30 R-dataset-specific DEGs. Gene Ontology analysis of these DEGs suggested that carbohydrate metabolism and transport, and stress response were the core pathways in citrus modulated by HLB. The 30 R-dataset-specific DEGs were mainly coded for LRR proteins, chitinases, CDR, miraculins, or lectins. Weighted gene co-expression network analysis of 2,499 DEGs revealed 21 modules with major hub genes. The miRNA nested network analysis suggested that *csi-miR167* and *csi-miR396* could affect citrus transporters and defense response pathways, respectively. Collectively, these meta-analyses suggested candidate genes for further gene expression analysis, over-expression analysis, or other genetic modification towards increased HLB resistance in citrus.

First steps to survive Huanglongbing in Puerto Rico

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Citrus Greening (CG) was first detected in Puerto Rico in 2009 affecting orange (*Citrus sinensis*) and lemon (*C. latifolia*). The causal agent was confirmed as *Candidatus Liberibacter asiaticus*. CG is transmitted by the Asian Citrus Psyllid (ACP) *Diaphorina citri* Kuwayana and by human-mediated grafting transmission. A survey conducted in 2010–2012 showed that the disease is widely spread in Puerto Rico. Consequently, efforts on an education program were established by the Extension Service to train agricultural agents on identification of CG and citrus orchard management practices. In order to maintain pathogen-free

budwood material, the Agricultural Experiment Station (AES) of the University of Puerto Rico, Mayagüez Campus has moved all the germplasm to insect-proof screenhouses. Initially, all the germplasm was located on AES at Isabela, Puerto Rico. Currently, new collections have been moved to AES at Rio Piedras and Adjuntas, Puerto Rico. AES is the only one that has protected citrus budwood that is certified CG free in Puerto Rico. In 2014, AES started a new institutional initiative to integrate various researchers to work with the critical citrus situation. The new initiative formed the project Production of Healthy Citrus Plants in Puerto Rico. This project combines the certification of disease-free citrus plants and the development of new methodology on the production of pathogen-free citrus plants in screen-protected houses. The certification will detect citrus greening, citrus variegated chlorosis, citrus leprosis, citrus exocortis, and citrus canker. On the other hand, the new methodology will explore different media components, fertilization, container design and size, different rootstock and varieties in order to accelerate root growth and whole plant development. Current status of the screen-protected houses, current germplasm collection, and practices will be presented.

Current status of Huanglongbing disease affecting Mexican lime in the state of Colima, Mexico

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In the state of Colima, Mexico, there are 20,000 hectares of Mexican lime (*Citrus aurantifolia*). The Asian citrus psyllid (*Diaphorina citri*) has been present there since 2004, while the ‘Huanglongbing’ (HLB) disease was detected in 2010. Seven months after the first detection, HLB positive trees were found in all producing areas of the state. In June 2013, a systematic sampling was done in 299 orchards checking 7500 trees, resulting that 100% of the orchards and 100% of the sampled trees had HLB symptoms, which had a canopy portion affected by the disease ranging from 25 to 75%. During 2010, 2011, and 2012, the number of fruits per square meter of canopy in different orchards was quantified. In these 3 years, the results were similar; it was observed that trees with HLB symptoms in over 75% of the canopy tend to reduce their production of fruit between 40 and 60%. Also, it was evident that asymptomatic sectors in HLB-affected trees present good production and good fruit size. And contrary to that, symptomatic sectors have chlorotic foliage, a reduced yield, and a fruit size slightly smaller in comparison with fruits from healthy trees. Until nowadays, in Mexican lime, it has not been detected misshapen fruit, inverted ripening, or an increased number of aborted seeds related to HLB. During 2013, an average yield of 14.4 t/ha was recorded, representing a reduction of 23.4 and 39.7% compared to that recorded in 2010 and 2012, respectively. This partly reflects the effect of HLB on Mexican lime production in Colima. It has been observed that an integrated crop management, with special emphasis on nutrition, although with a lower yield, allows HLB-affected trees to continue producing fruit. This represents an option for producers while HLB tolerant varieties are generated.

Cost-benefit analysis of severe pruning to rejuvenate HLB-infected trees

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Severe pruning has been suggested as a strategy to rejuvenate citrus trees which have been adversely affected by HLB. Pruning rebalances a tree's root to canopy ratio and thus allows an infected tree to increase its capacity to set fruit. An experiment was initiated at the UF/IFAS Southwest REC in February 2010 to measure the effects from severely pruning HLB-infected trees. Four years of data including shoot growth, canopy development, and fruit yield were collected from 2010 through 2013. Fifteen-year-old Valencia orange on Swingle citrumelo rootstock grown on two-row beds typical of citrus in the Florida flatwoods were selected, pruning one row and leaving the other row unpruned. Three foliar nutritional treatments plus a standard grower fertilizer program were applied to both pruned and unpruned trees. Seven replications of 10-tree plots received the pruning and nutritional treatment combinations. The harvestable yield from pruned trees was minimal in 2010, the year of pruning, and constituted the largest financial penalty when attempting to rejuvenate HLB-infected trees through severe

pruning. Production from pruned trees recovered during the second season (2011) and was statistically equal to production from unpruned trees. In 2012 and 2013, production from pruned trees surpassed the production from unpruned trees. Juice quality data generally showed no significant differences between pruned and unpruned trees. Cost effectiveness of pruning, however, depended on the enhanced foliar nutritional program. Pruned trees on two of the three nutritional programs produced nearly a box more fruit than unpruned trees receiving the same nutritional program. The increase in net returns was estimated to nearly \$4 per tree. The cost of severe pruning plus the value of yield loss in the first year after pruning was estimated to be less than \$2.30 per tree.

Costs and benefits of foliar nutritional amendments – Evidence from a 5-year trial

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Foliar nutritional programs have been adopted by nearly all Florida citrus growers as a strategy to maintain production from HLB-infected trees. Important questions arise, however, as to the relative importance and necessary quantity of micro- and macronutrients. Five years of production and cost data were collected from a trial on a commercial block of Valencia on Swingle on which various combinations of nutritional supplements were applied three times a year corresponding to the spring, summer, and fall flushes. Nine foliar treatments were designed and replicated five times over a 30-acre block. All treatments received a uniform ground fertilizer application and a uniform psyllid control spray program. The foliar treatments represented various combinations of micro- and macronutrients. Annual fruit yield and juice quality were recorded for each treatment and analyzed both by individual year and cumulatively across the 5-year period. Fruit revenue was estimated using average fruit prices reported in the Annual Citrus Summary (FASS). The costs of the individual nutritional treatments were itemized using 2013 retail fertilizer prices. Significant yield differences between all foliar nutritional programs and a standard were noted starting in the second year of the trial. Those treatments that combined both micro- and macronutrients yielded the greatest yield benefits. While the treatment with the greatest complement of micro- and macronutrients resulted in the largest numerical yield gain, the yield differences among most of the foliar nutritional programs were not significantly different. The costs of nutritional products, however, ranged significantly from \$100 to \$550 per acre.

Evaluating the likelihood of phloem mobility and using *Liberibacter crescens* to assess efficacy of possible treatments for citrus greening

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Huanglongbing (HLB) or citrus greening, caused by the phloem-limited bacterium *Liberibacter asiaticus* (Las), is threatening the viability of the citrus industry in the United States. The search for an effective treatment of HLB is imperative. Using the closest culturable relative to Las, *Liberibacter crescens* (Lc), as a model organism, we have quickly and inexpensively screened a variety of likely phloem mobile compounds for effectiveness prior to field experiments. An existing model describes the optimal chemical characteristics of phloem mobile xenobiotics as: an octanol/water partition coefficient (log P) between -1.5 and 2.5, a molar volume $\leq 300 \text{ cm}^3 \text{ mol}^{-1}$, and ionizability (pKa) between 2 and 15 (1). Following these assumptions, we selected and screened 145 compounds for Lc sensitivity using a high-throughput 96-well assay method. The percent inhibition was evaluated for multiple concentrations of each compound and the MIC90 and MIC50 was noted. Of the 145 screened compounds, 50 showed $\geq 90\%$ inhibition at one or more concentrations. Of those, 12 were penicillins, 16 were cephalosporins, 2 were carbapenems, 7 were tetracyclines, 6 were aromatic hydrocarbons, 3 were nitro compounds, and 4 were organic chemicals (not classified as antibiotics). The 27 quinolines and 20 sulfones tested showed little impact on Lc growth. Our next step is to test the phytotoxicity of those antimicrobials that are phloem mobile and inhibit Lc. Oxytetracycline fits these criteria and showed no phytotoxicity after repeated foliar sprays at 200 ppm. Using this selection and assay method, we can

efficiently sift through multiple treatment options and only select the most suitable for more laborious field-testing, and ideally provide a short-term treatment option for commercial citrus growers. Eventually, these results could help guide the production of effective novel compounds that will provide additional treatment options.

Tamarixia radiata: Mass production and release in Mexico

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The success story of the parasitoid *Tamarixia radiata* in the control of its host “*Diaphorina citri*” has promoted the establishment of a biological control program with the construction of two laboratories of mass production in 2010 by the Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), as a strategy to control the Asian citrus psyllid (ACP) in Mexico. The aim of this program is to produce parasitoids in order to perform *T. radiata* release in areas without chemical control and with the presence of *Murraya paniculata* plants (lemongrass or orange jasmine) or any kind of citrus, such as urban areas, backyards, reserve areas, or abandoned orchards (i.e., sites that serve as a reservoir for the pest). The mass production of *T. radiata* involved three steps of equal importance. The first step of this process is the mass production of *M. paniculata* plants; each plant must develop tender shoots of 10–15 cm in length before being used for host production (i.e., *D. citri*); at this step, plants previously selected are introduced into a cube of 70 cm with adult psyllids in order to infest the shoots with ACP eggs. In the course of the next 7 days, the process will get a massive production of nymphs (juveniles host), which were used in the last step of production. At this step, the appropriate nymphs were offered to the parasitoids in order to parasitize them during 10 days. During this period, the nymphs are screened to detect the emergence of the parasitoids. Finally, the parasitoids are extracted in containers, fed with honey, and prepared to be delivered to field technicians which are in charge of the *T. radiata* release in the different states with citrus crops. Currently, the mass production and release of parasitoid in Mexico reached an unprecedented worldwide level.

Nutritional status of citrus trees in relation to HLB infection: A case of deficiency or toxicity?

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Current strategies for managing Huanglongbing (HLB; citrus greening) include area-wide psyllid vector control, inoculum (infected tree) removal, use of clean planting stock, and foliar nutritional supplements to sustain productivity of groves with infected trees. Foliar nutritional supplementation has had mixed results, in part, because the basis for such supplementation to suppress HLB has either not been established or is useful in correcting specific nutrient deficiencies observed in trees. The possibility that other mechanisms such as toxicity and other nutrient interactions could be interfering with tree metabolism has not been addressed. In this multi-year field/lab study (2012-2014), the impact of HLB infection on leaf mineral contents of adequately-fertilized grapefruit (*Citrus × paradise*, var “Rio Red”) and sweet orange (*Citrus sinensis*, var “Valencia”) trees was investigated in Texas. Symptomatic and non-symptomatic leaves from known HLB-infected trees and leaves from non-infected trees, selected based on visual observations and qPCR tests, were analyzed for mineral composition (corrected for total non-structural carbohydrate contents – TNC). Leaves from HLB-infected trees had significantly higher TNC levels (23%) than leaves from healthy trees. HLB-infected trees also exhibited significant decreases (>10%) in leaf nitrogen (N), phosphorus (P), magnesium (Mg), calcium (Ca) and zinc (Zn), and significant increases in sodium (Na), copper (Cu), and boron (B) concentrations of symptomatic leaves compared to healthy trees, whereas asymptomatic leaves from HLB-infected trees had intermediate values. Significant correlations were obtained between leaf nutrient concentration and CT values. The present observations shed further light on the physiological and biochemical changes associated with HLB disease development. Since these groves were well fertilized, the observed differences seem to arise, at least in part, from an imbalance in uptake of soil minerals, suggesting that the decline in

leaf/tree physiological function may be due to toxicity associated with elevated tissue concentrations of Na and other trace elements.

Edge effects in the spatial distribution of Asian citrus psyllid in citrus groves: Consequence for the development of best management practices

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The spatial niche occupation of the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was evaluated to determine its field colonization and food resource exploitation strategies in citrus groves. Mature grapefruit and sweet orange groves were surveyed in 2009–2010 to determine *D. citri* population densities and between-tree distribution. In both cultivars, significantly more psyllids were found on perimeter trees throughout the study period, suggesting a strong edge effect in *D. citri* distribution in the groves. Using the selection index, *D. citri* exhibited a strong niche occupation preference for border trees. Asian citrus psyllid densities and infestation levels gradually declined from the edge to the center of grove. In detailed field studies, infestation of *D. citri* started from border trees in the grove where possibly one generation is completed before inner trees become infested. Given this edge effect in ACP niche occupation, the effectiveness of border treatment in ACP control was tested between major flush cycles. Border treatment provided comparable psyllid control to whole grove sprays for 2–3 weeks. This strategy can complement whole grove sprays for the development of best management practices for ACO and reduce the pesticide load in groves.

Control of *Diaphorina citri* by citrus tristeza virus (CTV) expressing dsRNA against psyllids key physiological pathways

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In 2011, we have demonstrated that feeding dsRNA targeting specific genes within the insect are toxic through RNA silencing mechanisms. This approach opened the door to use psyllid specific dsRNAs that high amounts are by citrus tristeza virus (CTV) in the phloem, the site of the viral replication and *D. citri* site of feeding. Some of the dsRNA molecules can move in the citrus once expressed in the phloem into the xylem a minor site of psyllids feeding. Two important targets were selected: a) important gut digestive enzyme and b) enzymes that control synthesis and metabolism of juvenile hormone, an important hormone that controls metamorphosis, egg development, and behavior. *D. citri* infected with *Liberibacter asiaticus* were allowed to feed on citrus trees that were transfected with CTV synthesizing dsRNA targeting digestive and juvenile hormone synthesis and metabolic enzymes. Adult *D. citri* were assayed initially by PCR for *L. asiaticus* and allowed to lay eggs on the leaves and the newly emerged adults were assayed for number of surviving adults and for *L. asiaticus* titers using PCR. A marked decrease in the number of the surviving adults was noted when compared with controls citrus trees that did not express dsRNA. The surviving adults when assayed by PCR were negative for *L. asiaticus* indicating that expressing specific dsRNA molecules in citrus can be highly effective against the spread of *L. asiaticus* by *D. citri*. A road map to control psyllid infestations and *L. asiaticus* spread will be discussed.

Bactericides as HLB therapy: Getting them where they need to go, when they need to be there

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Only two therapies have been shown to be effective for treating trees infected with “*Candidatus Liberibacter asiaticus*” (CLAs): (1) thermal therapy and (2) bactericide therapy. There have been reports of several

bactericides reducing CLAs titer and alleviating HLB symptoms. Injections of antibiotics have been the primary focus and several issues have hampered commercial development: (1) cost of treatment, (2) damage inflicted on the tree as a result of multiple injections, (3) phytotoxicity of antibiotic treatment, and (4) concerns over bactericide residue in the fruit. Cost can be high because of the labor intensive process of injecting each tree and/or the cost of the injection apparatus. Since the bacterium resides within the phloem cells, effective control requires the bactericides to first enter the tree and then move systemically throughout the vascular tissues. Damage from the injection process can create scar tissue that impedes normal transport of nutrients throughout the tree, and phytotoxicity is exacerbated because treatments require a high dose of the bactericide applied to a small application site. Our research was conducted to determine if effective treatment application strategies can be developed that reduce phytotoxicity and plant damage and also reduce the long-term cost by reducing labor costs of using bactericides in an HLB IPM strategy. We have shown that three bactericides—oxytetracycline, penicillin, and streptomycin—can be applied in ways that cause the molecules to penetrate plant tissues and move systemically throughout the plant. We have also developed a strategy for therapeutic delivery of the antibiotic in a “time-release” strategy that should reduce the phytotoxicity and cost of multiple applications. Encouraging results on greenhouse and research plots have led to current commercial grove application trials testing different application strategies and comparing effectiveness.

PAMP-triggered immunity is differentially induced in ‘Sun Chu Sha’ mandarin by *Candidatus Liberibacter asiaticus* and *Xanthomonas citri* subsp. *citri* flagellin 22

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Bacterial flagellin 22 (flg22)-induced PAMP-triggered immunity (PTI), mediated by plant cellular membrane localized receptor FLS2, is an important layer of plant defense. Our previous research on citrus canker (*Xanthomonas citri* subsp. *citri*, *Xcc*) showed that a strong induction of PTI by *Xcc*-flg22 was associated with the more resistant genotypes. RNA-Seq analysis of citrus response to CLAs-flg22 revealed that transcriptional reprogramming was triggered in ‘Sun Chu Sha’ mandarin (tolerant to HLB and resistant to canker) 24 hours after the treatment. However, the number of differentially expressed genes was much less than those induced by *Xcc*-flg22. Quantitative real time PCR analysis showed that CLAs-flg22 upregulated the expression of PTI marker genes *WRKY22* and *GST1* in ‘Sun Chu Sha’ mandarin, but to levels lower than those induced by *Xcc*-flg22. In addition, CLAs-flg22 induced a much weaker oxidative burst, if any, than *Xcc*-flg22. We also sequenced two FLS2-like genes (named *FLS2-1* and *FLS2-2*) from ‘Sun Chu Sha’ mandarin and other citrus genotypes. Expression analysis showed that both *FLS2-1* and *FLS2-2* were inducible by CLAs-flg22 treatment but again to lower levels than those induced by *Xcc*-flg22. In addition, *FLS2-2* expression was generally higher than *FLS2-1* after treatment with either flg22. Taken together, our results suggest that CLAs-flg22 induces a weaker PTI than *Xcc*-flg22 in HLB tolerant and canker resistant ‘Sun Chu Sha’ mandarin, which is possibly mediated by the FLS2-like genes identified.

Development of a reliable and highly sensitive, digital PCR-based assay for early detection of HLB

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Huanglongbing (HLB) is caused by a phloem-limited bacterium, *Ca. Liberibacter asiaticus* (Las) in the United States. The bacterium often is present at a low concentration and unevenly distributed in the early stage of infection, making reliable and early diagnosis a serious challenge. Conventional diagnostic techniques, including real-time PCR (qPCR), have often failed at early detection of HLB. We have demonstrated a promising novel diagnostic assay based on digital PCR (dPCR) for early and reliable detection of HLB. dPCR has revolutionized the detection of rare pathogens and nucleic acid molecules as it partitions samples into tens of thousands of picoliter wells in a single reaction. Each well carries

out an independent PCR reaction simultaneously. The number of negative and positive wells can then be fitted into a Poisson distribution to allow absolute and precise quantification of the target molecules in a sample without the use of a standard curve. The large number of independent reactions in a single assay makes it possible to apply statistical tools to estimate a level of precision and confidence interval of the measurement. Using probes targeting the Las 16s rDNA and the integrated prophage repeat sequences, we show that as few as 1–2 copies of the targeted DNA molecules per microliter can be detected, with the prophage probe providing the best sensitivity. The copy number measurement of the targeted DNA molecules can be statistically differentiated from the healthy sample and negative water controls. Furthermore, this assay can quantitate the copy number of the 16S rDNA and the phage repeat DNA simultaneously, permitting the tracking of lysogenic and lytic activities of the Las prophage/phage accurately. The dPCR-based assay will not only provide a reliable and early diagnostic tool but also an enabling technology to advance research on HLB therapies.

Antibody-based detection of HLB using CLAs-specific secreted proteins

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Accurate and timely detection is critical for the eradication of Huanglongbing (HLB). Unfortunately, early HLB detection is challenging because infected citrus plants remain asymptomatic, sometimes for years; furthermore, the HLB-associated bacterial pathogen *Candidatus Liberibacter* spp. (or CLAs) exhibits uneven distributions in infected trees with low titers. Very often, CLAs cannot be detected by quantitative PCR (qPCR) even in symptomatic branches or leaves. To date, reliable methods for HLB early detection and the ability to process a large amount of samples necessary to track HLB epidemic are urgently needed. In the past several years, we have identified unique secreted proteins of CLAs as markers for HLB detection and developed an antibody-based detection method using a simple branch imprint assay. The basic idea is that although CLAs has sporadic distribution in the phloem of infected trees, proteins secreted from CLAs cells into the phloem can be systematically distributed in the infected trees through the vascular flow. As such, serological detection methods based on CLAs-specific secreted proteins will better cope with the large variability in the distribution of CLAs cells within infected trees and the various degrees of disease progression, and thereby increasing the chances of HLB detection in a direct and highly specific manner. Our preliminary research suggest that this method holds promise for allowing early detection of newly infected trees and large-scale field surveys.

Pathogenicity of two entomopathogenic fungi isolated on *Diaphorina citri* and their potential of reducible effect on the vector population

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We collected cadavers of Asian citrus psyllids, ACP, which were parasitized and killed by entomopathogenic fungi in citrus groves of both Japan and Vietnam. In Vietnam, we collected cadavers on 30 trees in two groves once every month for one year. One dominant species from Vietnam and one from Japan showed high mortality on the psyllid in laboratory experiments. Partial β -tubulin and ITS rDNA sequences of these species were analyzed by the methods described in Luangsa-ard et al. (2005. Mycol Res. 109:581-589), identifying the Vietnamese fungus as *Purpureocillium lilacinus* (PL) and the Japanese one as *Isaria javanica* (IJ). The colony of the former was white to pinkish white on the medium and that of the latter was white. Their phialide forms were apical and their sizes were $6.89 \times 1.63 \mu\text{m}$ and $6.52 \times 2.03 \mu\text{m}$, respectively. Conidia were cylindrical in $2.72 \times 2.34 \mu\text{m}$ in the former and oval to cylindrical $4.95 \times 2.22 \mu\text{m}$ in the latter. Their morphological

characteristics thus corresponded to those reported in other papers. PL and IJ in a concentration of $10^6/\text{mL}$ conidia attained 96 and 97% mortalities, respectively, both being higher than *I. fumosoroseus* which showed 60% mortality in the same or higher concentrations. We further performed field experiments in southern Japan. IJ was sprayed at 10^3 to 10^7 conidia/mL to orange jasmine trees, *Murraya paniculata*, on which psyllid adults were released. All psyllids died at 10^7 conidia/mL in 3 days, while only half at $10^5/\text{mL}$ did. Computer simulations in which life history characteristics of the vector were incorporated suggested that wide area management of the vector by the fungi would reduce the vector population. The use of these fungi may reduce the expansion of the disease problem.

Characterizing the colonization and distribution of Asian citrus psyllid in an urban landscape: Role of roads and spatio-temporal patterns of occurrence

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Since its earliest known occurrence in California, the Asian Citrus Psyllid (ACP) has spread rapidly across the urban landscape of southern California. It is not surprising that much of the observed ACP infestation occurs within the urban areas of Los Angeles. However, to-date little is known about the factors that influence the spread and distribution of ACP at the landscape level. Our study focuses on understanding the colonization and distribution pattern of ACP across a predominantly urban landscape by using a combination of GIS tools and spatial models. More specifically, we first explore the role of roads in the colonization of ACP during early stages of spread and then develop several regression models to identify the key exogenous and endogenous factors that influence the eventual landscape-level distribution of ACP. Analyses of road-proximity patterns among ACP occurrences captured at different temporal stages of spread for each county showed that the initial occurrences of ACP were strongly and positively influenced by the network of roads for the counties of Los Angeles and San Diego. Logistic regression model highlighted the strong influence of 'percent impervious surface' and 'NDVI' as the key landscape factors along with 'rain in warm quarter' as the most influential bioclimatic factor in determining the occurrence of ACP in the urban landscape. Inclusion of spatial and temporal patterns of ACP occurrence as an autocovariate further improved the fit of regression models, suggesting the role of fine scale endogenous process like dispersal in determining ACP occurrence. Results from the best-fitting spatiotemporal auto-logistic regression model shall eventually be used to develop habitat suitability map for ACP.

Vigor in citrus orchard induced by neonicotinoids insecticides in the HLB management

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To induce vigor for early production has been a great strategy for fast payback and the search for high yields at a time when production costs have increased significantly and the estimated useful lifetime of orchards and production has reduced due to citrus Greening. The citrus production after HLB are restricted to producers most efficient. Same neonicotinoids insecticides that are acting on plant physiology produce greater vigor, shoot, and root development, improving the absorption of nutrients and water, enzyme activities, resistance, and productivity. The objective of this project starting in December 2007 was to compare the vigor and productivity in the field with 'Valencia' on 'Rangpur' lime, spaced $6 \times 2.5 \text{ m}$, and irrigated. The application of insecticides indicated for ACP management was tested with six treatments: T1, T2, T3-Thiamethoxam (Actara 250WG) in half, recommended, and double dose ($0.6, 1.0, \text{ and } 2.0 \text{ g}\cdot\text{tree}^{-1}$), respectively, T4-Imidacloprid $0.5 \text{ g}\cdot\text{tree}^{-1}$; T5-Aldicarb $10.0 \text{ g}\cdot\text{tree}^{-1}$, and T6-Control, these doses on first year, with drench applications in October and January of each year and the second year calculated as a function of tree height (T1,T2,T3: $1.25 \text{ g}\cdot\text{m}^{-1}$ canopy height; T4: $4.0 \text{ mL}\cdot\text{m}^{-1}$; and T5: $25 \text{ g}\cdot\text{m}^{-1}$). For analysis of four seasons (2010–2013), there was no significant difference (Tukey test $p < 0.05$) to height (m) and canopy volume (m^3); T3 thiamethoxam in recommended dose produced significantly in 2013, $605 \text{ kg}\cdot\text{ha}^{-1}$ soluble solids higher than the control. The cumulative production in 4 years was 243, 271, 224, 219, 228, and $212 \text{ t}\cdot\text{ha}^{-1}$, respectively, for T1-T6, and T2 produced

60 t·ha⁻¹ more than the control and differed from all others, besides having better production efficiency kg fruit·m⁻³ canopy, better leafiness and production within the canopy. Aldicarb and control produced plants with long branches, many canopy internal spaces. All neonicotinoids induce vigor, but the ACP management with thiamethoxam in recommended dose was more efficient in the production and payback expectation.

Induction vigor in young citrus plants is good strategy to have early yield after HLB

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Before HLB, the useful lives of the plants were larger than 20 years. Now it was estimated, even with modern managements, an average is 14 years. When plants are managed with HLB, eradicating the plant stand decreases, making low productivity and impeding the maintenance of the area, and making it necessary to eradicate all plants and plant new plants. A good strategy to have new citrus area is to induce vigor to have high productivity rates and earliness, getting “payback” in the first years. Some insecticides, like the neonicotinoids group, have demonstrated physiological action on treated plants - like increased vigor, shoot and root development, better absorption of nutrients and water, increased enzyme activity, resistance, and productivity. To evaluate vigor, healthy nursery trees and standardized Valencia orange single stem produced in greenhouse were planted in pots in randomized blocks and treated with systemic insecticides action indicated to manage the psyllid. The experiment was conducted with six treatments—three different doses of thiamethoxam, one of Imidacloprid, Aldicarb, and Check without insecticide. We evaluated the development of plants every 15 days until 150 days after planting, evidencing that all the treatments had an increase in trunk diameter, with the thiamethoxam treatments differing significantly from Control. Thiamethoxam at a dose of 1 g per plant produced fresh and dry weight of root differing from Check. This treatment also had higher weight of shoots. That way, treatment with thiamethoxam at a dose of 1 g per plant had the best results in all studied characteristics providing better vigor and plant development during the study period.

Repellent effect of *Psidium guajava* cultivars to the Asian citrus psyllid *Diaphorina citri*

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Considerable research has been conducted toward developing and implementing HLB and ACP management strategies. With respect to ACP control, of interest is that reports indicate guava, *Psidium guajava*, can be repellent to ACP. We conducted research to further assess repellency of guava to ACP. In one set of experiments, guava oil from five Brazilian cultivars (‘J3’, ‘Pedro Sato’, ‘Century XXI’, ‘Thailand’, and ‘Paluma’) was extracted from leaves (mature and immature) by hydrodistillation in a Clevenger apparatus and evaluated for psyllid repellency. In a second set of experiments, repellency of guava leaves to ACP was investigated using leaves (young flush leaves as well as mature leaves) from two guava cultivars, ‘Pink’ and ‘Thai White’. Repellency was evaluated by releasing ACP adults into a cage with two vials, one containing a young flush of *Murraya exotica* (a favored host plant of the psyllid, the flush of which is highly attractive to ACP) and one with *M. exotica* flush and the test material of interest (guava oil, mature leaf or flush). The adults were free to move throughout the cage and into the vials. After 24 hours, the number of psyllids in each vial was counted. The results obtained showed that all guava materials tested had at least some repellency to ACP. Mature guava leaves had a greater repellent effect than young flush. Each of the five oils exhibited repellency. There were no differences between males and females with respect to guava repellency. A report in the literature suggested that sulfur compounds associated with guava may be responsible for ACP repellency. Interestingly, the five guava oils we studied were repellent to ACP, but

three did not appear to contain any sulfur compounds. Identification of the constituents responsible could lead to new management tactics.

Soil applied systemic insecticides to control Asian citrus psyllid and slow spread of HLB in young citrus trees

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Orchard renewal is a special challenge where HLB is endemic. Young trees are especially susceptible to the disease and continuously attractive to the psyllid vector due to frequent flushing. Therefore, heavy reliance is placed on soil-applied systemic insecticides to protect young trees. We conducted a one year study to evaluate foliar concentrations of neonicotinoids and cyantraniliprole applications and two multi-year studies to assess long-term ACP control and consequent protection from HLB. In the first study, we found detectable levels of cyantraniliprole and significant reduction of ACP nymphs 295 days after a drench application to 2-month-old trees. In the first long-term trial, ACP adults averaged 1.4 per tap under heavy pressure on untreated trees over 2.5 years, compared to 0.06–0.11 on trees treated at 3-month intervals with rotations of neonicotinoid and cyantraniliprole drenches. Incidence of HLB reached 49% on untreated trees after 2 years compared to 6.3% on treated trees which later rose to 98% after 3.5 years. Trunk diameter on treated trees after 3.7 years was 37% larger and yield 6.5 times greater compared to untreated trees. During 15 months after planting the second long-term trial, shoot infestation averaged 60% and adults 0.9 per tap compared to 7.1% and 0.04 per tap for trees treated with a neonicotinoid – cyantraniliprole rotation. The numbers for repeated imidacloprid application were 12.7% and 0.08 per tap and repeated cyantraniliprole 18.0% and 0.08 per tap. HLB incidence was already 46% in the check compared to 12.5% for the rotation and 29 and 25% for imidacloprid and cyantraniliprole, respectively. Drip injection worked better than drench, once roots had established around emitters spaced at 30 cm.

Asian Citrus Psyllid behavior in dsRNA-treated versus not-treated plants

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Huanglongbing (HLB), the most devastating citrus disease worldwide, is threatening the citrus industry in several countries, mainly Brazil and United States. HLB is a bacterial-caused disease transmitted by the Asian Citrus psyllid (ACP). Currently there is no cure for this disease and no identified genetic resistance in the genus Citrus. An emerging technology, RNA interference (RNAi), which is a natural biological process that selectively down regulates the expression of a specific gene, is being developed as a more environmentally friendly approach to control insects. The feasibility of RNA interference (RNAi) strategies supports a method that would be highly specific ACP populations. One question that needed to be addressed was the effect, if any, on ACP response to plants treated with an RNAi product. To evaluate if ACP sensed the presence of an ACP-specific dsRNA in plants, the experiment examined psyllid response to dsRNA treated plants versus non-treated plants. Four groups of six plant flush each (3 treated and 3 untreated) were placed in each corner of a cage, and 100 ACP were released at the center. They were observed for 15 days, and the number of ACP on each flush recorded daily. A significant difference, using t-test analyses, showed more psyllids on the dsRNA treated plants than on untreated controls ($p > 0.05$). Psyllids appear not to be sensitive to dsRNA ingestion, as they may be for traditional chemical insecticides, thus were not repelled. These preliminary results suggest that RNAi-based products (at least those tested) may actually increase psyllid feeding from the treated trees, thus increasing RNAi efficacy, by increasing the amount of dsRNA each psyllid would ingest.

Does experience mediate host plant selection in specialist herbivores? A new look at the nature versus nurture debate

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The Asian citrus psyllid, *Diaphorina citri*, is an oligophagous herbivore that transmits *Candidatus Liberibacter asiaticus*, the devastating pathogen causing citrus greening disease. Efforts to manage *D. citri* populations and slow the spread of the pathogen may be aided by an increased understanding of the mechanisms underlying *D. citri* host plant selection. Although specialists are largely regarded to rely on innate responses for host selection, little research has been done to investigate the extent of learning in such organisms. Previous work has indicated that *D. citri* are capable of simple olfactory associations. However, it is currently unknown to what extent learned visual associations or the combination of olfactory and visual stimuli interact with innate responses to drive host selection. This study investigated the plasticity of a locomotor response after experience with visual and olfactory stimuli. Experienced *D. citri* were exposed to the novel test stimulus for 3 days while feeding on *Citrus × aurantium*. Naive *D. citri* encountered the test stimuli only during testing. Behavior was measured in a choice test using a modified y-tube olfactometer. Both male and female *D. citri* showed increased selection of the test stimuli after experience. Additionally, analysis of the latency to selection showed that experienced *D. citri* made decisions more quickly than naive *D. citri*. The results of this study suggest *D. citri* do acquire visual and olfactory information associated with the host plant environment and that experience with such stimuli can influence innate preference. Future research will investigate the extent to which such experience-mediated behavior influences host selection in the field. If consistent with laboratory experiments, such data could provide a basis for predicting regional *D. citri* movement, enhancing population monitoring, and potentially reducing the use of pesticides in 'low-risk' groves.

Variables in screening for resistance to Huanglongbing

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A series of experiments were initiated to assess factors which might permit more rapid screening for HLB resistance, using sweet orange in all experiments, and Carrizo and/or Temple as sources of resistance/tolerance. Numerous researchers working on HLB provided observations to identify study components. Consistency of CLAs titer and effectiveness in infecting target trees were evaluated using several source genotypes for graft inoculations: rough lemon and Valencia were much better sources of graft inoculum than citron or Volk, even though citron had very high titer levels. Effect of continuous versus diurnal lighting was assessed in factorial with scoring (to enhance starch accumulation) on symptom severity and rapidity—continuous light accelerated symptom development but scoring did not. Effect of oscillating stress on symptom development was assessed with inconsistent results. Levels of dead versus living CLAs were assessed in inoculum source budwood and levels of dead CLAs were never more than 50%, suggesting likelihood of little effect. Effect of feeding source genotype was assessed on inoculativity of Asian citrus psyllid (ACP): no major differences were observed. Several additional experiments will be completed before February 2015: inoculation by grafting versus 2 weeks of no-choice hot psyllid feeding versus continuous choice hot psyllid inoculation; effect of diverse (identified through sequencing) Florida CLAs strains on response of generally susceptible versus resistant/tolerant citrus cultivars; and effect of rootstock (including several reported to confer resistance) on CLAs titer and HLB symptoms. Consistent rapid screening is critical to efficiently assess disease resistance among plant materials. Despite the rapidity of Huanglongbing (HLB) spread in Florida, it often takes 8–10 months to observe a high level of *C. Liberibacter asiaticus* infection even in highly susceptible sweet orange. The observations from these experiments should increase efficiency in screening for HLB resistance and may contribute to more uniform testing between labs.

Field trial of Penicillin G trunk injection of commercial citrus for Huanglongbing control

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A field trial of three commercial citrus varieties in two separate locations was established in Central Florida by the scientists from Florida Department of Agriculture and University of Florida. Penicillin G potassium salt at two different rates, 1,000 ppm and 6,000 ppm, was injected into the trunk of Huanglongbing (HLB)-infected citrus. Both penicillin efficacy and residue level were monitored via qPCR, bioassay, ELISA, and HPLC/MS at different times prior to and after treatments until fruit were harvested. Efficacy was also evaluated through fruit size and yield. The trial was a randomized block design with split measurements. Data analysis from the trial should provide sufficient evidence to assess the efficacy of penicillin in curing HLB-infected trees and its residue level in fruit 3 months after the injection. The ultimate goal of the trial is to have penicillin registered through EPA for citrus trunk injections for HLB in Florida.

Frequent low volume applications of horticultural mineral oil for management of Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae)

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Huanglongbing (HLB) vectored by the Asian citrus psyllid (ACP), *Diaphorina citri*, can dramatically reduce yield and is incurable. Control of the HLB vector is the best strategy to limit infection but has resulted in greatly increased insecticide use. Horticultural mineral oil (HMO) application may represent a more sustainable alternative to broad-spectrum insecticides and is a traditional component of pest management programs in Florida. However, the effectiveness of frequent low volume applications of HMO against ACP had not yet been rigorously tested. Therefore, we initiated a 3-year trial in Feb 2011, at a commercial Valencia orange grove in Lee County, Florida to compare low volume (18.7 L ha⁻¹) sprays of HMO applied every 2 weeks with the grower standard (GS) (insecticide application) and an untreated control. Both the HMO and GS treatments significantly suppressed ACP adult and nymph populations, although adult suppression was greater for GS treatment. ACP egg densities were also reduced by GS treatments. Both treatments typically improved yields and the HMO-treated trees yielded significantly more than untreated trees in the final study year. A significant reduction in fruit drop associated with both GS and HMO treatments was apparent in 2013. Fruit quality was for the most part unaffected by treatments. Although a significant effect of either treatment on HLB symptoms or Ct values was not apparent, ACP suppression, comparable fruit yield, and eventual production gains associated with these treatments indicated that low volume application of HMO is a viable means of controlling ACP.

Citrus sinensis transgenic plants for resistance to *Candidatus Liberibacter asiaticus*

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Huanglongbing (HLB) associated with phloem-limited bacteria, *Candidatus Liberibacter* spp., is an important citrus disease affecting citrus production worldwide. Genetic transformation with genes that code for antibacterial peptides is an alternative to HBL management. Recently, *Citrus sinensis* cv. Hamlin, Valencia, and Pera plants were regenerated from genetic transformation experiments via *Agrobacterium tumefaciens*, with gene constructs containing the antibacterial attacin A gene (*atta*) under the control of phloem-specific promoters (AtSuc2, AtPP2, or CsPP2). The aim of this study was to perform the molecular characterization of these plants to confirm the transgene integration and expression. The *atta* gene integration was verified by Southern blot analyses and the transgene expression was quantified by RT-qPCR analyses, using F-box and ubiquitin as reference genes. The *atta*

integration was confirmed in the regenerated plants. The *attA* expression levels vary among different transformation events. Plants that showed high gene expression levels were propagated and inoculated with the pathogen in order to study the influence of *attA* gene expression in the pathogen multiplication.

Using Monte Carlo simulation to examine the economic cost and impact of HLB

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Crop budgets are a useful and integral tool for producers in making sound business decisions, although not without shortcomings. Typically, crop and enterprise budgets are static and examine prices at one point in time. In order to assess changing prices, for inputs and or production, it is typical to examine a range of scenarios – “Best Case”, “Worst Case”, and “Most Likely”. Utilizing Monte Carlo simulation, the static framework associated with budgets can be examined in a dynamic stochastic framework providing more accurate and rigorous analysis for producers. Data from citrus budgets for Florida producers is examined utilizing @Risk to generate Monte Carlo simulations of input and output prices. The stochastic results indicate the relative impacts associated with the respective changes in input and product prices. This framework identifies and allows producers to focus on input prices that have the largest impact, both positive and negative, on the firm’s gross and net returns. Additionally, dynamic product prices can provide guidance for producers with respect to establishing pricing targets risk minimizing strategies. The Florida analysis can provide a basis for examination and application for producers in Texas, Arizona, and California to gain insight into the key inputs and the impacts of these input prices. This is of utmost concern for areas that are establishing Citrus Health Management Areas (CHMAs). Dynamic stochastic budget analysis highlights the benefits from controlling input prices on those inputs that have the greatest economic impact on cost and returns.

Economic analysis of orange groves in the State of Sao Paulo under the impact of Huanglongbing

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Brazil is the World leader in the production of frozen and concentrated orange juice. However, the Brazilian citrus industry is threatened by Huanglongbing (HLB or greening) which is considered the most devastating citrus disease worldwide. Since its discovery in 2004, in the central region of the State of São Paulo (SP), HLB has been increasing in adjacent citrus regions. The HLB decreases productivity and can make trees unproductive, reducing the orchard life span and endangering the economic viability. The current management for the control of the disease is not enough to contain the spread of HLB in SP, especially regarding the late adoption by most producers since the onset of infection. This situation has forced the migration of orchards to other regions, aiming at economic viability through healthier plantings, while increasing transportation costs from production to processing industries located in the center of SP. There are doubts about the return on investment in new orchards in regions with high rates of infection, and there are few economic viability studies that can serve as tools for decision making for the investor. The objective was to calculate the economic viability of orange production in a region at high risk of HLB, within several proposed scenarios, including: economic return (based on the price of orange box), and the level of disease pressure (L = Low, M = Medium, and H = High pressure, according to the Gompertz model). Investment analysis was carried out for each proposed scenario and sensitivity analysis, adopting minimum real rate of attractiveness of 6%. Prices per 90 lb orange box, paid to producers and transported to the industry, necessary to ensure positive return on investment analysis are, within each proposed scenario: scenario L – US\$6.35 (BR\$14.14); scenario M – US\$6.53 (BR\$14.54); and scenario H – US\$6.93 (BR\$15.44), considered US\$1.00 = BR\$2.2281 on 07/03/2014.

Spectroscopic analysis for the rapid prediagnosis of citrus Huanglongbing in México

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Spectroscopy is a novel and useful bio-analytical technique, nondestructive, that allows the characterization of biochemical processes and identification of compounds. The method can be performed on living plants for characterizing the chemical structure of biological tissues; additionally, the spectrum obtained allows differentiation of the biochemical state of organs and cells, allowing distinguishing between normal and diseased tissue. The Coordination for the Innovation and Application of Science and Technology (CIACyT-UASLP) has been studying the citrus Huanglongbing by spectroscopy. The study was supported with the participation of Plant Health Committees who collected citrus samples from 116 trees of orange and lemon from Colima, Jalisco (HLB diseased), Veracruz and San Luis Potosí (Healthy). The samples were analyzed by qPCR in the laboratory of ENECUSA/CNRF according to the standard procedures and additionally the samples were analyzed by spectroscopy by LaNGIF. It was obtained approximately 2000 spectra; the data were preprocessed (normalization) and analyzed by Principal Component Analysis (PCA) considering the covariance matrix. Preliminary results of the PCA that best describes the phenomena showed separability of the symptomatic and asymptomatic HLB diseased and healthy trees. The qPCR results were similar to those obtained by spectroscopic analysis. The spectroscopic analysis, combined with a statistical PCA, confers a high degree of reliability in the pre-diagnosis of Huanglongbing disease widely distributed in México. The procedure is very low cost, and provides immediate results, in contrast to conventional methods of molecular analysis.

Productivity of HLB-infected trees enhanced by vector control of ACP and foliar nutrition

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Huanglongbing (HLB) has been endemic in Florida since 2005 and spread in the last decade throughout the commercial citrus-growing region, with incidence approaching 100% in many blocks. Statewide yields have decreased and production increased over the intervening years. Consequently, prices have increased and some operations continue to be profitable through aggressive psyllid control by insecticides supplemented by enhanced foliar nutritional sprays to compensate for reduced translocation of soil nutrients due to compromised root function. Nevertheless, the relative value of these practices to economically maintain yields is still subject to debate. A replicated field study employing a factorial design was initiated in 2008 in a 5.4-ha commercial block of young ‘Valencia’ orange trees to evaluate individual and combined effects of foliar nutrition and insecticide treatments. This report summarizes results over 6 years, supplementing a published account of the first 4 years presented at this meeting in 2012. Insecticide applications twice during the winter “dormant” season and subsequently threshold driven have consistently maintained significantly distinct ACP populations in treated and untreated plots, although with little effect on HLB incidence which has been close to 100% the last 5 years. Average threshold cycle (Ct) has generally decreased over time but with curiously higher values recently in untreated trees. Insecticide and nutrition factors continue to significantly affect production, with highest yields obtained from plots receiving insecticides plus foliar nutrients followed by those receiving only insecticides. Trees receiving no insecticide applications were least productive, irrespective of the last 2 years of the nutritional program. No significant differences were found in juice quality measures among treatments. After 6 years of treatments, the combined effects of foliar nutrition and insecticide treatments resulted in the most profitable

approach for juice prices over \$3/kg of solids; prices frequently reached during recent seasons.

The Citrus Greening (HLB) Bibliographical Database

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The Citrus Greening (HLB) Bibliographical Database was created in 2009 by the entomology group at the Southwest Florida Research and Education Center (SWFREC/IFAS) in collaboration with the Florida Center for Library Automation at the University of Florida and currently funded by the Citrus Research Board (CRB). The objective of the Greening Database is to centralize published information related to HLB, the vectors of *Candidatus Liberibacter* species, *Diaphorina citri* and *Trioza erytrea*, effects of the disease on plants and vectors, and management strategies for vectors and disease. It includes current and historic refereed and non-refereed publications, proceedings, presentations, reports, extension publications, periodicals, dissertations, book chapters, and abstracts. Entries are primarily in English, but Spanish, Portuguese, French, Japanese, and Chinese documents are also included. The database is continually updated and currently contains more than 3,400 entries of which 92% are linked to original sources. The database is open access and free of commercial content, thus facilitating bibliographical research tasks to any person or entity. It can quickly be found at www.imok.ufl.edu or by searching "HLB database" in Google. Basic searches can be conducted on key words of author, subject, title, or journal and advance searches on three different key words. Thus, the Greening Database provides easy access to a wealth of information useful to growers, consultants, researchers, and students interested in HLB related topics.

Distribution of *Candidatus Liberibacter asiaticus* in roots of Sour orange rootstock grafted with Valencia Sweet orange in Texas

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Huanglongbing (HLB), a disease with no known cure, has crippled citrus industries worldwide, and currently poses a serious threat to the survival of Texas citrus. HLB is associated with the phloem-limited gram-negative α -proteobacteria, *Candidatus Liberibacter* spp. The bacterium is unevenly distributed in the tree canopy and disease symptoms are commonly confused with general nutrient deficiencies and environmental stress which contribute to difficulty in identification. We found that roots serve as a more reliable diagnostic sample compared to leaves. The purpose of this study was to study the horizontal and vertical distribution of CLAs in roots of field grown sour orange rootstock grafted with Valencia sweet orange (8-year-old). Fibrous roots were collected from five HLB-infected trees, including asymptomatic trees, at many points; the samples included surface roots running horizontally up to 6 meters from the trunk and roots growing vertically down up to 1.5 m in depth. The results show that CLAs is evenly distributed horizontally and vertically in the root system, even in trees that were asymptomatic and PCR negative in the canopy. In addition to the distribution of CLAs, several DNA extraction and DNA amplification methods including conventional PCR (cPCR), quantitative PCR (qPCR), and Loop mediated isothermal amplification (LAMP) were employed in root and leaf samples to determine their efficacy. Comparison of qPCR and LAMP for their efficacy in CLAs detection in washed versus non-washed roots showed no difference.

Effect of the control of *Diaphorina citri* on the incidence of Huanglongbing disease and yield of Mexican lime in the state of Colima, Mexico

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The study was carried out in the state of Colima, Mexico, from April 2010 to December 2012, using a Mexican lime plantation that was 4 years old. The orchard had two blocks with a different Mexican lime variety for each. These varieties were 'Colimex' (thorned trees, seeded

fruit) and 'Lise' (thornless trees, seeded fruit). The populations of *Diaphorina citri* adults were monitored using yellow sticky traps and registering captures every 2 weeks. The management activities of the plantation were recorded. The plantation was inspected every month in order to determine the incidence of 'Huanglongbing' (HLB), which was confirmed by PCR. Also, the rainfall and temperature data were obtained. The results showed that the major vector populations occurred from November to May and the lowest populations from June to October during the rainy season. The effect of the rain and chemical control reduced the populations of HLB vector. During the first year occurred the maximum catch in 2 weeks that was 31.8 insects per trap in March 2010. From July to early October 2010, less than one insect per trap was registered. In the second year, the minimum capture occurred from September to October 2011 recording 0.2 to 0.6 insects per trap. The temperature did not present high variations (22–28°C) and had no influence on the populations of *D. citri*. The first trees with HLB symptoms were detected until May 2011, registering 0.34% disease incidence, which got a value of 100% sixteen months after the first HLB detection, and the yield reduction started to occur in 2012. The yields of variety Lise were 48.7 and 39.3 tons per hectare in 2011 and 2012, respectively, while the yield of variety Colimex was 42.0 tons in 2011 and 39.0 tons per hectare in 2012.

Analysis of a phytosanitary policy to control HLB within Key lime in Tecoman in the Mexican state of Colima (*Citrus aurantifolia* Swingle)

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Huanglongbing (HLB) or citrus greening is a disease that has spread worldwide and significantly adversely impacts citrus production. HLB first appeared in the Mexican state of Colima in April 2010. Despite a strong and well-grounded Federal strategy, HLB spread rapidly throughout the key lime groves in Colima. The Federal strategy was based on the international recommendations from and the US which promoted a three-part strategy: (A) eradication of symptomatic trees, (B) replanting trees from a certified nursery, and (C) controlling the population of ACP (Asiatic citrus psyllid), the vector of HLB. This paper documents the efforts of many agencies who were involved with the government effort, including the ministry of Agriculture SAGARPA under its different agencies such as the Agribusiness Subsecretariat, INIFAP, SENASICA, and the international agency IICA. These agencies worked to communicate and organize key lime growers to implement preventive and corrective measures during the phytosanitary crisis. Further, these agencies worked to integrate different levels of participants such as citrus growers and scouts (workers whose function is recognizing symptomatic plants and vectors), collaborated on identifying diseased plants, eradicating them and controlling vector's population. This paper explores the grower responses to the Federal government's efforts; important issues emerged while defining a symptomatic tree, even with a PCR analysis; the struggles for removing symptomatic but unproductive trees contributed to the whole worsening of the problem. The method adopted for this research was the Economic Analysis on Representative farms (Citrus RPU). The results allowed observing the economic viability under a before and after HLB economic scenario. In conclusion, the work enables understanding the constraints of the decision making process and its influence on aspects present while applying a Federal strategy on technical and collaborative solutions to the citrus groves.

Genes related to huanglongbing tolerance from transcriptome profiles of tolerant 'Jackson' grapefruit hybrid and susceptible 'Marsh' grapefruit

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Huanglongbing (HLB) is currently the most destructive disease of citrus worldwide. In this study, we examined the expression differences between HLB-tolerant 'Jackson' grapefruit hybrid and susceptible 'Marsh' grapefruit after HLB infection using RNA-seq. A total of 686

differentially expressed (DE) genes between two groups using FDR threshold of 0.1 were identified. Among them, 247 genes were up-regulated and 439 were down-regulated in tolerant citrus trees. We performed Gene Ontology (GO) enrichment analysis of DE genes. Genes associated with beta-amyrin synthase, cycloartenol synthase and Camelliol C synthase were significantly up-regulated in the HLB tolerant citrus trees while terpene synthase genes (CiClev10014707, CiClev10017785) were down-regulated in the tolerant citrus trees. Some PR-protein genes were significantly up-regulated in the resistant citrus trees, including several TIR-NBS-LRR genes. Many cell wall degradation-related genes, such as cellulose synthase/transferase, cellulase and expansins were up-regulated in the susceptible citrus trees. Some glucan hydrolase genes were also up-regulated in the resistant citrus trees. These genes may play important roles in symptom development. The DE genes were also enriched in two classes of RLKs, LRR-RLKs, and DUF26-RLKs. We have experimentally verified the expressions of 14 up-regulated genes and 20 down-regulated genes on three HLB-tolerant ‘Jackson’ and three HLB-susceptible ‘Marsh’ trees using real time PCR. Eleven of 14 up-regulated genes and 18 of 20 down-regulated genes were validated. Further characterization is underway for these differentially expressed genes and their potential roles in HLB progression.

HLB in Southern Paraguay. Current status of the outbreak

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Huanglongbing was detected in 2009 in the state of Parana, Brazil, at 40 km distance from the Paraguay border in the Northeast of the country. Survey of *Diaphorina citri* and HLB symptoms were intensified from that moment in that region and the rest of the country. Citrus and *Murraya paniculata* plants were inspected by the SENAVE personnel with the collaboration of the private sector, especially those from the Social Program for citrus planting for small growers. A total of 4000 ha of orange and grapefruit from 2700 small farmers and residential plants were assayed. These were in a 100 km radius in southern and central Itapua, Misiones, and Caazapa all Paraguay regions. Low incidence of *D. citri* was found, being in spring and fall the highest population. In March 2013 were detected the first positive plants in Paraguay in the Northeast of the country. In the region of Itapua (Southeastern region of Paraguay), 14 foci were detected in late fall and early winter of 2013; each focus consisted of 1–30 plants and they were separated by 1–20 km among them. All trees were 5 to 10 years old. Two plants of Rangpur lime were found in residences. The diagnosis was by conventional and real time PCR. All positive plants were eradicated as was done in the rest of the country. An active and strong program was initiated to control the insect vector with different insecticides and to survey the groves in a continuous form. In fall 2014, only three new positive plants were found in the previous foci and only one plant was in a new focus. The survey is at this time intensive with enforcement of protected nurseries and vector control.

Comparative genomics of multiple *Candidatus Liberibacter asiaticus* isolates reveals genetic diversity in Florida and provides clues to the evolution of the bacteria in citrus

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Understanding genetic diversity within and among the populations of an organism provides information about the potential diversity in pathogenicity and susceptibility to host defenses as well as sustainable effectiveness of control treatments. A near whole genome sequencing strategy was used to conduct a genome diversity study of US *Candidatus Liberibacter asiaticus* (CLAs). Two thousand PCR primer pairs were designed using the PSY62 genome. Overlapping CLAs genome fragments were amplified from total DNA of infected citrus from 35 locations (28 from Florida, 1 from California, 2 from Texas, and 4 international samples). All amplicons from each isolate were pooled as a

“pseudogenome” and analyzed by NGS (Ion Torrent). Sequences were aligned to the PSY62 reference sequence and compared. On average, the isolate pseudogenomes covered 86.8% of the reference genome at an average of 740X coverage of each nucleotide. A total of 14,342 variations were identified, including 13,319 single nucleotide variations (SNVs) and 746 indels. Most of the prophage regions failed to amplify in only three isolates (California, Japan, and Florida isolate FLCG-12). Three phylogenetically distinct genetic clusters of isolates were observed in Florida, with the Texas and California isolates being distinct but included in one of the three clusters. Seven isolates from adjoining trees in a single Florida grove, all clustered with PSY62, but displayed a total of 2189 variations, with non-reference alleles specific to each isolate and/or specific to this geographic group, suggesting rapid accumulation of variations. This analysis supports recombination and horizontal gene transfer between haplotypes within and among clusters. Among the variations, there was an excess of harmful mutations expected to be deleterious such as open reading frame shifts, stop codon gains, or start codon losses. Based on results, we hypothesize that genetic drift plays an important role in the evolution of this bacteria and could explain the fast accumulation of genetic variations.

The interaction between *Phytophthora nicotianae* and ‘*Candidatus Liberibacter asiaticus*’ damage to citrus fibrous roots

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Phytophthora nicotianae (*P.n.*) causes root rot of citrus, damaging the fibrous roots which depletes carbohydrate reserves and reduces water and nutrient uptake capacity. Huanglongbing (HLB) is a systemic disease that disrupts photosynthate transport, and is caused by phloem-limited ‘*Candidatus Liberibacter asiaticus*’ (Las). Elevated *Phytophthora* populations were reported as HLB spread throughout Florida citrus groves. To understand the possible interaction between these two pathogens and host tolerance, two rootstocks—Cleopatra mandarin (*Citrus reticulata*) and sour orange (*Citrus aurantium*)—were inoculated with Las, *P.n.*, both, or neither. *P.n.* infection, root loss, and carbohydrate content of fibrous roots were assayed. *P.n.* infection increased on both rootstocks indicating that Las reduced their tolerance to *P.n.* Differences in *P.n.* infection on Las positive seedlings at 5 and 11 weeks after inoculation suggest the interaction changed over time. Both pathogens caused significant root loss alone, but Las in combination with *P.n.* did not cause additional root loss compared to Las alone. Based on these results, we hypothesize that (1) early in disease development, Las increases susceptibility to *P.n.* infection by increasing zoospore attraction or facilitating penetration; (2) Las induces root loss resulting in a temporary drop in *P.n.* population by reducing available food supply; and (3) As new root flushes occur, *P.n.* starts the infection cycle again and this cycle repeats until there is a complete loss of fibrous root system.

Use of acibenzolar S-methyl to delay infection of *Ca. L. asiaticus* infection in sweet orange

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We evaluated HLB infection associated with *Ca. L. asiaticus* (Las) and the ability of resistance inducers (RI) to prevent or delay infection in nursery citrus trees. The first experiment aimed to evaluate the potential of salicylic acid, acybenzolar S-methyl (ASM), jasmonic acid, methyl jasmonate, gentisic acid, yeast mannan, citrus acids (commercial formulation) and hydrogen peroxide to induce resistance, avoiding Las-infection (n = 23 to 32). RI were applied four times after graft inoculation, every 20 days as foliar applications. Leaf sampling for Las detection was carried out every 30 days for 7 months. ASM reduced the percentage of Las-infected leaves from 60 to 210 days after inoculation (DAI). At 180 DAI, reduction of Las infection reached 58.5%. Other RI had no effect on Las infection. A second experiment evaluated the effect of ASM at rates of 10, 20, and 40 mg (four sprays). After 90 days of grafting, 90% of the non-treated trees were infected. Conversely, the highest rate of ASM prevented leaf infection in 50% of trees. After pruning, Las was detected in mature shoots from the majority of trees, without distinction of RI treatment (experiments 1 and 2). Additional

experiments assessed titer of Las in affected tree as well as the activation of resistance mechanisms. After infection, Las titer was similar in both ASM-treated and non-treated trees. Las titer increased over time. There was a slight reduction in Las titer when ASM was applied on HLB-symptomatic trees. Peroxidases, Polyphenol oxidases, Glucanases, and Quitinases activities showed a similar trend, irrespective of the ASM application. Overall, ASM delayed but not prevented infection of Las in sweet orange leaves, without affecting Las titer significantly. A field trial with 14 thousand trees that was set up in September 2011 to evaluate the effect of ASM on HLB epidemics is underway.

Genetically-modified *Citrus* plants with potential to control HLB disease

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HLB or citrus greening is a devastating disease in *Citrus* plants associated with the presence of *Candidatus Liberibacter* spp., an endogenous, sieve tube-restricted bacterium that is transmitted by the psyllid vector *Diaphorina citri*. Genetically-modified Mexican lime was transformed with genes coding for antimicrobial peptides, targeted to the *Citrus* vascular tissue. Both stable and transient gene expression was achieved by using the *Agrobacterium tumefaciens*-transformation method. mRNAs coding for the antimicrobial peptides, as well as the peptides themselves, were detected in functional phloem (i.e., mature sieve elements). GM plants expressing the aforementioned antimicrobial proteins were challenged in greenhouses with infectious psyllids collected from HLB-endemic areas. The quantification of live/dead *Candidatus Liberibacter asiaticus* (CLa) was achieved using real time PCR and digital PCR. The symptoms, as well as the bacterial titer, were significantly lower in GM plants when compared to non-GM isogenic lines. While GM Citrus should be evaluated in open field trials to assess the degree to which CLa could be controlled under natural infectious conditions, GM Citrus represents a suitable strategy to control HLB in Mexico, and likely worldwide.

Digital gene expression analysis of Huanglongbing infected Mandarins responding to heat treatment

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“*Candidatus Liberibacter asiaticus*” is the leading cause of Huanglongbing (HLB) in world citrus production. Heat treatment with high humidity has proved to be effective to eliminate the “*Ca. L. asiaticus*” titers of potted citrus trees. In this work, we attempted to figure out the curative mechanisms of heat treatment on Mandarin trees seriously infected by HLB. Comparison of transcriptome levels was done between heat treated and non-treated trees using digital gene expression (DGE) system. Our results showed that DGE sequencing outcomes were high quality and consistent between biological replications. Totally, 838 differentially expressed genes (DEGs) were obtained with 510 of which were down-regulated. The Gene Ontology (GO) analysis revealed that mainly enriched GO terms due to the heat treatment were oxidation-reduction process (biological process, BP), Chitin catabolic process (BP), cell redox homeostasis (BP), electron carrier activity (molecular function, MF), heme/iron ion/metal ion binding (MF), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (MF), and cell wall (cellular component). GO analysis of the up-regulated genes showed the GO terms with these descriptions were abundant: oxidation reduction, RNA modification, cell redox homeostasis, metabolic process, response to inorganic substance/abiotic stimulus/stress, and homeostatic process; while the down-regulated genes were related to transport, oxidation reduction, oxidation reduction, carbohydrate metabolic process, response to stimulus, and biological regulation. Strikingly, 141 genes encoding pentatricopeptide repeat-containing proteins involved in mitochondrion and RNA modification were all up-regulated. Whereas, the transcripts of

nine Auxin-induced protein genes were depressed, suggesting the effect of heat treatment on plant growth. Pathway analysis of the heat treatment induced DEGs indicated that phenylpropanoid/flavonoid/stilbenoid, diarylheptanoid and gingerol biosynthesis, biosynthesis of secondary/starch and sucrose/phenylalanine/galactose/porphyrin and chlorophyll metabolites, and plant-pathogen interaction were enriched. Our work provided a comprehensive basis for a better understanding of the curative mechanisms of heat treatment on HLB trees and the “*Ca. L. asiaticus*” biological regulation.

Transcriptional analyses of mandarins seriously infected by “*Candidatus Liberibacter asiaticus*”

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A range of leaf symptoms can be caused by Huanglongbing (HLB), a worldwide destructive disease on citrus, including blotchy mottle, yellowing, and small upright leaves with a variety of chlorotic patterns resembling those induced by zinc deficiencies. HLB is suggested to be caused by the phloem-limited fastidious prokaryotic α -proteobacterium “*Candidatus Liberibacter* spp.” Previous studies focused on the proteome and transcriptome analyses of citrus 5 to 35 weeks after “*Ca. L. spp*” inoculation. In this study, gene expression profiles were analyzed using mandarin of “Jiaogan” leaves after 2-year infection with “*Ca. L. asiaticus*”. Affymetrix microarray analysis explored a total of 2017 differentially expressed genes (DEGs), with 938 (46.5%) up-regulated. The 1364 genes in this extremely late infection stage with known functions were related to plant defense/stress response (21.63% of the total), carbohydrate metabolism (7.33%), photosynthesis (4.03%), plant growth and development (7.04%), structure related (5.94%), signaling transduction/translation factors (13.78%), transport (10.56%), oxidation-reduction process (3.20%), Other metabolic process (12.76%), and others (14.22%). Categories of genes related to photosynthesis, carbohydrate metabolic, and structure were mostly down-regulated, with rates of 92.7%, 61.0%, and 80.2% respectively. Whereas, 75.0% and 64.6% genes associated with oxidation-reduction process and transport, respectively, were up-regulated. Genes like ethylene responsive element binding protein, ethylene response factor, and ethylene-induced esterase were down-regulated, suggesting a depression of this signaling pathway after “*Ca. L. asiaticus*” infection. This study provides an enhanced insight into the host response of citrus to “*Ca. L. asiaticus*” infection at an extremely late stage.

Direct sequencing to obtain whole 16S rRNA gene sequence of ‘*Candidatus Liberibacter asiaticus*’ from environmental samples affected by citrus Huanglongbing

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Citrus Huanglongbing (HLB) (ex. Greening) is associated with three known species of ‘*Candidatus Liberibacter*’: ‘*Ca. L. asiaticus*’ (Las), ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’. Their 16S rRNA gene sequences have been used extensively in taxonomic identification and disease diagnosis. Partial 16S rRNA gene sequences are routinely obtained through the time-consuming cloning of the conventional PCR amplicon (1.1 kb) of OI1/OI2c, and the forward primer is located in the species signature region of the partial 16S rRNA gene sequence, which makes the sequenced amplicon less useful. Analyzing various Las strains’ full 16S rDNA sequences, including their upstream/downstream sequences, we have successfully designed and validated two novel PCR primers to amplify a 1.8 kb fragment containing the full-length 16S rRNA gene, and four internal sequencing primers for directly sequencing the fragment, using HLB-Las infected plant samples collected in the field in various states of the US All sequences obtained from the HLB-infected environmental samples were 100% identical in pairwise alignment to corresponding sequences of the NCBI reference genome (NC_012985.3). No bacterial sequences were obtained by the new PCR and direct sequencing primers from any HLB-negative samples collected in the field or from healthy greenhouse plants. The sensitivity of the 1.8 kb fragment PCR amplification and the sensitivity of the direct sequencing were the same as of the 1.1 kb fragment PCR amplification and the direct sequencing with OI1/OI2c. Whole 16S rRNA gene sequences were obtained by the new method from infected

citrus tissues with initial symptoms which produced Ct values of 31 for the 16S rDNA-based real-time PCR and had the equivalent of about 10^5 bacterial genomes (cells) per gram of fresh tissue. Reliable sequences could be acquired by direct sequencing from PCR amplifications without running a gel.

Effect of Huanglongbing on volatile components in peel oil of 'Valencia' oranges

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Citrus peel oil is widely utilized in food, cosmetics, and even the electronics industry. Orange fruit and juice from Huanglongbing (HLB) affected trees have been reported to be off-flavored, and this is the first report on volatile components of citrus peel oil affected by HLB disease. 'Valencia' oranges were harvested from commercial groves in South Florida. Fruit samples (26), each obtained from 3-5 visually HLB symptomatic or asymptomatic trees, were harvested and processed for juice, and peel was cold-pressed to extract oil. The juice of each sample was analyzed by qPCR for the HLB presumed pathogen (*Candidatus Liberibacter asiaticus*, CLAs) DNA, and cold-pressed peel oil volatile components were analyzed by gas chromatography—mass spectrometry (GC-MS). Based on qPCR Ct values using Li and LJ primers, samples were divided into three groups: 1) healthy (HLBh, 8 out of 15 asymptomatic samples confirmed as CLAs free), 2) HLB severe (HLBs, 5 out of 11 symptomatic HLB samples were confirmed with high CLAs titer), and the rest of the samples that were possibly HLB affected (HLBp), with low CLAs titer (6 symptomatic and 7 asymptomatic). A total of 57 volatile compounds were identified in peel oil samples, including 9 monoterpenes, 16 sesquiterpenes, 12 alcohols, 13 aldehydes, 1 alkane, 2 ketones, 2 esters, and 2 terpene oxides. Of those, 14 compounds were found to be significantly different among the three groups. Hexanal, (E,E)-2,4-decadienal, δ -cadinene, and α -copaene were significantly lower in HLBs samples than in the healthy samples, while sabinene, (E)-p-mentha-2,8-dien-1-ol, α -terpineneol, 3,7-dimethyl-6-octen-1-ol, (Z)-3,7-dimethyl-2,6-octadien-1-ol, carvone, cyclodecane, β -cubebene, (E)- β -farnesene, α -humulene, and α -farnesene were significantly higher in HLBs samples. The contents of those volatiles in HLBp were in-between. Principal component analysis discriminated HLBs from HLBh samples in PC2, with HLBp samples not separated from either HLBh or HLBs.

Optimization of an efficient transcuticular delivery system for control of citrus Huanglongbing

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We experimentally developed and optimized a transcuticular nanodelivery system for enhancing permeation of effective compounds against the HLB disease through citrus cuticles into the phloem by foliar spray or bark application. The results showed that two kinds of nanoemulsions (W/O and O/W) with the smallest drop size of 13.68 ± 0.26 nm were prepared for loading the antimicrobial compounds. The results also indicated that cuticles were more difficult to isolate from "Valencia" orange (*Citrus sinensis*) and from HLB-symptomatic leaves. Brij 35 was screened from a pool of eight candidate penetrants as an optimal penetrant for the HLB-affected cuticle, exhibiting an approximately 3-fold enhancement of cuticular permeability over the water control. Amp was loaded into nanoemulsions and coupled with Brij 35, resulted in higher inhibitory zone diameters (5.75 mm and 6.66 mm), when compared with those in the Amp loaded into nanoemulsions (3.02 mm and 4.90 mm), or Brij35 (4.34 mm) and Amp solution alone (2.83 mm) using transcuticular movement bioassay. And the above formulations more efficiently eliminated the *Candidatus Liberibacter asiaticus* in the potted-contained HLB-affected citrus *in planta*.

Field evaluation of tolerance to Huanglongbing (HLB) in *Citrus* × *Poncirus trifoliata* hybrids

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This study assessed the response of 116 accessions in a field trial at Fort Pierce, FL. A randomized single tree plot design with eight replications was used. Approximately 75% of the accessions tested were citrange progenies from crosses between sweet orange (*Citrus sinensis*) and *Poncirus trifoliata*, and the remainder included sweet oranges, pure *Poncirus*, and other hybrids, Volkamer lemon, and other miscellaneous citrus accessions. Stem diameter, HLB, and citrus canker symptom severity, and CLAs titer were determined in May and September of 2013, and May and August 2014 in a monitoring population. All 912 trees in the field were diagnosed for HLB by using RT-qPCR. The HLB infection rate was increased from 47% in October of 2013 and 49% in May 2014 to 61% of August 2014. Overall, the percentage of trees detected positive for CLAs was 100% for Navel sweet orange, 45% for Volkamer lemon, 67% for Temple, 50% for Carrizo citrange, and 65% for the hybrid *Poncirus* progeny. However, there were no positive trees found among seedlings of three pure trifoliata accessions (Argentina, Rubidoux, and Flying Dragon). Among 86 of *Poncirus* hybrid progeny, only three were found without any HLB infection, whereas six was found with 100% infection; the remainder ranged between 12% and 89% infection. There were significant differences in HLB infection among accessions and time points ($p < 0.001$). However, no clear relationships were found among stem diameter, canker, or HLB symptoms with the percentage of HLB positive trees. This study suggested that the phenotypic characteristics of tree growth and disease symptoms are not necessarily indicative of the true HLB infection rates.

Extension project for improvements of King mandarin cultivation under severe Huanglongbing circumstance in southern Vietnam

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Since October 2009, JICA and SOFRI have carried out the cooperative project for extension of appropriate techniques of King mandarin (*Citrus nobilis*) to citrus growers in southern Vietnam. In this region, most citrus trees are infected by Huanglongbing (HLB) within 2 years after being planting. Our project extended appropriate cultivation techniques to citrus growers for the improvement of King mandarin fruit yield in five provinces of southern Vietnam. The techniques consisted of (1) 4 m planting distance which is wider than the usual one (ca. 1.5 m), (2) training and pruning for lowering tree height, and (3) periodical neonicotinoid applications to control the vector, Asian citrus psyllid (*Diaphorina citri*). For the first step of the technique extension, we provided training courses to extension officers of five local governments. Secondary, the trained officers transferred the techniques to citrus growers one to three times per month and had monthly meetings, in which they discussed technical problems and gave suggestions to the growers. HLB infection in five farms was eventually lowered, 5.1 to 19.1% in 42 months, compared to other non-trained farms, usually > ca. 50%. The former attained a fruit yield of 34.6 kg/tree on average, being higher than the latter, 10 kg/tree. Nonetheless, two farms of our project suffered from heavy invasion of the disease, reaching 41.5 to 57.0% tree infection in the corresponding period. Asking growers, we suspect that the failure in the cultivation due to high disease infection could be attributed to the delay in the insecticide application and the management of pre-existing infected trees around or near the farms. The success of the cultivation thus depends on transferring of precise techniques by the extension officers and practice of correct techniques by the growers.

No evidence of the Huanglongbing transmission on young King mandarin tree by using scissors used for infected trees

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In the JICA-SOFRI project for the extension of the appropriate cultivation techniques of King mandarin (*Citrus nobilis*), pruning of citrus trees is one of the key techniques to increase fruit yield. Growers might have used scissor shears which had been used for infected trees and consequently contaminated with the disease pathogen. We attempted to evaluate the risk of the disease transmission to intact trees by using such contaminated scissor by experiments, in which we examined (1) detection of the HLB pathogen on the blades of scissors used to cut HLB-positive blanches of King mandarin trees; (2) changing both the number of the cuttings; (3) leaving the scissors for a fixed time after the cuttings; and (4) either treating the scissor for disinfection after the cutting or not. The HLB pathogen was detected only on blades used 15 times for cutting HLB-positive blanches, whereas no HLB transmission was confirmed one year for any scissors. The results indicate that HLB transmission to intact trees was unlikely to occur in ordinary uses of scissors that had been used for cutting HLB-infected trees.

Field trials of the integrated approach to control citrus Huanglongbing in Florida

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Developing strategies/approaches for managing HLB-affected trees in the field is the most urgent need facing the Florida citrus industry. Based on our screened compounds and optimized nanoemulsion formulations, three independent field trials were conducted using an integrated approach to combat citrus HLB, including: (i) Exp. 1: Four compounds of SDX, AMP, ZS, and VA were prepared and loaded in the W/O and O/W nanoformulation for trunk-injection using injectable bag and bark-application; (ii) Exp. 2: Antibiotics of SDX and AMP were prepared and loaded into the O/W nanoformulation coupled with insecticide and fertilizer by trunk-injection; and (iii) Exp. 3: Carv and PCY were prepared and loaded into W/O nanoformulation for foliar spray. Before application, all HLB-affected citrus were trimmed and pruned, and then fertilized in December 2013. All compounds were applied once every 2 months. Following initial treatments, all trees were tested for *Ca. L. asiaticus* using qPCR once every 2 months. The effects of the combined treatments on citrus will be investigated. The preliminary results showed that trunk-injection using injectable bags was good for water-soluble antibiotics. Uptake of one liter of solution in 24 hours could be accomplished by improving the injection technology. Compared to other compounds, Pen was one of the most effective to eliminate the Las bacterium. Bark application was good for SDX loaded into O/W nanoemulsion. Pen and SDX promoted the growth and emergence of new flushes or leaves with longer and more new branches. Foliar spray of Carv and PCY in W/O nanoemulsion were not effective to suppress the Las bacterium in the HLB-affected citrus. The results also indicated that supplemental insecticide or fertilizer in the injectable bag caused temporal phytotoxicity, thereby it was better to be applied by foliar spray.

Efficiency of chemotherapy coupled with thermotherapy against citrus HLB

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Six independent experiments were carried out to evaluate the effectiveness of the chemotherapy coupled with the thermotherapy on pot-contained HLB-affected plants based on our previous results from graft-based methods. Three-year-old potted HLB-affected citrus plants were exposed to four thermotherapy regimes in a CMP5000 growth chamber, with fluorescent lamps at 60% intensity, a 12-h photoperiod, and 50% relative humidity, including: (i) 12-h @ 40°C/12-h @ 30°C for one week; (ii) 12-h @ 42°C/12-h @ 30°C for one week; (iii) 12-h @ 45°C/12-h @ 30°C for one week; and (iv) 12-h @ 45°C/12-h @ 30°C for shoot, but the roots were kept at constant temperature of 25°C for one week. After heat treatment, the plants were moved to the greenhouse for chemical treatments. A total of 18 chemical treatments from 10 compounds or their combinations were applied once every 2 weeks.

Water was used as chemical control. Following treatments, all trees were tested for *Ca. L. asiaticus* using qPCR each every 2 months. The results indicated that heat treatments promoted the growth of new flushes and leaves and eliminated the Las bacterium in the new leaves with increase of the treatment temperature. The chemical effectiveness of the tested compounds and their combinations against citrus was varied. Some compound combinations (ACT+ZS and ACT+VA) effectively eliminated the Las bacterium, which resulted in the recovered growth of the HLB-affected plants in the greenhouse.

Engineering mobile RNA in Carrizo to enhance plant defense responses in both rootstocks and nontransgenic mature scions to control citrus greening

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Programmed cell death (PCD) or apoptosis is a plant defense response that can be triggered upon pathogen infection. There are many apoptosis triggers such as Reactive Oxygen Species (ROS) which activate plant defense signaling pathways over long distances. There are also some anti-apoptosis proteins in plants which dampen plant defense responses against pathogens. By suppressing expression of these anti-apoptosis proteins, PCD should occur in a more rapid manner and with augmented plant defense responses upon pathogen infection. Huanglongbing (HLB) is arguably the most damaging disease on citrus and threatens citrus production worldwide. It is caused by *Ca. Liberibacter asiaticus* (Las), which grows strictly in the cytoplasm of living citrus phloem cells and produces a predicted peroxidase enzyme that may dampen the ROS signaling pathway. Here, we employed a gene silencing strategy to suppress an anti-apoptosis gene in Carrizo rootstocks in an attempt to enhance plant defense responses against Las. Five silencing clones were constructed and used to transform Carrizo rootstock. Average silencing efficiencies ranged from 77.30% to 82.35%. Using a Las flagellin protein fragment (flg22) as a proxy for Las inoculations, expression levels of three citrus defense response genes were compared in silenced plants and in nontransgenic (NT) controls after inoculating with 10 μM of the Las flg22 elicitor. The expression levels of all three citrus defense response genes were significantly higher in silenced lines than in NT controls: *NDR1* (Non-Race Specific Disease Resistance-1) was 6–30X higher, and both *PRI* (Pathogenesis Related-1) and *EDSI* (Enhanced Disease Susceptibility-1) were up to 6X higher. Selected lines have been grafted to mature Hamlin, Valencia, and Midsweet scions, and potential systemic movement of the silencing RNAs from rootstock to NT scions will be examined, and challenge inoculations with *Xanthomonas citri* (causal agent of citrus canker) and with Las are planned.

High incidence of pre-harvest colonization of *Diplodia* in HLB-symptomatic orange, its exacerbation of postharvest fruit decay, and implication for HLB-associated pre-harvest fruit drop

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Huanglongbing (HLB), presumably caused by *Candidatus Liberibacter asiaticus* (CLAs), is a devastating citrus disease associated with excessive pre-harvest fruit drop. *Diplodia natalensis* (*Diplodia*) is the causal organism of citrus stem end rot (SER). The pathogen infects citrus fruit under the calyx abscission zone (AZ-C), and is associated with ethylene and cell wall hydrolytic enzymes involved in abscission. By means of DNA sequencing, *Diplodia* DNA was found in CLAs positive juice from HLB-symptomatic fruit (S), but not in CLAs negative juice. Therefore, the incidence of *Diplodia* in on-tree fruit tissues and its impact on postharvest decay and HLB-associated pre-harvest fruit drop were investigated in Hamlin and Valencia oranges. Analysis using qPCR and primers specific for *Diplodia* β-tubulin gene revealed significantly ($p < 0.001$) greater incidence of *Diplodia* in AZ-C of the HLB-symptomatic (S, CLAs Ct value <30) than in asymptomatic (AS, CLAs Ct value ≥30) fruit. In agreement with the qPCR results, 2 weeks following exposure to ethylene, the incidence of SER in S fruit was 66.7% (Hamlin) and 58.7% (Valencia); while for AS fruit, the decay rates were 6.7% (Hamlin) and 5.3% (Valencia). Colonization of *Diplodia* in S fruit AZ-C was observed by scanning electron microscopy and confirmed by qPCR and morphology of conidia in isolates from the AZ-C after surface sterilization. *Diplodia* Ct values were negatively correlated with ethylene production in S fruit, and positively correlated with fruit detachment

force (FDF), suggesting that *Diplodia* colonization in AZ-C may contribute to the HLB-associated pre-harvest fruit drop. Therefore, a fungicide spray trial was conducted on Navel and Early Gold orange trees in an HLB-affected grove. The preliminary data showed lower *Diplodia* titers and higher FDF for fruit from sprayed trees compared to controls for both varieties, implying that the fungicide application to control *Diplodia* may alleviate the HLB-associated pre-harvest fruit drop problem.

Whole genome sequencing of “*Candidatus Liberibacter asiaticus*” Strain A4 from Guangdong, China, and Strain HHCA from California

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“*Candidatus Liberibacter asiaticus*” is associated with citrus Huanglongbing (HLB) in both China and the United States. While HLB has been known in Guangdong, China, for over a hundred years, the disease was not found in California until 2012. To better study the “old” and “new” HLBs, the whole genomes of “*Ca. L. asiaticus*” Strain A4 from Guangdong, and Strain HHCA from California were sequenced. The A4 genome was sequenced from an infected periwinkle plant through dodder transmission from an HLB-affected citrus. The HHCA genome was sequenced directly from infected citrus DNA. To overcome the problem of low bacterial titer and interference from host plant DNA, “*Ca. L. asiaticus*”-infected plant DNA was first treated with MBD2 protein to increase the bacteria/plant DNA ratio. Then, total DNA was enlarged through multiple displacement amplification and subjected to Illumina (MiSeq) sequencing. Sequence reads of “*Ca. L. asiaticus*” were identified through a standalone BLAST with the available “*Ca. L. asiaticus*” whole genome sequences as queries. Bacterial reads were extracted through a Perl script and assembled using Velvet 1.2.10 on a Linux platform. After annotation by RAST server, the A4 strain was determined to have a genome size of 1,208,625 bp, G+C content of 36.4%, 1,107 predicted open reading frames (ORFs), and 53 RNA genes, and the HHCA strain has the genome size of 1,118,244 bp, G+C content of 36.6%, 1,191 ORFs, and 51 RNA genes. Unlike the previously published psyllid-derived genome sequences, the sequences of both A4 and HHCA were directly from plant hosts. Strain A4 is currently maintained in a screen-house as a continuous source of “*Ca. L. asiaticus*” DNA for future research.