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Abstracts of Presentations

Abstracts presented at the 5th International Research Conference on Huanglongbing in Orlando, Florida, USA, March 14-17, 2017. 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: Aiken G, Gottwald TR, Kainz J, Poole GH, Truett J. 2017. Development of the Hydro-Solar Thermotherapy (HSTT) for treatment of HLB infected citrus [abstract]. J Cit Pathol. iocv_journalcitruspathology_34714.

Development of the Hydro-Solar Thermotherapy (HSTT) for treatment of HLB infected citrus

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The use of thermotherapy to treat HLB-infected citrus trees has been demonstrated by various systems over the past few years, which basically fall into two primary categories – passive solar systems (tents), and active heated water/steam spray systems. The system envisioned here is a combination of the two – a solar-heated water system, that is active but requires no power or user input, or expendable materials that need to be replenished. Circulating heated water is used to transfer heat to the tree by means of a thermal insolated jacket placed around the trees trunk, or on larger trees, around main scaffold branches. Multiple trees can be linked to the water source in series or in parallel subunits. Preliminary tests to determine the maximum temperature achievable along with feasibility of operation were done with a solar heating system, similar to the envisioned final design. Water temperatures in excess of 60C can be generated, and under bark temperatures reached the low to mid 40's. A pulsed system has also been tested which runs for only 5 hrs and produced higher temperatures at the trunk, but not in excess of 45C. Once the proof of concept was completed, tests beds were designed to determine the optimum temperature of operation. The first test bed was basic and only provided heated water at a constant temperature, for extended periods of time. Studies showed that the maximum temperature that a diseased tree could tolerate was 48-49C over 48hrs. The second test bed, known as the Mobile Thermotherapy Lab, or MTL, is more versatile and designed to provide heating and cooling of water to simulate what might be achieved through solar heating and supplemental cooling with a 40C differential in temperature. The goal of the MTL is to look more in depth at the parameters, and to test the effect of sequential heating and cooling on CLas bacteria survival in the phloem. Results indicate that a passive solar water heating system can produce higher temperatures under the bark of the trunk, as well as increased temperatures into the canopy. This heat generated is below the thermal tolerance level of the tree, but also may not be hot enough to significantly decrease the bacterial population in situ as determined by pre- and post-PCR assay. We will discuss the question, 'If thermotherapy does not decrease bacterial populations, why do treated trees display disease remission for multiple months?". Temperature of 49C would be the maximum usable temperature due to the thermal tolerance of citrus trees, however optimal duration at that temperature has not been determined.

Genetic diversity of the locus CLIBASIA 05640-05650 in strains of *Candidatus* Liberibacter from Mexico

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The bacterium associated with huanglongbing (HLB) in Mexico, *Candidatus* Liberibacter asiaticus (*C*Las) is present in 23 citrus growing regions, where its dissemination is due to the presence of its vector, *Diapohrina citri* Kuwayama (Hemiptera: Liviidae). In Mexico, some reports have documented differences in the severity of symptoms in infected trees according to the type of citrus (sour or sweet); those differences could be associated with *C*Las genetic variation. In order to probe this hypothesis, we analyzed a hypervariable region of the *C*Las genome from infected citrus leaves and *D. citri* adult samples from the Yucatán peninsula, and the Southeast of Mexico, as well as, the coastal zone of the Pacific Ocean and Northeast Mexico. Analysis of the samples yielded at least seven electrophoretic profiles; one is widely distributed among the states of the coastal zone of the Pacific Ocean and Northeast Mexico, and the rest is in the Yucatán peninsula and the Southeast part of the country. Our results suggest the presence of two *C*Las populations in Mexico, one of them showed genetic diversity and characteristically restricted to the Yucatán peninsula and the Southeast; meanwhile, the other lacks such diversity, the records suggest wide distribution in the country, and is infecting the Mexican lime in the citrus region of the Pacific Ocean. Our data provide insights into the understanding of the epidemiologic development of HLB in Mexico, and for the improvement of the current management strategies.

Micro-CT scanning of Asian citrus psyllid, *Diaphorina citri*, anatomy and feeding

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An international collaboration to establish an interactive Digital Video Library for a Systems Biology Approach to study Bacteria transmission by the Asian citrus Psyllid, linking psyllid genomic/proteomic interactions is demonstrated. Advances in micro-CT, digital computed tomography (CT) scan uses X-rays to make detailed pictures of structures inside of the body, can now visualize very small insects with great detail. Combining micro-CT imaging which permits digital section of an insect with Digital Video Library systems which links information from other databases enables a new visualization and interaction by the viewer. 'Interactive Information' systems are changing the way researchers, entomologist, and the public search for and use complex information. *Diaphorina citri*, spreads a plant-infecting bacterium linked to Citrus greening disease, a worldwide threat to citrus sustainability. The anatomy was elucidated using a high resolution Bruker Skyscan 1172 micro tomography system, micro-CT (www.Skyscan.be) Department of Zoology, University of Granada, Spain. Live psyllids were prepared by overnight fixation in 4% glutaraldehyde with 2.5% paraformaldehyde made with sodium cacodolate buffer pH 6.5. Samples were rinsed three times, 10 min each, with 30% ethanol, and dehydrated in an ethanol series, 30 min per step, 50%,70,80,90,95, three times at 100%. Samples were chemically dried by placing in 2 mL of 100% Hexamethyldisilazane (HDMDS) for 2 hrs, drying overnight at 35C. High resolution scans combined with computer software enabled rotation, 'digital sectioning', and coloring of specimens providing an innovative, interactive system to explore insect anatomy. Video http://www.youtube.com/watch?v=LLz5QWyNM98. In-depth explana-

tions of sample preparations of leafhoppers and other insects, plus operation of the software for the Bruker SkyScan system at: www.skyscan.be/company/usersmeeting2014a.

Changes in primary metabolism of citrus rootstocks following infection with *Candidatus* Liberibacter asiaticus

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A recent study investigated leaf metabolite profiles of six citrus rootstock cultivars grown as seedlings, with different responses to HLB using untargeted GC-TOF MS analysis. The study found that tolerance to HLB does not seem to be associated with an accumulation of larger amounts of protective metabolites in response to infection with *Candidatus* Liberibacter asiaticus (Las), but rather with different concentrations of specific metabolites independent of infection. The highest number (166) of metabolites responding to infection with Las at 12 months after graft inoculation (mai) was found for the susceptible rootstock cultivar Cleopatra mandarin (*Citrus reticulata*), but the majority of compounds (122) were of unknown chemical identity. The present study focuses on the chemically known metabolites and their involvement in the different pathways associated with primary metabolism. The study was conducted on greenhouse-grown rootstock seedlings at three different stages (8, 10 and 12 mai) of disease progression. Among the metabolites most strongly affected by Las in Cleopatra were those associated with arginine and proline metabolism, specifically the amino acids proline, hydroxyproline, ornithine, and citrulline. Numerous roles for proline have been suggested, and include osmoprotection, stress adaptation, reactive oxygen species (ROS) generation, and phytohormone-induced senescence. Other important functions of the amino acids related to this pathway are involvement in nitrogen metabolism and root architecture. Interestingly, whilst compounds of arginine and proline metabolism were up-regulated in response to Las at 8 and 10 mai, a significant down-regulation was observed at 12 mai, suggesting collapse of defense responses at this later stage of disease, and resulting in intensification of disease symptoms and plant decline. Also down-regulated at this stage of disease were metabolites associated with other sugar metabolism pathways, namely raffinose, galactinol, melibiose, sorbitol, glucose-1-phosphate, glucose, and fructose. Sugars are important components of plant signaling reactions and defense and provide important sources of energy for pathogens. Comparison of responses of Cleopatra with the more tolerant rootstock cultivar Carrizo citrange (*C. sinensis x Poncirus trifoliata*) showed a similar down regulation for several pathways at 12 mai, most notably pathways associated with arginine and proline metabolism, galactose metabolism, and propanoate metabolism. The similarity of metabolic responses of Cleopatra and Carrizo is surprising, but may be associated with the foliar disease symptoms which were similar in manifestation in both cultivars at this stage of infection. It is important to note that except for galactose metabolism, other pathways of carbohydrate metabolism as well as TCA cycle reactions were not affected in Carrizo, which may explain the better performance of this rootstock cultivar under HLB pressure. In contrast, the tolerant rootstocks US-897 and US-942 which did not show any disease symptoms at 12 mai responded to infection by changes in the amounts of only three metabolites. This suggests that different and rootstock-specific mechanisms are associated with tolerant responses to HLB.

Citrus flowering and vegetative growth characteristics in relationship to Asian citrus psyllid control in HLB management

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Due to concern for pollinating bees in citrus trees in the spring, spraying for psyllid control with harsher, but often more effective pesticides is suspended from when 5 to 10 % of the citrus flowers are open until 95 % petal fall is reached. After use of dormant sprays at least one spray on emerging flush is desired before bee-friendly sprays are applied during the 'flowering period'. Data was not available for how long a period existed from various stages of vegetative growth until 5 to 10 % open flowers is reached. Hamlin, Valencia, Sunburst and Murcott blocks were monitored during the 2015, 2016 and 2017 bloom periods to determine vegetative bud break, leaf feather stage and unfolded leaves as well as pinhead, popcorn, open and petal fall stages of flowering. In the first two years the average time period from bud break to 10 % open flowers was 39 and 45 days, respectively, and the days from 10 % leaf feathers until 10 % open flowers was 26 and 20 days. This provided 13 and 25 day intervals between bud break and feather leaves in the first two years. Round oranges and mandarins differed in time from bud break to full bloom in 2015, 72 versus 57 days, but were similar in 2016, 57 and 56 days. Petal fall (95 %) occurred 8 to 20 days after full bloom. Weather data will be evaluated to determine if any differences in time for vegetative development between the three years was due to daily temperatures, similar to the temperature response for flower development. More details including 2017 data will be discussed, particularly as pertaining to the best time to apply the first vegetative spray before the 10 % open flower stage is reached. The predicted date on which vegetative flush may be optimum for applying a psyllid spray could be obtained from the 'Citrus Flowering Monitor System', which is on-line: http://disc.ifas.ufl.edu/bloom. Another aspect of interaction between the citrus tree and HLB is the increase in off-season flowering. This is most pronounced in early- to mid-winter under Florida conditions and has consistently occurred over the last four years. This flowering reduces the available buds for normal spring flowering and probably contributes to carry over of fungal inoculum for postbloom fruit drop (PFD). The role of fall tree water stress on the induction of this off-season flowering, and the association of this tree water stress with debilitation from HLB will be discussed.

Comparison of distributions of psyllids and plants that were found positive for *Candidatus* Liberibacter asiaticus in Florida

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Diaphorina citri samples from many different venues in peninsular Florida were tested for presence of *Candidatus* Liberibacter asiaticus (Las) between 2005 and 2008. The positive samples were geocoded and plotted on a map of the state. Independently, suspect plant samples also were collected from many different venues and tested for Las at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry between 2005 and 2010. The positive samples also were geocoded. The distribution of positive plant samples was compared with the distribution of positive psyllid samples to determine whether the two sets of samples were correlated, and whether positive psyllids predict positive plants or vice versa.

Evidence for a dominant huanglongbing tolerance gene in citrus

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Huanglongbing (HLB) is a devastating bacterial disease of citrus and has been detected in most citrus producing areas worldwide. Breeding efforts to produce trees that are tolerant to this disease are ongoing. However, genetic sources of resistance/tolerance to this disease are extremely limited and little information is known about the inheritance of HLB tolerance. Several F1 populations derived from crosses between an advanced breeding selection and commercial germplasm are being evaluated under high HLB disease pressure conditions in Fort Pierce and Gainesville, Florida. These seedling populations are exhibiting clear segregation for HLB tolerance in the field. Pedigree analysis of this trait originating from *Poncirus trifoliata* fits a Mendelian dominant inheritance pattern. These populations present an opportunity for identifying loci controlling HLB tolerance in citrus.

Establishment of Asian citrus psyllid isofemale lines from Florida that are efficient or non-efficient in acquisition and transmission of the huanglongbing pathogen *Candidatus* Liberibacter asiaticus

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Genetic variability in insect vectors can be a valuable tool to study vector competence determinants and to select or establish non-vector populations that may help reduce the spread of vector-borne pathogens. We collected and tested more than 20 isofemale lines of Asian citrus psyllid (ACP) *Diaphorina citri*, vector of *Candidatus* Liberibacter asiaticus (CLas). CLas is the bacterial pathogen associated with huanglongbing, which is the most serious citrus disease worldwide. Individual ACP adult males and females were collected from *Murraya* plants in various parts of Florida and reared in pairs. After mating, females laid eggs on *Murraya* plants before being tested for CLas by qPCR. Nymphal progeny from non-infected parents were tested for CLas acquisition from infected citrus plants for three successive generations. So far, we identified one line (Laurel 8) as a 'good acquirer' with 26.8-48.6 % of CLas+ adults. We also identified several poor acquirer lines (e.g. Laurel 16) with 0-10.2 % CLas+ adults. The 'good' and 'poor' acquisition phenotypes were stable over the three successive generations, showing that variation in CLas acquisition has a genetic basis in the psyllid. 'Good' and 'poor' acquirer lines were selected and further tested for CLas transmission into healthy citrus, using our excised leaf assay method (J. Econ. Entomol. 2013, 106:25-35). Interestingly, CLas transmission assays indicated that Laurel 8 is also a good vector line (16.7-26.7% transmission rate) and Laurel 16 is a poor vector line (0% transmission rate), which suggests that both acquisition and transmission of CLas are correlated at least in these ACP populations. We are continuing these tests and studying the genetic composition of both good and poor vector lines using molecular biology methods to study vector competence determinants of CLas in ACP that could be valuable in developing new tools for combating this devastating citrus disease.

BAC-based reconstruction of *Candidatus* Liberibacter asiaticus revealed a genetic locus associated with genome instability

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As sequencing technologies rapidly advance, the successful application of other cutting edge molecular technologies has become increasingly reliant upon the availability of a complete and accurate genome sequence. In an effort to verify the accuracy of the Las bacterial genome while not being subject to the identical limitations encountered with the original sequencing method, a BAC library was constructed from the DNA of Asian citrus psyllids collected from Huanglongbing-affected citrus. Of the 61,440 clones contained within the BAC library, 27 clones specific for *Candidatus* Liberibacter asiaticus (Las) were sequenced in their entirety and used to reconstruct the genome. During the reassembly, a novel ~8.3 kb DNA fragment containing 9 putative ORFs was revealed. Although smaller segments of the 8.3 kb fragment were found dispersed amongst the other sequenced Liberibacter strains, 2 of the putative proteins did not share any homology with any previously identified Las protein. Moreover, 1 of these proteins is not homologous to any protein currently in the NCBI database. Further examination regarding the fragment demonstrated that it was absent in 20% of the Las-infected citrus samples and 5% of the Las-infected ACP samples collected from across the state of Florida, suggesting it is functionally important in the life cycle of the bacterium although not required for survival since the entire 8.3 kb fragment was absent in the genomic DNA used to sequence *Ca.* L. asiaticus strain psy62. Taken together, the data suggest that the fragment is involved in the generation of a heterogeneous population and may contribute to the virulence of the bacteria through a type of phase variation. It also demonstrates the plasticity of the Las genome and provides a possible marker for monitoring genetic shift in the bacterium.

Symptoms of huanglongbing-affected oranges and associated effects on volatile profiles in peel oil

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Huanglongbing (HLB)-affected oranges are typically green or yellow in color, rather than orange, and the latter is more common in the Florida citrus groves. The yellow color is often associated with insufficient accumulation of carotenoids (the reason for lack of orange color) in the flavedo, lack of natural shine, and shriveled peel (due to water loss). The green color is an indicator of maturity due to HLB-associated phloem malfunction and resulting retarded growth and development of the fruit. HLB-affected Valencia fruit together with healthy fruit, were harvested in the middle of the harvest season, and peel oil extracted from flavedo tissues using methylene chloride/pentane (5/3) solvents. Volatile compounds were analyzed by GC-MS. All HLB-affected fruit produced a lower volume of peel oil compared to healthy fruit, however, only green fruit showed substantially different volatile profiles from healthy fruit. The green fruit contained low concentrations of most of sesquiterpene hydrocarbons and derivatives, such as, nootkatone, valencene, α-selinene, 7-epi-α-selinene and caryophyllene oxide, some monoterpene hydrocarbons and derivatives with orange/citrus characteristics, such as, limonene, myrcene, α-pinene, neral, geranial, and α-terpineol, and straight-chain aldehydes, such as, octanal, nonanal and decanal. Eight compounds were determined in the green fruit peel which were not present, or present in very minor quantities, in the healthy fruit. Their retention indices (RI) on a DB-5 column were RI 1137, 1144, 1149, 1189, 1320, 1334, 1346 and 1387. The preliminary identifications based on MS and the NIST database RI comparisons were (E)-p-mentha-2,8-dien-1-ol, 1(7),8-p-menthadien-2-ol, 3-[(E)-3-methyl-1-butenyl]-cyclohexene, 6-methyl-bicyclo[3.3.0]oct-2-en-7-one, p-mentha-2,8-diene-1-hydroperoxide, an unknown, p-menth-8-en-2-ol and p-mentha-[1(7),8]-diene 2-hydroperoxide, respectively. Other compounds in HLB-affected green fruit were cis-3-hexenol, cis-pinocarveol, carvone, and α-cubebene.

Citrus greening disease or huanglongbing (HLB) impacts on flavor compounds of oranges with compromised abscission zones with secondary infection by the fungus, *Lasiodiploida theobromae*

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Trees affected by HLB exhibit excessive fruit drop, which is exacerbated by a secondary infection of the fruit calyx abscission zone by the fungus *Lasiodiplodia theobromae*. 'Hamlin' and 'Valencia' orange trees, both healthy and HLB-affected, confirmed by qPCR analysis for *Candidatus* Liberibacter asiaticus (*C*Las), from two harvests (Hamlin) and one harvest (Valencia) were shaken, the dropped fruit collected, and the retained fruit harvested. Many of the dropped fruit had no calyx, while most of the retained fruit maintained their calyx after harvest. Subsequent qPCR analysis of the fruit abscission zone revealed that the dropped fruit from HLB trees had higher titers of *C*las and *L. theobromae* (causal organism of postharvest stem end rot) than the retained fruit, and were the only fruit that produced ethylene. Earlier studies with scanning electron microscopy showed fungal hyphae in the abscission zones of fruit from HLB-affected trees. The harvested fruit were washed, juiced, pasteurized and frozen for later chemical and sensory analyses. For chemical analyses, soluble solids, titratable acidity, individual sugars, individual acids, flavonoids, limonoids and aroma volatiles were quantified. Results showed that sugars and acids were lowest in dropped HLB fruit in December, but not in January, except for ratio. The bitter limonoids were generally higher in HLB fruit juice and highest in dropped HLB 'Hamlin' samples in December and January. This was repeated for 'Valencia' in April with similar results. For aroma volatiles, dropped fruit had less fruity ester volatiles and generally more terpenoid-type volatiles. Principal component analyses of all the flavor compounds separated healthy, HLB retained fruit and HLB dropped fruit for the December 'Hamlin' and April 'Valencia' but not late season January 'Hamlin'. Confirming other studies that, generally, flavor affects from HLB are more prevalent earlier than later in the season. Overall, there were less differences for sugars and acids than for bitter limonoids and astringent flavonoids, but the chemical analyses generally explained the poor flavor of the HLB fruit, later determined by a sensory panel, and especially for the dropped fruit that had formed an abscission zone.

Update on the Hot Spot Cluster Analysis of Ct-values from Asian citrus psyllid samples

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The large scale surveys for detecting Asian citrus psyllids and HLB in Texas and California are on-going. We continue to analyze the spatial pattern of the diagnostic Ct-values from psyllid samples and our goal is to help focus the survey effort for HLB infected citrus trees. The real-time polymerase chain reaction diagnostic methods used to detect HLB are set to run for 40 cycles. The reaction must surpass a threshold of ≤ 32 prior to the completion of the run to be considered positive for HLB. Currently, reactions that surpass the threshold at 33 or more cycles have proven impossible to acquire a confirmatory PCR bands and DNA sequence data and are considered inconclusive. The question we are trying to answer is whether there is additional information in the spatial pattern of inconclusive psyllid samples for predicting locations with HLB infected trees. Based on analysis of the spatial pattern of psyllid Ct-values proximate to HLB positive tree detections in both Texas and California, there appears to be a biological process underlying indeterminate Ct-values in the range of 32 – 38. Analyses indicate significant clustering of indeterminate Ct-values of psyllid samples, and some clusters in both Texas and California were around known positive HLB trees. The San Gabriel area infections in California were found after more intensive survey around an inconclusive psyllid sample. Analyses have also revealed significant space-time scales of pattern in the California psyllid data, such that inconclusive samples tend to cluster within 1.5 miles and 15 months of each other. A secondary cluster was found for inconclusive samples > 4.5 miles and > 3 years (San Gabriel area) from the first detection in Hacienda Heights. These analyses may serve as early warning indicators for HLB positive trees, as well as informing dynamic spread models in development.

Temporal and spatial HLB progress in citrus areas maintained under strict management are highly influenced by neighboring non-commercial citrus plants

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A long period of data about the occurrence of HLB in commercial citrus farms located in São Paulo state, Brazil, was used to evaluate the disease progress and its management. Approximately 260 Sweet orange plots from five farms (~2.5 million trees), naturally affected by HLB and under strict disease management (≥4 HLB-tree removal/year and ≥12 insecticide sprays/year), were evaluated considering the proportion of HLB-affected trees, occurrence of *Diaphorina citri*, plot location, and presence of neighboring commercial and non-commercial citrus plants. The logistic model better explained, than Gompertz, the yearly proportion of HLB-affected trees. The cumulative disease incidence (per plot) varied from <4.0% to 80% and highly correlated with area under disease progress curve, independently of plant age, but not with the disease rate of progress (logistic model). Adults of *D. citri* were poorly detected by shoot evaluation conducted 2 to 5 times/month in 1% of trees in each plot. In contrast, higher frequency of *D. citri* identified in yellow stick cards occurred in the most HLB-affected plots. However, the majority of yellow cards presented no adults for most of the time. Spatial heterogeneity was observed for both HLB-tree incidence and *D. citri* detection (by shoot observation and using yellow stick cards). In all farms, a strong concentration of HLB-affected trees occurred in some plots located in their borders. The border effect was not only observed in relation of plot location. In the majority of plots, especially those presenting higher disease incidences, >95% of HLB-trees occurred up to 200 meters from their borders. The disease incidence in each farm varied from 7.1% to 25.9%, though the similar disease management adopted for HLB (tree eradication and insecticide sprays). Recently, surveys around those farms have been started to identify neighboring citrus and *Murraya* sp. plants that could may act as HLB-sources of inoculum. Their location, represented by commercial and non-commercial citrus plants, strongly suggests that non-commercial trees are very effective sources of HLB, even when represented by a few plants.

Vector-pathogen interactions: Metabolome changes in *Diaphorina citri* associated with infection by *Candidatus* Liberibacter asiaticus.

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The Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama is the insect vector of *Candidatus* Liberibacter asiaticus (*C*Las), the causative agent of the citrus greening disease. Understanding the interactions between the insect vector and the bacterial pathogen is essential for the development of efficient strategies aimed at limiting the spread of the infection and inhibition of bacterial growth. Furthermore, better knowledge of insect-pathogen interaction at the metabolite level will help develop *in vitro* culture of *C*Las, a much needed tool for studying its metabolism and designing means to combat it. In this study, we compared the primary metabolite and lipid profiles between *C*Las-infected and healthy psyllids using GC-TOF-MS and LC-TOF-MS, respectively. We found that both feeding on infected trees and the actual infection with *C*Las cause dramatic changes in monitored compounds' accumulation. The results of metabolomic analyses and the possible implications for the pathogen-vector interactions will be presented and discussed.

Phosphatidylcholine synthesis pathway in *Liberibacter crescens* is missing from *Ca.* L. asiaticus and contributes to culturability

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Huanglongbing is a devastating disease of citrus caused by the *Candidatus* Liberibacter asiaticus (Las). The inability to sustainably culture and reinoculate Las into any host has prevented functional genomics and impeded research and development of disease control methods. Phosphatidylcholine (PC) has recently been established as an important membrane component of those few bacterial species that associate with eukaryotic membranes, such as *Legionella pneumophila*, *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. For many of these species, PC is required for virulence. Las colonizes living citrus phloem cells, and infects psyllid hosts systemically, moving both intra- and inter-cellularly, crossing through multiple membrane layers. We hypothesize that Las PC is similarly required for colonization of psyllids. *Liberibacter crescens* (Lcr), the only cultured Liberibacter to date, is a useful and genetically tractable proxy for functional genomics of Las. It carries two predicted pathways for synthesis of PC: 1) import of exogenous choline and subsequent conversion of choline to PC utilizing phosphatidylcholine synthase (Pcs), and 2) *de novo* synthesis of PC utilizing phospholipid *N*-methyltransferase (Pmt). Notably, Las is missing the *de novo* pathway, and must import choline, which is abundantly present in psyllid hemolymph and phloem sap, to make PC. We created individual marker interruption mutations of both Pcs and Pmt in Lcr. Rescue of the Pmt mutant in Lcr required 0.1% choline supplementation in the medium. The standard culture medium for Lcr is BM7 which contains $6X10^{-6}$ % choline, an amount that appears to be insufficient for culturing Las. Other genes found in Lcr but not found in Las and that should significantly limit Las culturability are: glyoxylase I and the ATP/ADP translocase. Phage lytic cycle genes found in Las but missing from Lcr also likely significantly limit Las culturability.

Assessment of growth media requirements for Liberibacters and making *L. crescens* more Las-like

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Despite the availability of multiple complete genomic DNA sequences of *Candidatus* Liberibacter asiaticus (Las), inability to sustainably culture, manipulate or re-inoculate Las has seriously impeded the identification of virulence factors and subsequent development of disease control methods. *Liberibacter crescens* (Lcr), is a culturable proxy for Las, but is not pathogenic. Given that the Las genome (1.26 Mb) is even more reduced than Lcr (1.5 Mb) and several Lcr genes are suspected of contributing to its culturability, one long term goal of our lab is to sequentially delete multiple Lcr genes not found in Las and to make its morphology and metabolism more comparable to Las, and make informed incremental adjustments in the growth medium to help compensate for the loss of the Lcr functions. To date, the best growth of Lcr has been obtained in the complex growth medium BM7, containing fetal bovine serum (FBS), ACES, α-ketoglutarate, and TNM-FH insect medium. We have found that replacing FBS with 1 mg/mL of methyl-β-cyclodextrin (MβCD) reduced growth, even though such replacement has been reported to improve culture of the fastidious pathogenic

microbes *Coxiella burnetii*. In addition, both BSA and glucose, major components of FBS, inhibited growth of Lcr. Both MβCD and BSA are hypothesized to inhibit Lcr growth by sequestering fatty acids and other small hydrophobic molecules that are important to Lcr growth. Lcr growth was unaffected by the presence of cholesterol in the medium. Replacing TNM-FH with Schneider's insect medium resulted in removal of sucrose and marginally improved growth of Lcr. Eliminating sucrose from the culture medium allows use of conditionally lethal DNA cloning vectors such as pUFR080 carrying *sacB* that can be forcibly and reliably evicted by adding sucrose to growth media. Such a system is important to make sequential mutations. We report here that Lcr is able to import isotopic sucrose in a time- and dose-dependent manner, enabling use of the *sacB* eviction system. We have successfully created 2 marker interruption mutations in Lcr that make Lcr more Las-like and that will likely inform culture media components. Other genes found in Lcr but not found in Las and that should significantly limit Las culturability are: glyoxylase I; ATP/ADP translocase and the sucrose transporter. We are currently attempting a series of conditional knockout mutations involving a *sacB* vector with genes suspected of enabling Lcr growth in media, each time evaluating different rescue strategies to keep the resulting mutant strains alive.

A gas chromatography coupled with olfactometry reveals few changes in aroma-active compounds in peel oil extracted from oranges affected by huanglongbing (HLB)

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Fruit from trees with severe symptoms of Huanglongbing (HLB) produce juice that is described as less sweet, more sour with some bitterness and harshness together with typical off-flavor in comparison with juice made with fruit from healthy trees. Commercial juice processing includes separation and recovery of peel oil from the fruit, which is then used in flavor manufacturing including add-back to orange juice to standardize flavor. To this date, there is no published data on the aroma quality of peel oil from fruit affected by HLB. Hamlin and Valencia fruit from healthy and HLB-infected trees (as confirmed by qPCR of *Candidatus* liberibacter asiaticus) were harvested in December 2013 and April 2014, respectively. Fruit were washed and juiced using a JBT industrial extractor. Peel was separated and the oil was expressed using several cycles of manual cold pressing followed with centrifugation. Peel oil was then analyzed by gas chromatography mass spectrometry and olfactometry (GC-MS and GC-O), as well as presented to a trained taste panel for aroma differences. Juice from the same fruit were also presented to the trained taste panel for sensory descriptive analysis. For peel oil from Hamlin, 14 compounds had higher peaks by GC-MS in healthy samples, including 1-octanol which was perceived by GC-O. Further, three unidentified peaks with mint (Retention Index – RI=1065 on DB-5 column), citrus (RI=1555) and pungent odors (RI=1620) were perceived only in the oil from healthy fruit by GC-O. For Valencia oil, differences by GC-O were for peaks with odors of fatty/plastic (RI 1185), □-cadinene (RI = 1543) (citrus) and metallic (RI=1645) which were only perceived in HLB samples. Other differences were for RI=955 (fruity floral), α-phellandrene (mint, citrus), and terpinolene (fatty), which had higher intensities in Valencia healthy samples. Overall, those differences detected by GC-O were not translated to aroma differences between oil samples by a trained panel. The same trained panel found differences when tasting the juice of Hamlin, with HLB juice having more 'typical HLB flavor', 'bitter', 'metallic', 'tingling' and 'astringent', likely due to differences in non-volatile compounds. There were no difference between HLB and healthy samples for Valencia juice.

Performance of 'Valencia' sweet orange on twelve rootstocks in two Florida locations indicates rootstock field tolerance to HLB

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Some new hybrid rootstocks grafted with commercial citrus scion cultivars produce trees with reduced huanglongbing (HLB) symptoms and higher fruit production over a period of many years, following infection by *Candidatus* Liberibacter asiaticus (Las), as compared with trees on other common commercial rootstocks in the same trials. In one replicated trial with 'Valencia' scion severely affected by HLB, highest yields over five consecutive harvests were with 'US-942' (*Citrus reticulata* × *P. trifoliata*) and 'US-1516' (*C. grandis* × *P. trifoliata*) rootstocks. Other components of tree health, including fruit quality, tree growth, and tree survival, differed greatly among the rootstocks, and provided additional evidence of improved field tolerance to HLB for some rootstocks. In a second replicated long-term field trial with 'Valencia' scion severely affected by HLB, highest yields over four harvests were with 'US-942' and 'US-802' (*C. grandis* × *P. trifoliata*) rootstocks. In both trials, some other rootstocks that are commonly used in commercial production performed poorly, probably indicating less tolerance of those rootstocks to HLB. Similarities and differences between the two trials will be discussed, as well as the value of different metrics for assessment of relative field tolerance of rootstocks to infection by Las. The implications of these observations for the choice of rootstocks to be used in commercial plantings will also be discussed.

Impact of geography, cultivation method, and *Liberibacter* infection on the Citrus microbiome

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Interactions between plants and their associated microbial communities (microbiomes) are complex, dynamic, and of varying impact. These interactions include plant-microbe and microbe-microbe relationships for each member of the community and can vary from positive to negative on each species. The structure of these communities can alter plant defense against other microbes (pathogens) and herbivores, plant productivity, and uptake of nutrients. Given these impacts, it is of interest to characterize the variation in microbial communities associated with crops facing major disease. The most devastating disease of Citrus is Huanglongbing caused by infection with at least one of *Candidatus* Liberibacter asiaticus, *C.* L. americanus, or *C.* L. africanus. Current methods of disease control involve disease vector suppression through conventional and biocontrol methods, detection of infected trees, and inoculum removal. Despite the success in slowing disease spread, these methods haven't stopped the spread of disease. Methods to inhibit pathogen invasion may involve manipulations of microbial communities. In preparation for manipulative experiments, the microbial community of Citrus was characterized using 16S amplicon sequencing. Samples were collected from multiple locations within Florida, Texas, and California and consisted of trees cultivated using conventional and organic methods. Leaf midribs, fibrous roots, and soil were collected from trees infected with and trees with no detectable *C.* L. asiaticus. Results are discussed in terms of their impact on future microbial community manipulation experiments as well as their impact on disease detection.

Psyllid vector-Liberibacter interactions at cellular and molecular interfaces

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Ca. Liberibacter asiaticus (CLas) and solanacterum (CLso) are the putative fastidious bacterial causal agents of Huanglongbing and Zebra chip, which are important diseases of citrus and solanaceous crops, respectively and are transmitted by psyllids in a circulative manner. Identification and functional characterization of the gene products specifically involved in *Ca*. Liberibacter infection and acquisition transmission processes are crucial to the development of successful management strategies, which are currently lacking for both disease agents. The 110,937 and 83,231 Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama and potato psyllid (PoP) *Bactericera cockerelli* Sulc midgut and salivary gland transcripts, respectively, were used for the identification of psyllid proteins obtained by nano- liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis of adults and excised midguts and salivary glands. Approximately 500 unique proteins were identified from infected and uninfected samples. *In*

silico profiling of ACP and PoP midguts and salivary glands identified a number of transcripts and proteins with altered expression in response to *Ca*. Liberibacter infection and tissue specificity. Several of these transcripts were selected as bait to detect protein-protein interactions using the yeast-2 hybrid system. Results indicate that transcripts involved in the immune response, endocytosis, and cytoskeleton assembly are important to *Ca*. Liberibacter infection of the psyllid host and/or in psyllid-mediated *Ca*. Liberibacter transmission. *Ca*. Liberibacter asiaticus (CLas) is the obligate, fastidious bacterial pathogen that causes citrus greening disease, or Huanglongbing. CLas is transmitted in a circulative, propagative manner by Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama adults. Identification and functional characterization of effectors involved in invasion of the psyllid gut and its presumed entry into the salivary glands was investigated using transcriptomic, proteomic, yeast-2 hybrid (Y2H) and co-immunoprecipitation (Co-IP) analyses. *In silico* annotation and differential expression analysis of contigs from ACP nymphs and adults, and adult midgut and salivary gland tissues identified transcripts and proteins with altered expression in response to CLas infection. Several differentially expressed transcripts were selected and used as bait for Y2H detection of protein-protein interactions. Those positive by Y2H were subjected to verification by bait to prey co-transformation and Co-IP. Proteins positive in one or both assays were tested in 'knock down' experiments using dsRNA to induced RNA-interference (RNAi) and quantified by qPCR. Candidates of the greatest interest have been those with a predicted role in virulence and invasion, that if disrupted by RNAi could abate CLas accumulation in and exit from the gut, circulation in hemolymph and systemic infection, and acquisition e.g. in salivary glands. Collectively, the results suggest a model for invasion in which CLas- and prophage-encoded effectors transform the endocytic host pathway into a 'pathogen-mediated phagocytic scenario' utilizing membrane ruffling in CLas interactions with the gut, leading to bacterial exit into the hemocoel and systemic invasion of other psyllid host tissues and organs. *Ca*. Liberibacter asiaticus and solanacterum are the causal agents of Huanglongbing and Zebra chip disease of citrus and solanaceous crops, respectively. Bacterial effectors that interact with psyllid vector proteins during invasion of and exit from the gut, and that facilitate entry into the salivary glands were identified using functional genomics, proteomics, yeast-2 hybrid (Y2H), co-immunoprecipitation (Co-IP), and electron microscopic analyses. *In silico* annotation and differential expression studies of psyllid nymphs and adults, and midgut and salivary glands identified transcripts and proteins with altered expression in response to *Ca*. Liberibacter infection. Differentially expressed transcripts were used as bait in Y2H studies to corroborate protein-protein interactions. Those positive by Y2H were subjected to verification by bait to prey co-transformation and Co-IP. Electron micrographs of the PoP midgut revealed the presence of CLso in the ventricular lumen, apical and basal epithelial cytosol, and in the filter chamber periventricular space. CLso were also prevalent in salivary gland pericellular spaces and head epidermal cell cytosol. Collectively, the results suggest a model for invasion in which *Ca*. Liberibacter- and prophage-encoded effectors manipulate the endocytic host pathway into a 'pathogen-mediated phagocytosis scenario' leading to bacterial exit from the gut into the hemocoel, and systemic invasion of other psyllid host tissues and organs.

Recovering value-added co-products from culled HLB Symptomatic and pre-harvest dropped fruit

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Beyond the devastating effects of HLB on tree health and crop size are the secondary impacts of preharvest fruit drop and the quality degrading flavor profile of symptomatic fruit. A precise number for the increasing percentage of the smaller, symptomatic fruit being sent to processors for juice extraction is not readily available. But inspection of data provided by NASS does indicate the number of fruits per box has been trending higher over the last five seasons. As previously noted increasing disease severity will increase the percentage of symptomatic fruit being used for juice extraction. In a 2015 survey of growers, production managers and other associated occupations 81% of the responders reported that 80% - 100% of their acreage was infected and the average percent of infected trees was 90%. As more and more fruit become symptomatic their detrimental effect on juice quality will become more and more pronounced. Beyond a blending ratio of 3:1 (healthy/non-symptomatic to symptomatic juice) off-flavors in the product become discernible. The incidence of preharvest fruit drop also has been increasing with a recently reported 10% - 20% rise. Enabling a technology to utilize these two groups of HLB damaged fruit and recover lost value would be beneficial to the citrus industry during this transition period before resistant trees are available, especially if that technology also could provide increased value for all juice extracted biomass. Here we report on a pilot-scale continuous process to release and recover multiple value added co-products from culled fruit, preharvest dropped fruit and juice extracted biomass. Using steam-explosion followed by a simple water wash we have been able to recover pectic hydrocolloids, bioactive phenolics and fermentable sugars. Limonene is captured by condensation of the recovered steam upon depressurization. Pectic hydrocolloids are functional polysaccharides that can be used to modify the rheology of water and as a hydration control or ion capture agent. Citrus phenolics contain a number of compounds that possess bioactivity against chronic human illnesses. Sugars can serve as a feedstock for a variety of synthetic options as well as an energy source in varied fermentation schemes. Limonene has a variety of established commercial applications. Summing these potential co-products could promote the dropped or flavor degrading fruits as an alternative value-added resource and recover increased value from juice extracted biomass vs. a traditional feed mill operation.

'*Candidatus* Liberibacter asiaticus', causal agent of citrus huanglongbing, is reduced by treatment with brassinosteroids

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Huanglongbing (HLB) constitutes the most destructive disease of citrus worldwide, yet no established efficient management measures exist for it. Brassinosteroids, a family of plant steroidal compounds, are essential for plant growth, development and stress tolerance. As a possible control strategy for HLB, epibrassinolide was applied to as a foliar spray to citrus plants infected with the causal agent of HLB, '*Candidatus* Liberibacter asiaticus'. The bacterial titers were reduced after treatment with epibrassinolide under both greenhouse and field conditions but were stronger in the greenhouse. Known defense genes were induced in leaves by epibrassinolide. With the SuperSAGE technology combined with next generation sequencing, induction of genes known to be associated with defense response to bacteria and hormone transduction pathways were identified. The results demonstrate that epibrassinolide may provide a useful tool for the management of HLB.

Host marking and possible effects on mass rearing of *Tamarixia radiata*

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Tamarixia radiate (Waterston) (Hymenoptera: Eulophidae) established and spread throughout Florida following first release in 1999 for control of the Asian citrus psyllid (ACP) *Diaphorina citri* (Kuwayama) (Hemiptera: Psylloidea). Nevertheless, persistence of the pest and continued losses from citrus greening disease have created the need for more effective biological control, including mass rearing and

augmentative release of *T. radiata*. However, information is lacking on ways to manipulate host density to optimize rearing efficiency. Three release rates of *D. citri* per shoot, and four host: *T. radiate* ratios were evaluated. Results indicated that 20 psyllid adults per flush provided optimal host density without excessive stickiness from honeydew. Sixty *T. radiate* females per cage of approximately 4,800 nymphs provided most economical use of wasps and hosts. However, only 60% of the hosts were utilized for progeny production under these conditions, possibly due to competition between females mediated through deterrent host marking. In order to determine the existence of a host mark on parasitized hosts, a three-day-old *T. radiate* females was released in a petri dish with six parasitized hosts (either parasitized by herself or by a conspecific) and six clean hosts randomly distributed on an orange jasmine shoot. The number of hosts probed and parasitized in each host category was noted. To evaluate volatility of the host mark, 10 parasitized 4th instar *D. citri* nymphs and 10 clean nymphs were randomly assigned to one of the arms of the T- maize olfactometer. Ten *T. radiate* females were released at the releasing chamber to make a choice of the two odor sources. Results showed that *T. radiate* females probed and parasitized significantly more on the clean hosts versus parasitized hosts but showed no a preference for either of the odor sources. Thus, *T. radiate* females were able to discriminate between clean hosts and host parasitized by herself by perceiving a non-volatile host mark. Future research will focus on determining whether the mark also has a deterrent effect on nearby hosts. The ultimate goal is to improve mass rearing efficiency and biocontrol effectiveness in the field.

Metabolomics, Transcriptomics, and Proteomics analysis of *C. sinensis* (L.) Osbeck trees graft-inoculated with *Candidatus* Liberibacter asiaticus

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Candidatus Liberibacter asiaticus (*C*Las) is the bacteria associated with the economically-important citrus disease, Huanglongbing (HLB; citrus greening disease). Understanding the pathogenicity of *C*Las and its effects on the citrus host is challenging, as *C*Las has yet to be cultured. To understand the impact of *C*Las infection on host leaf metabolism, Parent Washington Navel orange (*Citrus sinensis* (L.) Osbeck) greenhouse plants were graft-inoculated with *C*Las+ (from Hacienda Heights, CA (treatment)) or *C*Las- (control) budwood, and leaf samples were collected longitudinally until tree death. Samples were analyzed with metabolomics (^{1}H NMR), transcriptomics (RNA-seq), and proteomics (mass spectrometry) to identify *C*Las-associated changes in the plant. Changes in metabolite content could be observed as soon as 6 weeks post-infection, before symptom development. Further changes in the metabolome, transcriptome, and proteome were observed throughout the study, with the most drastic differences noted at the last time point (46 weeks post infection) as the trees were nearing death. *C*Las affected both primary and secondary metabolism, including photosynthetic processes, cell wall synthesis, and stress responses. These data show that early changes in tree metabolism occur in asymptomatic plants with *C*Las infection, and shed light on how *C*Las impacts the citrus host throughout the disease.

Field validation of a system for autodissemination of an entomopathogenic fungus, *Isaria fumosorosea*, to control the Asian citrus psyllid on residential citrus

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The citrus industries of California and Texas share a pressing problem with the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and huanglongbing (HLB) spreading in residential citrus near commercial groves. Insecticidal treatment of residential trees for the psyllid is problematic and not cost effective; therefore, control of this insect vector in urban areas needs to rely heavily on natural enemies such as entomopathogens. The entomopathogenic fungi *Isaria fumosorosea* (*Ifr*) Wize (Hypocreales: Cordycipitaceae) is highly pathogenic towards *D. citri* and available as a commercial blastospore formulation (PFR-97) for use in autodissemination management strategies. "Autodissemination" is the dispersal of an entomopathogen by an insect to conspecifics and *Ifr* blastospores are ideal for autodissemination because they are desiccation tolerant and germinate quickly on suitable hosts. Autodisseminators can be deployed in residential citrus trees to attract and infect *D. citri* adults with PFR-97 blastospores for the purpose of inducing epizootics that will decimate psyllid populations on these trees. During the fall and winter of 2015, we deployed autodisseminators treated with PFR-97 formulation on residential citrus trees in the Rio Grande Valley of Texas. For one study, we recovered autodisseminators after 1, 7, 14 or 21 days and tested their PFR-97 formulation for infectivity against *D. citri* adults. For a second study, we monitored *D. citri* populations for 12 weeks on trees without autodisseminators (controls) and trees with 1, 2, or 4 autodisseminators replaced every two weeks. We found that PFR-97 formulation recovered after 1 to 14 days of weathering could still infect adult psyllids. Mean reduction of psyllid populations on trees with autodisseminators ranged from 30 to 79 %. Based on our results, we determined that a single autodisseminator per tree and replacement every two weeks was optimal for psyllid control. Our findings show that autodissemination of *Ifr* could be a key component of a sustainable management system for *D. citri* in residential landscapes.

A growing degree-day model for assessing flush shoot ontogeny in citrus

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The concept of growing degree-day (GDD), which integrates the rate of development of an organism as a linear function of air temperature to which it is exposed, has been widely used in studies of crop and insect phenology for scheduling agricultural practices. In citrus, however, these studies have focused on the reproductive development and on the physicochemical characteristics of the fruits but not on vegetative shoot ontogeny. This is important because vegetative shoots are strictly linked to the biological performance of *Diaphorina citri*. Although the thermal requirements of the psyllid have been established in growth chambers, little is known about the thermal requirements for the psyllid in natural environments or about the cumulative heat units for the emission and development of new shoots, taking into consideration their ontogeny. To assess this, experiments were carried out in growth chambers using 2.5 year-old potted 'Valencia' orange grafted on 'Swingle' citrumelo. The plants were pruned 20–30 cm above the grafting line and evaluated for several flushing cycles. Daily assessments of shoot size, new leaf emergence and leaf area were carried out on a single shoot per plant. The same variables were evaluated for three to four successive natural flushing cycles that appeared on the same plants. The experiment has been repeated in the field with mature trees. Upper and lower threshold temperatures for the new shoots were respectively 9.88 and 32.19°C. For each 1°C below the lower or above the upper thresholds the developmental time needed for the shoots to reach 50% of final size (T_{50}) increases >30% or decreases <5%. Preliminary data suggests that shoot elongation and increase in leaf area can be described by the sigmoid logistic curve while leaf emission by linear regression. T_{50} correlated positively with the final shoot size but not with the final leaf area. T_{50s} were similar for all leaves regardless of their position on the shoot. From the pruning date, a total 451 (±7.4) GDD had to accumulate for the shoots to reach full maturation, with 22.4% of the total needed for their emergence, 45.1% for development, and 32.4% to complete the entire maturation phase. Leaf emission started after the first 34.01% and lasted 151.6 (±17.2) GDD. The GDD concept was then useful in modelling the emergence and development of new shoots in citrus. Since new shoots are the preferred sites for the multiplication of *D. citri* and other insects, it may represent an important tool in the management of HLB and other pests and diseases that affect citrus orchards.

A CLas effector targets a specific family of secreted proteases in citrus

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Gram-negative bacterial pathogens, like the Huanglongbing (HLB)-associated pathogen, *Candidatus* Liberibacter asiaticus (CLas), possess secretion systems that deliver virulence proteins into the host. These "effector" proteins can aid infection by manipulating plant physiology and subverting host immunity, thereby promoting bacterial colonization and disease progression. We predicted Sec-delivered effectors (SDEs) from the genome sequence of the CLas isolate psy62 and analyzed their expression in infected citrus tissues. One effector, named SDE1, has 40-folds higher expression in citrus relative to in psyllid, implicating a possible role in HLB disease progression. We then performed yeast two-hybrid (Y2H) screening using a citrus cDNA library to identify the interacting proteins of SDE1, and found that SDE1 directly interacts with several members belonging to a specific family of proteases, known as papain-like cysteine proteases (PLCPs). PLCPs are upregulated during CLas infection; homologs of PLCPs in other plant-pathogen systems have also been shown to contribute to defense against microbial pathogens. Our current hypothesis is that SDE1 affects the activity of these proteases in order to promote CLas infection. We will discuss recent progress on the characterization of SDE1 virulence function and the role of PLCPs in disease resistance.

Streptomycin and oxytetracycline-resistance profiles in Liberibacter crescens suggests a management strategy HLB on citrus

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Emergency use of 2.23 million pounds of oxytetracycline and streptomycin on 388,534 acres of citrus has been approved for the control of HLB in Florida in 2016. As a result, future use of these two antimicrobials for HLB control will require management strategies that reduce the frequency of resistance. As a first approximation of the resistance problem, the frequency of resistance to both antimicrobials, singly and together, was determined in the closest cultured relative of the HLB pathogen, *Liberibacter crescens*. Spontaneous streptomycin resistance was easily obtained in the lab at a frequency of <1 in 10 million cells. All nine of the streptomycin resistant mutants obtained were also resistant to oxytetracycline despite having no past exposure to oxytetracycline, suggesting a common resistance mechanism. In contrast, all attempts to date to generate spontaneous mutants to oxytetracycline have failed when exposing *L. crescens* to oxytetracycline alone. Attempts to obtain spontaneously resistant mutants to both antimicrobials by treating a large number of cells with both was successful at about the same frequency as spontaneous streptomycin mutants. This also agrees with the hypothesis that spontaneous resistance to streptomycin also confers spontaneous resistance to oxytetracycline by the same resistance mechanism. These results suggest that treating citrus with streptomycin greatly increases the prospects of resistance to oxytetracyline as well. Treatment with oxytetracycline alone induces resistance at much lower levels, if at all.

The discovery of citrate as an important carbon source for culturing *Liberibacter crescens* suggests an HLB management strategy

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The inability to culture *Candidatus* Liberibacter asiaticus, the causal agent of HLB, limits our understanding of the molecular bases of this disease and further improve treatment methods. *Liberibacter crescens* is the only species from the genus *Liberibacter* that has been cultured to date, and is used as a surrogate for culturing methods and the identification of antimicrobials suitable for testing against HLB. The only medium available to date for the culturing slow-growing *L. crescens* is the complex medium BM7, making it difficult to understand its metabolism. Here, a chemically defined medium is described, M12, which includes the known components present in BM7 medium plus other compounds. With the availability of M12, two carbon sources, α-ketoglutarate and citrate, were identified as each being able to substitute for all other carbohydrates in the medium. Citrate provides longer sustained growth while α-ketoglutarate allows faster growth early in the growth phase. In contrast, other organic acids, including malate, succinate, and fumarate, could not substitute for other carbohydrates. Citrate, but not α-ketoglutarate, is present in mM levels in citrus phloem. The genes for citrate metabolism are identical between *L. crescens* and *Ca*. L. asiaticus. Thus, these results are expected to be applicable to the pathogen. This discovery allows for the simplification of a defined medium for *L. crescens*. Further simplification will come from similar experiments with other classes of compounds including amino acids and vitamins. A simplification of the medium will increase our understanding of the growth requirements of *Liberibacter*. In addition, the importance of organic acids for the growth of *Liberibacter* suggests that nutritional approaches that discourage phloem loading of organic acids in plants may starve *Ca*. L. asiaticus in citrus phloem leading to suppression of HLB symptoms.

Huanglongbing in Texas 2012-2017 – an update

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Citrus Huanglongbing (HLB) was confirmed in Texas in January 2012, 11 years after the first report of its vector, the Asian citrus psyllid (ACP) in the state. Texas has put in place a number of steps to counter the threat, including regulations requiring the screening of citrus nurseries, the establishment of an area wide psyllid management (AWM) program, improved orchard care, surveys to determine the spread of the disease by qPCR tests of both psyllids and suspect trees, and, where possible, tree removal. Since 2009, over 80,000 tree and 74,000 ACP samples have been tested, with 4.8% tree and 1.9% ACP testing positive. Recently, incidence appears to be increasing more in residential sites where ACP control is less aggressive. The disease has spread to all three counties in the commercial citrus area of South Texas, and to two counties around Houston. There has, thus far, been little evidence of severe tree decline except in some residential sites where tree care is minimal, and in orchards where other issues such as *Phytophthora* infections are impacting tree health. The voluntary AWM of psyllid combining two dormant sprays and aggressive ACP management during the active growing season implemented in > 92% of citrus acreage has resulted in significant vector population suppression which may be slowing HLB spread in Texas. Other factors which may be affecting the slower development of HLB in Texas include the discrete flush cycles that are not allowing continuous availability of resources for ACP reproduction, and the long (4 to 5 months), dry and hot summers with mean daily temperatures exceeding 90 ºF from May to September.

A deeper look into the causes of off-flavor in orange juice affected by huanglongbing (HLB)

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Huanglongbing (HLB) compromises citrus fruit and juice flavor resulting in reduced quality for citrus producers and the food industry. Stress caused by HLB induces the production of secondary metabolites associated with bitter and astringent tastes in orange juice. The objective of this study was to identify which compounds, aside from limonin and nomilin, are responsible for the bitterness and astringency in juice made with oranges harvested from trees symptomatic for HLB. Alcohol soluble compounds were extracted from healthy and symptomatic orange juice and fractionated using chromatography into five fractions. The experiment was performed with food grade equipment and the organic solvents were eliminated before tasting. Three subjects tasted each fraction and documented their observations. Harsh, bitter and astringent tastes were identified in the first two fractions. Both of these fractions included hydroxycinnamates, polymethoxylated flavones, flavanone rutinosides, limonin and nomilin. These fractions were further separated by HPLC into 13 and 11 sub-fractions, respectively. Each was tasted and the majority of the compound groups contained in the sub-

fractions were identified. In general, the sub-fractions of healthy orange juice presented descriptors of a high quality juice, such as: flavors of citrus, caramel, honey, fruity, floral etc. On the other hand, the sub-fractions of juice made from oranges harvested from trees symptomatic for HLB presented descriptors such as: acrid, strong bitterness, smoky, astringent aftertaste and lingering irritation in the back of the throat.

Quantifying *Diaphorina citri* invasion dynamics in Southern California citrus groves

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For the first three years following the initial detection of *Diaphorina citri* in Southern California, it was limited exclusively to urban and suburban environments. However, starting in 2011, the first *D. citri* detections occurred in citrus groves and since then it has become prevalent in commercial citrus throughout region. We leveraged a statewide monitoring database to conduct a spatial analysis of the factors contributing to *D. citri* invasion into commercial citrus. Specifically, using a survival analysis framework, we modeled the effects of select grove characteristics (i.e. area, amount of edge, degree of perforation), surrounding landscape characteristics (i.e. proximity to major roadways, urbanization intensity), and neighbor effects (i.e. proximity to residential detections or other grove detections) on the rate at which individual citrus blocks became invaded. Invasion rate depended on grove characteristics, with larger and more convoluted-shaped blocks being at higher risk, and landscape context. But, the strongest predictors of invasion rate were proximity to residential detections early on, and then proximity to other invaded groves in more recent years. These results suggest that there is a strong linkage between urban infestations and spillover into commercial groves. Yet, they also suggest that, as more groves became invaded by *D. citri*, these infestations drove the majority of new grove invasions. This “tipping point” phenomenon may have implications for how to prioritize the various elements of a larger disease management strategy.

ELISA detection for HLB using a pathogen-secreted protein as the biomarker

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We have identified detection markers from the HLB-associated bacterial pathogen, *Candidatus* Liberibacter asiaticus (CLas), and developed an enzyme-linked immunosorbent assay (ELISA) using CLas-specific antibodies. The antibodies specifically recognize epitopes from a Sec-secreted protein of CLas (called SDE1), which is presumably present in the phloem of infected trees. As such, this biomarker may have a greater distribution in the trees than the bacterial DNA, and thereby allow for more robust HLB detection. Using this antibody, we have developed a competitive indirect ELISA protocol, which is based on a competition for the HLB-specific antibody, between the purified antigen coated on the ELISA plate and the CLas biomarker presumably present in the HLB-infected citrus tissues. The protocol has been applied for HLB detection using freeze-dried field samples from Texas and Florida, which were simultaneously analyzed by qPCR. Our data showed that the ELISA protocol gave comparable results with qPCR and in some cases could provide more definite diagnosis, e.g. for samples with qPCR results higher than the established threshold (Ct value>38). Our work demonstrates that competitive ELISA is a valuable tool for detecting CLas secreted proteins and thus HLB diagnosis.

Asian citrus psyllid predator *Ceraeochrysa valida* (Chrysopidae) tolerant to insecticides. Production, release and effectiveness in field.

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The trash-carrying larvae of *Ceraeochrysa valida* (Banks) (Neuroptera: Chrysopidae) have been found preying on the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, in citrus grove of Central America even after pesticides application. There are different efforts of citrus growers to control populations of *D. citri*, as it vectors *Candidatus* Liberibacter spp., a disease that is present in Costa Rica science 2011. The objective of this study is to provide the Costa Rica citrus industry with a new biological control agent of the vector. *C. valida* is produced in Ticofrut science February 2014 with 200.000 eggs average by month. We present a form of eggs production and release; evaluation of four dosages: 0, 400, 800 and 1.000 eggs by hectare, its effectivity and the effect of pesticides on larval stage in field. For the production, *C. valida* larvae are rear on *Sitotroga cerealella* eggs and adults with pollen, honey bee and yeast in controlled conditions (25±2ºC, 80-85% RH, 12:12h). A pot of production lined with paper consist in 50 adults with 7:3 female-male proportion, water and food; than is changed daily to collect the eggs. Each pot produce eggs for 45 days, with 6.82$\pm$2 eggs in average by female. The paper fragments of 20 eggs with three days after oviposition caused 84% mortality in ACP population 15 days after field application; which was better than larval and adult stages (gl: 4; p=0.000). In 100% of infestation with ACP eggs and 1-3 nymph was not significant differences between the different evaluated dosages 15 days after application (gl: 3; p=0.0518); but 21 days after, dosage of 800 eggs was better (gl: 3; p=0.000). Similar results were found in presence of leaf miner, scale bugs and mites. In presence of aphids, the ACP control by *C. valida* varied and the higher dosage was better (83%). Thanks to its trash, *C. valida* larvae after three days of emerged are 78% tolerant to Zeta-cypermethrin, 60% to dimethoate and was not affected by systemic insecticides (Thiamethoxam). This study presents a new tool for the integrated management of ACP compatible with the actual practices.

Characterization of a ribonuclease of the causing agent of HLB *Liberibacter asiaticus*

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Liberibacter asiaticus, the causative agent of HLB, is an endogenous and seive-stricted Gram-negative bacterium, transmitted from tree to tree by citrus psyllid insect vector *Diaphorina citri*. In absence of laboratory conditions that allow the propagation of *L. asiaticus*, the functional characterization of proteins with putative essential activities is a priority for the development of effective management practices. YbeY has been reported as a specific endoribonuclease and its homologs are present in almost all sequenced bacteria. In *Sinorhizobium meliloti*, YbeY is involved in the regulation of small RNAs, as well as in the ribosome quality control. YbeY was also shown to play a critical role in the processing and maturation of the three rRNAs in *V. cholerae* and in *E. coli*, and to be required for expression of various virulence genes in *Yersinia enterocolitica*. The aim of this work is to analyze the role of YbeY homolog in *L. asiaticus*. Preliminary results showed that the heterologous expression of $YbeY_{Las}$ in the *E. coli* BW25113Δ*ybeY* genetic background partially restores the defective growth of this strain at 43°C. *In vitro* assays, aimed to test the RNAse activity of YbeY on ribosomal RNA, showed full degradation of the substrate, suggesting that $YbeY_{Las}$ may have exonuclease activity. Since YbeY has been described as an endoribonuclease involved in maturation of 5’ end of 16S and 23S rRNA, the 5’ ends of 16S and 23S rRNA from *L. asiaticus* were synthesized and assessed for YbeY’s enzymatic activity. Our results suggest that YbeY may perform a similar activity as reported in other Gram-negative bacteria, and may be essential for *L. asiaticus* growth within the host, which makes it a suitable target for the development of new management strategies.

Citrus cropping systems under the huanglongbing expansion in Bhutan

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Citrus industry in Bhutan enjoys many advantages over the other domestic crops. It is one of the major export commodities that fetch foreign revenue and has a well established marketing chain that provides growers with assured export and domestic market. However, Huanglongbing (HLB) is currently a major threat to citrus cultivation in Bhutan. We assessed change in the directives and the citrus cultivation

systems in recent years in awake HLB in Bhutan. We reviewed the past minutes of meetings convened by the Department of Agriculture, Ministry of Agriculture & Forests, Bhutan in past four years (2013 to 2016) and the physical infrastructural facilities developed. We also randomly surveyed and interviewed a few citrus growers from the two southern districts of Bhutan (Tsirang and Dagana) to assess their interests towards citrus cultivation and evaluated the change in citrus cropping systems. The Royal Government of Bhutan and the foreign funded projects have emphasized on production of HLB free seedlings as the major step to mitigate HLB in Bhutan. High priority was accorded for infrastructural development required in production of HLB free seedlings and in development of legal framework for providing biosecurity guidelines. On the other hand, citrus growers expressed differential views and interests to citrus cultivation although they directly depended on citrus for their livelihood. Citrus growers in low altitude areas (300 to 700 meters above sea level) showed lesser interest in citrus cultivation and many left their orchards unattended while those in mid altitude areas (700 to 1000 meters) resorted to other cash crops particularly mung bean (*Vigna radiata*) and fruits (mango, litchi, Banana etc.). A great majority of the citrus growers with their orchards located above 1000 meters had inter-cropped citrus with large cardamom (*Amommum sabulatum* Roxb.). Although, steady progress has been made in terms of infrastructure development to sustain citrus industry in Bhutan, much remains to do in terms of enhancing human capacity of stakeholders with respect to HLB at various levels. While citrus still remains as a main cash crop in Tsirang and Dagana districts, citrus farming system varies with location of the orchards and its altitude range.

Differential coexpression analysis using RNA-seq data of citrus infected with '*Candidatus* Liberibacter asiaticus' (*CLas*)

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Huanglongbing (HLB) is considered as the most devastating disease of citrus in the world. In our previous study, transcriptome data of HLB-susceptible sweet orange (SO, *Citrus sinensis*) and HLB-tolerant rough lemon (RL, *C. jambhiri*) were compared to identify differentially expressed genes. However, alteration in gene coexpression relationships was not studied. In this study, six coexpression networks were constructed separately for six RNA-seq datasets from two treatments [mock-inoculated (CK) and *CLas*-inoculated (HLB)] of SO and RL. In total, 2781 differentially associated pairs (DAP) were identified by comparing the CK and HLB networks, 1141 decreased and 1640 increased coexpression interactions under HLB. Gene ontology (GO) analysis showed that programmed cell death, defense response, cell growth and differentiation were accompanied with increased association under HLB. However, GO terms such as root morphogenesis, protein translation and carbohydrate metabolic process were over-represented with decreased association under HLB.

Symptom variations and molecular markers that illustrate the HLB complexity

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Huanglongbing (HLB) is a devastating bacterial disease of citrus worldwide due to its intracellular and systemic infection. Various HLB symptoms are observed on different species/varieties of citrus plants: from yellow shoots to blotchy mottle on the leaves, from vein yellowing/vein corky to mosaic/green islands similar to zinc deficiency on the leaves, from whitish discoloration to stunted green leaves, etc. These variations of symptoms, which result from a combination of biotic and abiotic stresses, are not only present on individual plants from a variety but also exist on individual branches of an infected plant. Our results indicated that the adaptation of the bacterial populations, such as the dynamics of '*Candidatus* Liberibacter asiaticus' (Las), plays an important role in the induction of various symptoms and that Las mutations as well as the number and recombination events of Las prophages/phages affect this phenomenon. In addition, the selection of the host plants (resistance/tolerance) for the bacterial populations is also critical for symptom expression during disease progression. Based on severity, we divided HLB symptoms into four grades. It is worth noting that the grades of HLB symptom severity show a positive correlation with our newly identified biomarkers from host plants, and that gene expression profiling of different grades of infected leaves rationalized the differentiation based on the dynamics of these biomarkers. Because of these findings, we propose new approaches that allow for rapid selection of variant citrus plants, including bud sports with greater HLB resistance/tolerance.

Can engineered trees protect citrus against huanglongbing (HLB)?

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Most commercially cultivated citrus cultivars are vulnerable to *C*Las and develop disease symptoms within a few years of infection. While improved production techniques are able to keep an infected tree alive, the only promising long term strategy to manage this disease is to utilize HLB resistant trees that can thrive in an endemic HLB environment. Genetically engineered citrus plants are an important component in the citrus improvement toolkit and offers promise of durable HLB resistance. Incorporation of gene(s) via genetic engineering can potentially confer resistance in susceptible cultivars, while maintaining the varietal fidelity. We have tested several antimicrobial peptide gene constructs (AMP's), Pathogenesis-related (PR) genes and the SAR inducing genes NPR1 and SABP2 in commercial sweet orange cultivars with varying degree of success. Field trials in an HLB endemic environment as well as greenhouse based insect vector transmission trials have identified a few genes that can potentially confer tolerance to HLB. We have evidence that an engineered rootstock can potentially protect the non-engineered scion. We will report on our current citrus improvement strategies to provide durable long term resistance to both scion and rootstock cultivars. In addition strategies to develop consumer-friendly engineered trees will be detailed.

A handheld, smartphone based optical sensor for high-throughput, early-detection of huanglongbing disease and a platform for managing citrus health

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We have developed a low-cost, hand-held, smartphone based spectrophotometer platform for detection and early diagnosis of Huanglongbing (HLB) as well as differentiating between other underlying health conditions of citrus. The spectrophotometer clips onto a leaf, and nondestructively interrogates the internal physiology of a leaf using broadband diffuse reflectance spectroscopy (DRS) in the near-infrared wavelength (600 - 1000 nm), and records the resulting spectrum on a smartphone device. Diseases and other plant health conditions modify the physiological state of the leaf, thereby changing the optical scattering and absorption properties and creating a DRS spectrum uniquely correlated with each health condition. The integration of the optical sensor with a smartphone and cloud based computing enables recorded DRS spectrums to be analyzed by deep learning analytical algorithms and affords real-time diagnosis of the plant's health with a high-degree of accuracy. The resulting platform can be an economical and geographically tailored solution for early stage detection of HLB, which requires collective community efforts and timely response for managing the spread of the disease. Results demonstrating differentiation among healthy, HLB, citrus stubborn disease (CSD), and citrus tristeza virus (CTV) graft infected rough lemon and sweet orange greenhouse trees are obtained with greater than 99% accuracy. Progress with on-going longitudinal studies for early stage disease detection will also be discussed.

The Citrus Clean Plant Network in Puerto Rico: Accomplishments

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Citrus spp. production in Puerto Rico has declined due to Huanglongbing (HLB), first found in Isabela, PR in 2009. The disease associated with *Candidatus* Liberibacter asiaticus (CLas), is widespread

from the coastal areas to the mountainous area (2). Since HLB was found, orange and mandarin orchards were severely affected in San Sebastian, Lares, A*ñasco*, Utuado, Jayuya and Las Marias. *Citrus* spp. in the island are also affected by *Citrus tristeza virus* (CTV), which in old orchards can be detected at high percentages (~50%). Within the goals of the Citrus Clean Plant Network (CCPN) in Puerto Rico is the use of pathogen-tested citrus propagative materials for nursery production and plantings and protect the local citrus germplasm collection composed of 50 *Citrus* spp. varieties. In 2015, testing for HLB, CTV and Citrus Variegated Chlorosis (CVC) was conducted in five commercial nurseries using serological and molecular methods. Valencia, Hamlin, Tahiti, Encore, Cara-Cara, Navel, and Rhode Red, grown under protected structures at Adjuntas and Isabela Experimental Stations were inspected for graft-transmissible diseases and found free of HLB, CTV and CVC. Foundation blocks with the new released rootstocks; 'Swingle citrumelo' [*Citrus paradisi Macf. x Ponsirus trifoliata* (L.) Raf.], 'Carrizo' [*C. sinensis x P. trifoliata* (L.) Raf.], HRS 812 [mandarina 'Sunki', *C. reticulata x P. trifoliata* (L.) Raf.], were tested in the Isabela nursery using conventional PCR. Testing of *Citrus* spp. in commercial nurseries in Puerto Rico, and vector management will benefit the industry in the island.

Laser light enhances the delivery of antimicrobials and ds-RNA to citrus trees

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In the age of HLB, the use of antimicrobials and other foliar agrochemicals has emerged as one of the few effective treatments against HLB infected citrus trees. Aerial sprays are preferred as a means to deliver agrichemicals over large areas given that leaves are the most readily accessible tissues and represent a significant proportion of the total plant body. The main obstacle in using foliar applications of aqueous solutions on citrus trees is that penetration into the leaves is severely hindered by the presence of the protective waxy cuticle. Penetration into leaves is only possible through the stomata openings (found almost exclusively on the abaxial side in *Citrus* leaves). Nevertheless, the collective surface area of stomatal openings that would allow for penetration of externally supplied solutions into the leaf is minimal, even under optimum circumstances, since stomata often close under a variety of biotic and abiotic conditions. To increase penetration of externally supplied substances across the leaf cuticle, and subsequently into the phloem, we examined the use of laser (Light Amplification by Stimulated Emissions of Radiation) light as a tool to increase the permeability of the cuticle without extensive damage to the underlying leaf tissue. Our investigation demonstrated the effectiveness of laser light technology in drastically enhancing the penetration of antimicrobials and double stranded RNA into citrus leavtractes.

Engineering a CRISPR-based antimicrobial system for the treatment of *Liberibacter* infections

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Vector management has failed to contain the spread of *Liberibacter*. Although approved for use for the control of *Liberibacter*, the broad-acting antibiotics Oxytetracycline and Streptomycin are far from ideal and likely to receive significant pushback from consumers. An ideal antimicrobial system would be 1) highly effective against the target organism 2) with a narrow range and 3) minimal risk of resistance development. Toward this end, our group is exploring the recently-elucidated prokaryotic immune system, CRISPR-Cas for its engineering potential as antimicrobial for the control of *Liberibacter* pathogens. CRISPR-Cas targets DNA in a sequence dependent manner making it highly specific and easily programmable. We have demonstrated that the CRISPR-Cas system will induce cell death when a bacterial cell is compelled to target its own genome and have achieved selective removal of individual strains from a bacterial community. Furthermore, we are engineering a broad-host range bacteriophage to deliver this antimicrobial system to the target bacteria. The availability of multiple genomes of pathogenic *Liberibacter sp.* facilitated a construct design that selectively removes specified strains but will leave the remaining bacterial community undisturbed. Initially, single genomic regions of the pathogen have been targeted; however, the system can accommodate multiple genome-targeting spacers to diminish the chance of resistance and/or encompass diverse strains. We envision a construct ultimately able to target multiple regions within the bacterial genome, multiple pathogens within the same host, and the endosymbionts of the insect vectors; leading to comprehensive, engineered citrus crop protection.

Attractiveness of *Diaphorina citri* Kuwayama to constitutive volatiles emitted from two citrus genotypes: 'Natal CNPMF 112' and 'Natal Folha Murcha'

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Huanglongbing (HLB) is caused by fastidious phloem-restricted bacteria (*Candidatus* Liberibacter spp.) and vectored by *Diaphorina citri* Kuwayama (Hemiptera, Liviidae), the Asian citrus psyllid. As there is no cure for the disease, methods for reducing psyllid population are required. The understanding of the interactions between insect vector and its host plants may contribute to development of alternative methods of insect control. This research aimed to evaluate the attractiveness of two citrus genotypes ('Natal CNPMF 112' and 'Natal Folha Murcha') to *D. citri*. 'Natal' plants with young shoots, grafted onto *Poncirus trifoliata*, were used. The insects were originated from the rearing kept in the Laboratory of Entomology of Embrapa Cassava and Fruits. Freshly-emerged females were individualized in glass tubes containing a small branch of *Murraya paniculata* for 4 to 7 days, until they were used in a bioassay. Before a bioassay, the females stayed without food for 1 to 2 hours. In total, 87 females were tested individually using a multiple-choice (Pettersson) olfactometer. The treatments were volatiles from plants of the two varieties (two arms treated with each variety). The positions of treatments follow a completely randomized and was alternated every three bioassays. Means for residence time and number of entries were subjected to t test ($p<0.05$). The residence time spent in 'Natal CNPMF 112' arms was higher than in 'Natal Folha Murcha' ($p=0.04$). The insects also presented a higher number of entries in arms containing 'Natal CNPMF 112' compared to 'Natal Folha Murcha' ($p=0.04$). The behaviour responses of *D. citri* may result in a low preference for 'Natal Folha Murcha' plants under field conditions. The identification of the chemical profile of volatiles of each cultivar evaluated in bioassays are in progress. These studies can provide information to understand the host selection mechanisms based on the attractiveness of citrus genotypes evaluated to *D. citri*.

Screening of rootstock citrus genotypes for resistance to *Diaphorina citri* Kuwayama

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Huanglongbing (HLB) has emerged as the most severe disease worldwide. HLB-symptomatic trees must be eradicated, causing a very high socio-economic impact on the citrus industry. In Brazil, Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera, Liviidae) is the insect vector. Currently, HLB management depends on the sanity of seedlings, plant eradication and efficient vector control. This research was carried out aiming to identify rootstock citrus genotypes regarding to the attractiveness and host selection to *D. citri*. A completely randomized design experiment was used to evaluate 24 genotypes under greenhouse conditions with five replicates for each genotype. There were two experiments in September 2015 and in June 2016. For infestation, *D. citri* adults were evenly distributed inside the greenhouse at a proportion of 20 adults per plant for the first experiment and 56 adults per plant for the second experiment (total of 720 insects and 2016 insects for the first and second experiment, respectively). The number of adults, eggs and nymphs were counted. For the first experiment, the number of eggs was assessed through a scale from 0 (without eggs) to 3 (more than 20 eggs). Data were subjected to analysis of variance and the means were grouped by the Scott-Knott test ($p<0.05$). The variables number of adults and number of nymphs were transformed to root square ($x+0.5$). The least preferred genotypes were TRBK (trifoliata

'Benecke'), LCR x TR - 001, HTR - 069, LRF x (LCR x TR) - 005, HTR - 051, TSKC x (LCR x TR) - 040, TSK x TRBK - CO and TSKC x CTCM - 008. Otherwise, LAJC (laranjeira 'Azeda Jacarandá'), LVK x LCR - 038 and tangerineira 'Sunki Tropical' were the most preferred genotypes. It is concluded that there is a genetic variability for selection of rootstock genotypes to *D. citri*. Further studies are necessary in order to identify the mechanisms of differential preference.

Visualizing bacterial pathogenic-symbiotic metacommunities of the Asian citrus psyllid using fluorescent in situ hybridization (FISH) and mass spectrometry-based proteomics

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Candidatus Liberibacter asiaticus (CLas) is a phloem-limited, gram-negative, fastidious bacterium that is associated with the development of citrus greening disease; Huanglongbing (HLB). CLas is transmitted by the Asian citrus psyllid (ACP) *Diaphorina citri*, in a circulative manner mainly through the psyllid midgut and the salivary glands. Interactions among ACP and its microbial endosymbionts are likely to impact transmission of CLas. *Ca.* Carsonella ruddii (Gammaproteobacteria) and *Ca.* Profftella armatura (Betaproteobacteria) are primary/obligated symbionts and housed in specialized cells known as the bacteriocytes. Genome sequencing data for these symbionts indicate that *Carsonella* has a nutritional symbiosis with the psyllid and *Profftella* may be a defensive symbiont. More than 10% of the genome of *Profftella* has been reported to be involved in the production of a polyketide toxin called diaphorin. *Wolbachia* (Alphaproteobacteria) is a secondary/facultative symbionts reported in the ACP. Our proteome data revealed that CLas negatively regulates the expression of *Wolbachia* signaling proteins in the psyllid gut. In the bactericytes, proteins involved in polyketide biosynthesis by *Profftella* are up-regulated and the production of diaphorin and a diaphorin-like polyketide are changed in CLas-exposed insects. To investigate changes in the psyllid microbiota at the cellular level, we performed a thorough confocal microscopy analysis within the ACP to determine the localization of CLas in insect vector and the possible effects of the pathogen on the ACP microbiota. For this purpose, we used dual and triple hybridization of symbiont-specific, fluorescently labeled DNA probes in healthy and infected ACP coupled to confocal microscopy for probe visualization. Consistent with previous reports, we observed CLas along the alimentary canal. *Wolbachia* was also found to be in alimentary canal. Intriguingly, CLas and *Wolbachia* were detected within the same cells of the midgut but localized to distinct subcellular compartments. CLas-exposure did not change the distribution of *Carsonella* and *Profftella* in the psyllid. Our studies provide foundation for the development of symbiont-based control strategies against CLas.

Proteome mining: using peptidomics to identify biomarkers of CLas infection in the Asian citrus psyllid

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One of the major challenges in the fight against Asiatic huanglongbing (HLB), caused by '*Candidatus* Liberibacter asiaticus' (CLas), is that current diagnosis methods are unreliable. Detection of the pathogen relies on qPCR analysis of DNA extracted from citrus leaves, often chosen based on the presence of symptoms. However, due to the extended incubation period observed in CLas-infected plants, the nonspecific nature of disease symptoms, and the uneven distribution of CLas in the tree, this method often results in false negative diagnostic results. Furthermore, recent research indicates that newly infected, young citrus leaves can become infectious after receiving an inoculum of the bacterium from an adult psyllid in just 10-15 days. Therefore, detection of psyllids harboring CLas may provide a more reliable assay for growers to use from the initial detection of invasion and throughout the asymptomatic period. The peptidome is comprised of a dynamic set of small polypeptides that are continuously produced by proteolysis and other cellular processes, and has been a source of biomarkers for cancers and neurodegenerative diseases in humans. This study uses a similar workflow to screen the peptidome profiles of psyllids reared on CLas-infected or uninfected citrus for biomarkers unique to infected or CLas-free psyllids. To that end, proteins were extracted from sets of ~1000 infected or uninfected adult psyllids collected within one week of molting. Native peptides were separated by centrifugal ultrafiltration and analyzed by high resolution mass spectrometry. We have identified candidate peptides that predict infection state of psyllids, which will be validated by testing a diverse collection of psyllid samples from Florida and California and by using an array of sample preparation methods to minimize any geographical or genotypic biases in the psyllid peptidome phenotype. As the peptidome reflects the physiological state of an organism, biomarkers of infection are expected accumulate long before bacterial titers exceed the threshold for detection by qPCR and symptoms appear. The information obtained from this work will be used to engineer field-deployable diagnostic tools using synthetic biology approaches. These simple-to-use tools will aid ongoing HLB and epidemiological studies on the spread of HLB as well as rapid detection and response to the appearance of CLas-positive insects in a grove.

Biological control of the Asian citrus psyllid, *Diaphorina citri*, in the Lower Rio Grande Valley of Texas using the ectoparasitoid, *Tamarixia radiata*

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Tamarixia radiata Waterston (Hymenoptera: Eulophidae), is a biological control agent of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), that is being used as a tool to help reduce psyllid populations in urban environments of citrus growing areas in Texas. The USDA APHIS PPQ S&T CPHST Mission Laboratory has developed methods to produce large numbers of the beneficial insects for the biological control of ACP. Releases were made in citrus trees where plant tissue testing positive for HLB has been detected. In 2010, before we began our releases, we were detecting up 43 immature psyllids per flush in residential citrus. Since our biological control releases began, we have seen the populations gradually decline. The latest finds in 2016 indicate we are observing only 3.8 immature psyllids per flush. This is a reduction about 91.2% of the psyllid population.

Systems biology resources for the citrus greening disease complex

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Huanglongbing (HLB), considered to be the most devastating of all citrus diseases, involves citrus host trees, the Asian citrus psyllid (ACP) insect and a phloem restricted, bacterial pathogen *Candidatus* Liberibacter asiaticus (CLas). We have designed a web portal with information for consumers and growers with genomics and bioinformatics resources for the host citrus (*C. clementina* and *C. sinensis*), the vector Asian citrus psyllid (*Diaphorina citri*) and multiple pathogens including CLas. We have implemented JBrowse to provide the context for expression data and features annotated on the genome. We have also used Biocyc Pathway Tools databases to model biochemical pathways within each organism and which can be used to explore the entire disease complex. DiaphorinaCyc, the pathway database for D. *citri*, is avalilable at http://ptools.citrusgreening.org/ and users can upload multi-omics data sets from transcriptomics and proteomics experiments and analyze pathways for differentially expressed genes. Micro-CT analysis of the ACP will be combined with transcriptomics data from different tissues, life stages, conditions and sexes to create a 3D atlas that will reveal the internal anatomy of ACP overlaid with the expression profile of different tissues across major life stages. All tools like JBrowse, Biocyc, Blast and the Atlas connect to a

central database containing gene models for citrus, ACP and multiple Candidatus Liberibacter pathogens. The portal includes user-friendly manual curation tools to allow the research community to continuously improve the knowledgebase as more experimental research is published. Bulk downloads are available for all genome and annotation datasets from the FTP site (ftp://ftp.citrusgreening.org). The portal can be accessed at https://citrusgreening.org/.

Investigating the role of secreted proteases during HLB progression

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The inability to culture the huanglongbing (HLB)-associated pathogen *Candidatus* Liberibacter asiaticus (CLas), has provided significant challenges for understanding the molecular mechanisms controlling disease progression. To provide insight on how CLas manipulates citrus during infection, we used a comparative proteomics approach to identify dynamic changes in the phloem proteome of navel sweet oranges. Navels were mock or graft inoculated with the California CLas strain HHCA. At ten months post inoculation, crude phloem was extracted from uninfected and infected navel trees. Differentially expressed proteins were identified using label-free quantitative mass spectrometry. We identified various protease classes such as glycosidases, serine proteases, cysteine proteases, and aspartic proteases as significantly induced. Homologous proteins act as immune-related proteases in *Arabidopsis*, tomato, and maize and are targeted by diverse pathogens to promote infection. The overexpression or deletion of specific proteases in crops such as maize, tomato and potato have shown to have enhanced resistance and susceptibility to pathogens, respectively. To identify whether the identified proteases are also induced in citrus field samples, we acquired uninfected and infected samples from a citrus grove in Texas. We used mass spectrometry to quantify protease abundance. Additionally, we assayed for activity using activity-based protein profiling (ABPP). ABPP permits the quantification and identification of active proteases. Our current data demonstrates that the abundance and activity of several proteases is increased in infected samples. A specific group of proteases display enhanced abundance but no altered activity during infection. Based on these observations, we hypothesize that CLas suppresses citrus defense responses by targeting host protease activity. Identifying the role of citrus proteases will contribute to the development of HLB biomarkers and specific proteases could be manipulated to enhance resistance to CLas.

Genomic and proteomic investigation of the interaction between citrus and *Candidatus* Liberibacter asciaticus

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Huanglongbing (HLB) is a widespread and devastating citrus disease. The HLB-associated pathogen, *Candidatus* Liberibacter asiaticus (CLas) is transmitted by the Asian citrus psyllid, a piercing-sucking insect. The Asian citrus psyllid feeds on citrus phloem and can acquire CLas by feeding on an infected tree. In this study, we used mass spectrometry and quantitative proteomics to identify proteins that were differentially expressed in navel phloem during infection. Multiple differentially expressed proteins were proteases and we were able to identify the same classes of proteases as differentially expressed in infected navel trees sampled from the field in Texas. These proteins have the potential to serve as biomarkers for HLB as well as targets that can be manipulated for disease control. We have also investigated the genetic diversity and conservation of predicted secreted proteins present in CLas. We analyzed eight currently available genome sequences and sequenced two new strains from China and Florida. Using stringent criteria, we identified a group of CLas secreted proteins that are conserved across all sequenced strains. Collectively, the completion of this research has enabled a greater understanding of how the plant responds to CLas infection as well as identified plant and bacterial components with promise for pathogen detection and control.

Recent progress of development on culture medium for *Candidatus* Liberibacter asiaticus

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Up to now, cultivation of citrus greening (huanglongbing) pathogens has been attempted in many laboratories. "*Candidatus* Liberibacter asiaticus (Las)" is known as a fastidious bacterium that is unculturable in many traditional culture media. To facilitate basic research and establish definitive diagnosis, we are developing a new selective culture medium for Las and the other related species. At first, we examined whether Las can multiply in some traditional media and Las specific media reported previously. Living Las cells were extracted from the infected citrus leaves according to our procedure which was developed in our previous reports. The multiplication of Las cells was evaluated from the copy number of Las DNA amplified using real-time PCR. The results indicated that almost existing media do not support the multiplication of Las cells enough. Thus we explored constitutions of a new Las selective medium. By comparing with other bacterial genomes, inability of Las genome to produce essential metabolic pathway (amino acid synthesis, carbohydrate catabolism, etc.) was predicted. Then based on this prediction, we have developed a culture medium by adding the compounds to replenish the shortage of nutrients for Las. And a new medium could induce the multiplication of Las cells. But multiplication was very slow and there was no visible typical colony on a solid medium. In spite of lack of visible colonies, after 14 days of incubation, Las cells were detected by *in situ* hybridization. Moreover, RNA-seq, RT-PCR and partially DNA sequencing could be performed from the invisible agents on the medium. Towards establishing the practical and reproducible method of cultivation, we continue to improve the medium.

Responses of *Candidatus* Liberibacter asiaticus to antibiotics in *in vitro* culture assays

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Response of *Candidatus* Liberibacter asiaticus to antibiotic treatment *in vitro* was demonstrated experimentally using a new medium. Primarily, a metabolic pathway analysis of *Ca*. L. asiaticus genome was carried out using KEGG database. Comparative approaches between *Ca*. L. asiaticus and closely-related bacterial species allowed us to identify a group of genes that do not remain in the pathogen's genome and select essential nutrimental components for the preparation of a new culture medium, including sugar, amino acids, fatty acids and vitamins that cannot be synthesized by the pathogen. Quantification of *Ca*. L. asiaticus was investigated for a month using real time PCR assays and *in situ* hybridization. *Ca*. L. asiaticus became detectable two weeks after initiating incubation and thereafter. Very small colony-like patterns appeared in the media, but the further growth was not observed. Furthermore, *in-vitro* assays to examine pathogen's responses to eight antibiotics (ampicillin, kanamycin, streptomycin, polymyxin B, chrolamphenicol, rifampicin, tetracycline, and oxytetracycline) was conducted using the new medium. The results showed that *Ca*. L. asiaticus did not proliferate in the culture medium supplemented with polymyxin B. On the other hand, supplement of tetracycline or oxytetracycline in the medium resulted in accelerating the growth of the pathogen. The mode of action of tetracycline and oxytetracycline on *Ca*. L. asiaticus remained unknown. In short, this study suggests that a culture-dependent approach may give us vital insights into further understanding of the behavior of *Ca*. L. asiaticus and contribute to the screening of potential agents and chemicals for citrus greening disease management.

The *Ca.* Liberibacter Culturing Consortium (*C*LCC): Developing methods to culture Ca. Liberibacter asiaticus – a game change in HLB research

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Development of methods to fight Huanglongbing (HLB, citrus greening disease), caused by "*Candidatus* Liberibacter asiaticus" (*C*Las), has been hampered by lack of ability to culture *C*Las in the lab, which precludes the testing of gene functions that may be involved in *C*Las viability or virulence. Identification of such gene functions will have an enormous impact on the ability to control *C*Las and eventually allow development of integrated strategies to stop HLB spread by the Asian citrus psyllid (ACP) and the resulting economic devastation to citrus agriculture. Development of the ability to culture *C*Las *in vitro* will open up new avenues of research that are guaranteed to provide a game change in knowledge of *C*Las biology and transmission, including a culturing platform necessary for development of an amenable genetic system. The *Ca.* Liberibacter Culturing Consortium (*C*LCC) was established to develop such an *in vitro* culture system and make it available to the citrus industry and academic research community by establishing a system for host cell-free culture of *C*Las, providing standard operating procedures for culture of *C*Las to the research community, and providing cultures through standard repositories. Progress to date in these efforts will be reported.

Putative secreted proteins of *Candidatus Liberibacter* asiaticus are localized in specific cellular compartments and triggered different responses in plants

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Candidatus Liberibacter asiaticus (Las), the most prevalent causal agent of huanglongbing citrus disease in North America and Asia, is a phloem-limited and unculturable bacterium. However, during the past few years, genomes of several *Liberibacter* species were sequenced suggesting that members of this genus have suffered a significantly genome reduction. Comparative genomics of Las has allowed the prediction of the putative effector proteins, potentially involved in virulence. In this scenery, where small secreted protein could play crucial roles between plant and pathogen interaction, effector proteins identification is a complex objective. Plant defense can be triggered by the host perception of conserved pathogen-associated molecular patterns (PAMPs). Pathogens in turn can suppress PAMP-triggered immunity, causing disease. Identification of Las effector proteins is a key step to understand the mechanisms by which Las disrupt the host response. To provide a functional basis for this hypothesis, eight of these sequences were cloned and expressed fused to green or red fluorescent proteins under the control of 35S promoter. Agrobacterium-mediated transient expressions were performed in *Nicotiana tabacum* and *N. benthamiana* leaves. Bacterial protein expressions were monitored over a 10-day period by fluorescence and confocal laser scanning microscopy. All tested genes shown expression in plant cells, and revealed different subcellular distribution patterns respect to the control. Expression of two of them produced particular phenotypes in plants. *CLIBASIA_04560* encoded a hypothetical protein which is localized in nucleus when it is transiently expressed in *N. benthamiana.* More interesting, this protein triggers an increase of H_2O_2 production respect to the control without necrotic or cell death phenotype in *N. benthamiana* after 3 dpi. Now, we are studying if Clibasia_04560 may act as an effector protein modulated the energetic metabolism in citrus plants.

Postharvest management of Asian citrus psyllid in bulk citrus – potential use postharvest fumigants

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Asian citrus psyllid (*Diaphornia citri* Kuwayama) is a known vector of Huanglongbing (HLB) disease that is an imminent threat to the California citrus industry. At present, controlling the psyllid vector is the best management strategy to delay the spread of HLB, while disease management tools are developed). In recent years ACP finds from trap catches have increased in the San Joaquin Valley especially in residential areas and at packinghouses and juice plants. Researchers have attributed HLB spread in Florida to human-assisted transport of infected psyllids and infected plant material including bulk citrus. ACP quarantine boundaries and HLB quarantine boundaries have been established in California and preharvest treatments are required to ship insect-free bulk citrus between quarantined zones to mitigate the spread of ACP and eventually HLB. It is clear from the finds at juice plants and packinghouses that current methods of orchard disinfestation (preharvest insecticide sprays) are not reducing psyllids sufficiently and psyllids are being moved in bulk citrus In the context of identifying a postharvest treatment to control ACP after harvest and before shipping to packinghouses, University of California Researchers in collaboration with USDA-ARS and Fruit Growers and Supply have begun testing the potential of fumigation on Asian Citrus Psyllid to develop a treatment plan for disinfesting bulk citrus. We discuss the prospects for use of postharvest fumigation to disinfest bulk citrus prior to shipping to other regions of the state.

Correlation of electronic monitoring and stylet pathways elucidate the role of sclerenchymatous ring as a barrier to phloem feeding on citrus leaves by Asian citrus psyllid

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Asian citrus psyllid (ACP, *Diaphorina. citri*) feeding behaviors play a significant role in the transmission of the phloem-limited *Candidatus* Liberibacter asiaticus (CLas) bacterium that causes the economically devastating citrus greening disease. Recent studies have shown a fibrous ring of thick-walled sclerenchyma around the phloem in mature citrus leaves that is more prominent on the lower compared with the upper side. We performed Electrical Penetration Graph (EPG) studies on ACP adults placed on lower or upper surfaces of young or mature Valencia orange leaves. Feeding sites on the same leaf tissues were then sectioned and examined by epifluorescence microscopy. Based on the EPG recordings and histological correlations, we found that thick-walled fibrous sclerenchyma located around the phloem of mature citrus leaves significantly reduced phloem ingestion by psyllids placed on the lower leaf surface compared with ingestion from the upper surface of mature leaves or on young leaves. The longest duration of phloem ingestion was observed from the upper side of young flush leaves that had the least developed sclerenchyma. Bouts of phloem salivation (E1 waveform), however, were significantly longer on mature leaves compared with young flush. ACP adults made consecutive phloem feeding attempts (bouts) on the lower side of mature leaves and those bouts resulted in unsuccessful or shorter periods of phloem ingestion. ACP adults also made more frequent and longer bouts of xylem ingestion on mature leaves compared with psyllids placed on young leaves. Our results support the hypothesis that the presence of a thick, well-developed fibrous ring around phloem tissues of mature leaves acts as a barrier to frequent or prolonged phloem ingestion by ACP from citrus leaves. This may have an important role in limiting or preventing CLas acquisition and/or transmission by ACP, and could be used for identification and development of resistant citrus cultivars.

An attract-and-kill strategy for Asian citrus psyllid

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Phytophagous insects including Asian citrus psyllids (*Diaphorina citri* Kuwayama) (ACP) use multiple sensory modalities [vision, olfaction, contact chemoreception, gustation (taste), perception of auditory or vibrational stimuli] to locate host plants or conspecifics. Many studies have reported that ACP is strongly attracted to bright yellow and light green. Several chemical blends have been suggested as ACP attractants, but only modest attraction has been demonstrated in the field. George et al. (2016. Chem Senses. 41:325-338) found that acetic and formic acids were highly excitatory to antennae of male and female adult ACP. These two compounds occur in the atmosphere surrounding citrus orchards and originate as breakdown products of common plant volatiles including *β*-ocimene and citral (geranial and neral) that oxidize spontaneously in air. A mixture of these compounds incorporated into SPLAT induced increased probing by ACP. Addition of a third compound (para-cymene) further increased probing in a 3-component blend. This blend acts as a phagostimulant by increasing the number and the length of probes into a wax substrate containing the 3-component blend. Phagostimulants may be useful to deliver "payloads" such as an engineered RNAi construct or to enhance ingestion of toxicants as part of an attract-and-kill strategy. Furthermore, ACP infected with the citrus greening pathogen probed the 3-component phagostimulant blend more often compared with healthy ACP. A number of sensory stimuli (cues) will be incorporated into the design of an attract-and-kill device to optimize attraction of ACP and mortality after psyllids land on the device. The goal of this project is to develop an economical and environmentally appropriate management strategy for ACP that exploits what we have learned about the psyllid behavior including its response to visual, olfactory, and gustatory stimuli.

Evaluation of the presence of HLB in Rutaceae in nature reserves in Misiones, Argentina

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The disease Huanglongbing (HLB) is currently the most serious threat for Argentina's Citrus industry. Early diagnosis of affected tissues requires conventional molecular methods, polymerase chain reaction (PCR) or quantitative real time PCR (qPCR), due to the inability to grow any of the bacteria involved in the disease etiology: *Candidatus* Liberibacter asiaticus (CLas), africanus (CLaf), and americanus (CLam). Because HLB is incipient in Argentina eradication of all infected plants is recommended, however the presence of HLB in wild plants cannot be ruled out or has not been locally studied. The aims of this study were to localize native and feral Rutaceae in forested areas of Misiones province, characterize suspicious symptoms associated with HLB, and identify the presence of vector (*Diaphorina citri* Kuwayama) and its parasitoids. After two sampling campaigns performed during August/October of 2016 spanning more than 20 conserved areas in Northern and Central part of Misiones, we found neither HLB positive plants nor presence of adults or immature psyllids stages within the provincial nature reserves. All HLB positive plants identified were located nearby backyards and citrus commercial plots, which indicates a strictly anthropogenic influence for HLB spreading in that area. Forestry parks of Misiones appear a complicated place for HLB settling because extremely low population level of vector. From several species of Citrus identified, the most abundant were Rangpur lime (*C. limonia* Osbeck) and Criolla tangerine (*C. reticulata* Blanco), which are widely distributed in all parks surveyed. Several other native Rutaceae which cohabit with feral Citrus were identified: *Helietta apiculata* Benth, *Fagara rhoifolium* Lam., *Balfourodendron riedelianum* (Engl.) Engl., *Esembeckia grandiflora* Mart. Samples tested from native Rutaceae were also negative for CLas/CLam. All feral Citrus located in conserved areas of Misiones represent a remarkable source of germplasm for future breeding programs.

Risk based HLB survey for Hacienda Heights and San Gabriel in Southern CA

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After the discovery of a single residential HLB tree in the Hacienda Heights area in 2012, another HLB positive tree was confirmed in San Gabriel early July 2015. This is the second time the disease has been identified in Los Angeles. Subsequently 17 additional locations have been discovered with HLB within 800 meters of the initial infected tree. To prevent further spread of the disease, it is necessary to design a high intensity residential survey to quickly identify additional HLB-infected trees in the area. An extended quarantine with approx. 5 mile radius (i.e. 87 square miles) was created around the properties on which HLB was recently detected in the city of San Gabriel. Residential population density does not have a uniform distribution in this part of the Los Angeles basin. For balanced and efficient sampling like the Hacienda Heights area, San Gabriel is divided into eight radiating segments (defined as N, NE, E, SE, S, SW, W, NW) around the centroid of recently found HLB positive properties. Six inner buffer zones (i.e. 400m, 800m, 1200m, 1 mile, 2 miles & 5 miles) were also created to adapt different sampling strategies if needed. The overall sampling proportion in each segment is determined by the number of households and previous ACP finds. Percent sampling effort ranges from 9.2% to 15.6% among the eight directional sectors. Within each segment, sampling locations (i.e. census blocks) are selected based on an overall risk calculation, which is a combination of predetermined residential HLB/ACP risk, distance from HLB finds, census travel risk from Asian connections, previous ACP density, and distribution of CLas+ ACP locations (note, inconclusive CLas+ ACP locations were also considered with less weighting). The residential property density also influences the chance of location selection and modulates sampling intensity. According to the manpower availability and sampling efficiency, total sampling capacity of 52,800 properties is calculated through the survey period. A total of 948 moderate to high risk census blocks (i.e. risk>0.8) were identified within the eight sectors through the risk-based model. Using the overall risk calculation we define, 81% of census blocks in the San Gabriel quarantine area are sampled at least once. Approximately 10% of the properties within the risk-defined census blocks are surveyed to give a reliable representation of HLB dispersal. To maximize HLB/ACP detection in San Gabriel, we provide a detailed sampling plan for three temporally contiguous sampling cycles with approximately the same duration. Because of the intensity of risk in some areas, there is some overlap in the selection of census blocks to sample, which will provide multiple opportunities to detect the disease in higher-risk areas as the tree phenology changes due to flushing and climate. The designed survey methodology can be used to monitor actual HLB situation, check whether HLB has spread out of the quarantine area, and determine the possible pathway for HLB spread. The survey methodology is flexible and dynamic. If additional HLB locations are found outside the original HLB epicenter, adjustments can be made to address sample intensity in the new areas.

Census-travel risk model to predict points of disease/pest introduction

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There is increasing concern that invasive pests and diseases can be introduced into new areas due to international travel. For early detection of pathogens and their vectors, we have developed a predictive-system methodology that maximizes the prediction points of introduction and identifies high-risk areas for local transmission at an early epidemic phase. The model is versatile and independent of pathosystem, meaning it can be applied to various hosts, pathogens and vectors, and other insect pests. The model is constructed based on US Census and international travel data, partnered with knowledge of the epidemiological characteristics of the pathosystem. Combining existing foreign population habitat and international pathway data, the model generates a risk index map to identify locations (at various spatial scales) with ranked introduction potential for the disease. The foreign travel distribution is estimated for each of 176 individual source countries. This is then coupled with actual travel statistics from national travel and tourism offices to calculate the risk. The risk weighting for each

individual source country is adjusted by the number of confirmed disease cases (e.g. log transformed) or the percentage of active area. The census travel model estimates the likely destination(s) of foreign travelers (e.g. connectivity to family, relatives & friends) in order to place a risk-bias for survey of interested areas. The census travel model is currently implemented in Shiny (a web application framework for R) to bring greater ease-of-use for regulatory agencies to detect high risk pathogens of agricultural significance at the early epidemic phase. The census travel model can parameterize and weigh risk contribution of international travelers in various categories (i.e. seasonal travel volume, visa class, age and gender) given there is a valid disease-associated concern. The census travel front-end allows users to run the model that best suits their needs without strong knowledge of background code and data. Risk maps have been generated for plant diseases (e.g. huanglongbing, citrus black spot, plum pox virus) and other human viruses (e.g. Ebola, malaria, dengue, Chagas and Zika). The model has been incorporated into risk-based Multi-Pest Survey (MPS) programs to provide an augmentative risk factor for continued risk introduction from human-mediated disease spread. In addition, the model can be linked with survey scenario estimators to balance efforts and costs within agency constraints and among emergency programs.

Canine assisted early detection of HLB

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The underpinning for HLB control is early detection and early response, especially in Texas and California where incidence is low and Arizona where the disease has not been detected. Eventually Florida, which has been devastated by the disease, will likely replant large areas to reestablish the citrus industry. In all of these situations, the optimal control strategy is to inhibit HLB from entering and establishing in commercial plantings. Infected tree removal, to reduce inoculum in the early stage of the epidemic, remains the most effective deterrent to epidemic development. The earlier the detection of Candidatus Liberibacter asiaticus (CLas) infections, especially when asymptomatic or better yet subclinical, the more efficacious infected-tree removal can be. A number of early detection technologies are being explored among which canine detection of CLas infected trees, shows considerable promise. Twenty dogs were trained for early detection via a USDA, APHIS HLB Mac grant. Using 10 dogs, each tested against 1000 trees in replicated randomized “field trials” in a gridded array with varying HLB-incidence, resulted in 99.17% overall detection accuracy. All dogs performed very well with statistically insignificant trends toward false negative or false positives. Each dog has its own personality and interacts slightly differently depending upon the trainer-handler. However, there was no statistical difference in CLas detection by trainer-dog combination. We also explored the use of multiple dogs for confirmation of CLas infections. When two or more dogs alert on the same tree, the tree statistically has a 100% probability of infection. Dogs were also capable of accurately detecting CLas-infected trees exclusively from 5-gm feeder root samples. Using 10 dogs in a time course experiment with ACP inoculated trees, dogs began to detect CLas infections within 2-3 weeks of inoculation, whereas, none of the trees became PCR-positive for CLas until three months post inoculation and the majority of CLas-infected trees required multiple months prior to PCR detection. This confirms that dogs are indeed a very early detection methodology; able to detect CLas in trees with subclinical infection, i.e., before symptom expression and considerably prior to the ability of PCR for detection/confirmation. In field trials of young and mature citrus plantations, canines trot along the rows with an average interrogation time of ~1 tree/sec, most rapid of all detection methodologies. Dogs were also effectively utilized for detection of CLas infected trees in residential areas. Dogs are rewarded for detections by verbal praise and short duration play with handlers. Various deployment strategies will be discussed based on the known spatiotemporal distribution of CLas infected trees.

Use of HLB detection canines in real world settings

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Using canines to detect HLB in citrus has shown extreme promise in the research environment. Tests on different aged infections, different cultivars, roots, and infections from both grafting and psyllids have all shown the dogs abilities to detect and differentiate an HLB infected tree from clean trees at accuracies exceeding 99%. The next logical step is the introduction of the canines to “real world” environments, i.e., commercial citrus plantings and residential or “dooryard” trees to determine if detection accuracy continued. The dogs were introduced to a young grove in Florida first, and trained over the course of several months. However, due to the high incidence of infection in Florida, finding a suitable low disease incidence training area for dogs in a mature grove is almost impossible. The dogs were therefore taken to other states, especially Texas, for training and testing. This also allowed the dogs to work in different environments, different grove management practices, and different citrus species. Another aspect of early canine detection is the use of the dogs in residential environments. Again, using other states for a test bed, the dogs were run through various types of residential areas, including residential neighborhoods, mobile home parks, and even a Buddhist monastery. The dogs showed the adaptability to successfully work in these exceedingly diverse and sometimes environmentally hostile environments. *Specificity* (in statistical terms the true negative rate) includes the ability of dogs to accurately discriminate between *Candidatus* Liberibacter asiaticus (CLas) infections and infections by other pathogens or extraneous scent profiles. To accomplish this, a group of the dogs were taken to Beltsville, Maryland and tested against trees held in the international pathogen collection composed of a number of different viral and bacterial citrus pathogen accessions from around the world. The canines consistently differentiated CLas-infected trees and did not alert on trees infected with other pathogens. One unique finding was that dogs trained on CLas were also intrinsically capable of detecting Liberibacter africancus (CLaf) and Liberibacter americanus (CLam) infected trees as well, without additional sensitization or training.

California strategies for managing Asian citrus psyllid within groves and for limiting human-assisted transport

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Asian citrus psyllid continues to spread in California both by natural means and human-assisted means. In areas of the state where the psyllid is well established, such as Southern California, growers are applying coordinated insecticide treatments to reduce populations. In these regions, urban areas receive parasitoid releases to establish *Tamarixia radiata* and *Diaphorencyrtus aligarhensis*. Insecticide treatments are only applied to residential citrus trees within 400 meters of commercial citrus. Studies are underway to determine the efficacy of these treatments and to improve the areawide programs in various regions of the state. In regions of the state where the psyllid is not well established, eradicative treatments of pyrethroids and neonicotinoids are applied to both commercial and residential sites around trap finds. These finds have a high correlation with traffic corridors, packinghouses and juice plants, indicating that humans are assisting psyllid spread by moving infested bulk citrus, equipment and plant material. Industry-led changes in how the commodity is handled are expected to reduce the impact of human-assisted psyllid spread. Control of psyllid movement around the state is critical to prevent spread of huanglongbing.

Soil and water acidification sustain root density of huanglongbing-infected trees in Florida

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Early symptoms of HLB include fibrous root loss and leaf blotchy mottle, followed by premature fruit and leaf drop, and yield decline. As a consequence of initial bacterial infection of fibrous roots, a 30-50% reduction in fibrous root density and elevated soil Phytophthora populations were detected in field surveys. Continued sampling of Hamlin and Valencia orange trees on Swingle citrumelo rootstock in different stages of HLB decline revealed that root loss occurs in two stages. The second phase of root loss (70-80%) begins at the early stage

of tree canopy thinning resulting from leaf drop and branch dieback. A more extensive survey of HLB-affected groves indicated that greater decline in fibrous root health and expression of HLB symptoms is observed where irrigation water is high in bicarbonates (> 100 ppm) and/or soil pH > 6.5. HLB symptom expression of trees on different rootstocks follows the known intolerance to bicarbonate (Swingle citrumelo > Carrizo citrange > sour orange > Cleopatra mandarin). Acidification of irrigation water in central ridge and south central flatwoods Valencia orange groves on Swingle citrumelo rootstock for three seasons has maintained soil pH below 6.5 on the flatwoods and 6.0 on ridge. Over the last three seasons of survey, root density as an index of root heath has been sustained. Phytophthora populations remain below the damaging level in ridge groves and in flatwoods increase to damaging levels coincident with the fall root flush but drop back to non-damaging levels for remainder of the season. Compared to the 2013-14 season, yields in the ridge blocks have increased up to 4% and on the flatwoods have increased up to 22%. Growers using acidification treatments with sulfuric and/or N-phuric acid for the last 3 seasons report an average cost of $60 per acre. This cost will analyzed in relation to yield response to provide a cost benefit of acidification

'Hybrid' nutrition programs featuring soil-applied Controlled Release Fertilizer (CRF) or frequent treatments with liquid nutrition can improve the health, growth and productivity of HLB-infected trees

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Our field and greenhouse data shows that secondary and micronutrient deficiencies are much greater in roots than in leaves of HLB-impacted trees. Not only is there severe feeder root loss, but the ability of the remaining roots to mine and translocate essential nutrients is compromised. Restoring 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 and more recently TigerSul micronutrients in the field and greenhouse. In a previous greenhouse study, HLB-infected Valencia sweet orange on UFR-3 rootstock (Nova+Hirado Buntan pummelo x Cleopatra+Argentine trifoliate orange) showed greater feeder root growth when grown with Harrell's CRF supplemented with a 3x overdose of TigerSul manganese, or the Schumann blend of TigerSul manganese, iron and zinc (nutrients in sulfate form, embedded in clay prills). Results suggested that trees in the HLB world have higher specific micronutrient requirements than what are currently being recommended. PCR testing of the greenhouse trees suggested that an overdose of boron could suppress the bacterial titer in the midribs, whereas an overdose of manganese could suppress the bacterial titer in the roots. Several experiments are ongoing in the field to test hypotheses developed from the above information. We define 'hybrid' nutrition programs as any program that combines multiple sources of nutrient delivery with a goal of providing a constant supply of all required nutrients year round (including winter!) at an affordable cost. Successful field experiments with industry cooperators to be discussed include the St. Helena Project, where we are testing 100% CRF; and the Post Office Block, where we are testing a 50% CRF/50% traditional dry fertilizer program with micronutrient overdoses. Other successful 'hybrid' programs successfully developed by citrus growers will also be presented, including the Ed English program and the Duda program. Data from these trials will demonstrate that enhanced ground nutrition can restore health and sustainable profitable production to HLB-infected trees. Additional fine-tuning of fertilizer composition, type and delivery method could result in less need for psyllid control and thus lower production costs.

Breeding rootstocks to prevent or mitigate HLB in commercial trees

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Genetic variability for HLB tolerance/resistance has been identified (grafted trees) in existing experimental rootstock germplasm (not pre-screened for HLB tolerance) 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. Such rootstocks are also showing a stronger recovery following infection in commercial trials. This information suggests that it should be possible to substantially increase the level of tolerance by conventional breeding at both the diploid and tetraploid levels, with focus on direct selection for HLB tolerance in progeny from carefully selected parental combinations. For the past 5 years, we have developed and utilized our 'Gauntlet' screening program described below. Each year, crosses of superior parents are made at both the diploid and tetraploid levels. 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 Valencia 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. To date, several thousand individual hybrids have been screened. The oldest 'Gauntlet' trees have now been in the field for approximately 4 years, and several promising new rootstocks have been identified. Although PCR+, individual trees appear to have a reduced bacterial titer and are showing normal healthy growth and fruiting patterns. Seed trees of two of the most promising selections are producing abundant polyembryonic seed, and propagations for large-scale field trials are underway. Patterns are emerging regarding successful genetic combinations, and this will be discussed. 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.

Longevity of imidacloprid soil drench on citrus nursery stock for sale at retail stores in Florida

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Florida requires that citrus plants for sale be treated with an imidacloprid-based soil drench (ISD) to control *Diaphorina citri* (Kuwayama), the vector of the pathogens that cause huanglongbing. Nursery producers are required to tag the plants with the date of treatment. Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS/DPI) inspectors visit retail citrus venues regularly to look for colonies of *D. citri* on citrus plants for sale. If inspectors find psyllids, the treatment has failed. Between January 2007 and September 2016, FDACS/DPI inspectors have collected 1,872 *D. citri* samples throughout the state. Although it is impossible to know the percentage of failed treatments (inspections are variable and total numbers of plants is unknown), the frequency distribution of *D. citri* samples among cohorts of months past ISD treatment should not vary with samples size or inspection frequency. On average 7.3 % of infested citrus trees had been treated within the past 30 days, and 17.3 % of the infested trees had been treated between 31 and 60 days prior to sampling. This increase suggests that some treatments could be failing in the second month after treatment. The percentage of infested plants with expired (>6mo since treatment) or missing tags declined from more than 30% in 2009-2012 to less than 10% in 2016.

ImmuneTissue Prints: a simple and scalable complement to PCR-based assays for the detection of '*Ca*. Liberibacter asiaticus'

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Antibodies form the basis for various assays designed to detect plant pathogens in many pathosystems, but until recently have not been available to detect '*Ca*. Liberibacter asiaticus' (CaLas). We have used genome sequence data to identify epitopes on the surface of CaLas. Genes that encode these epitopes have been cloned in *E. coli*, and the proteins expressed, purified and used to immunize rabbits. The major outer membrane protein A (OmpA) has been used as the basis of a immune tissue print assay for CaLas. A problem for assays of CaLas is

the well known erratic distribution of the pathogen in infected citrus trees. DNA based detection methods require extraction and purification of the DNA prior to the assay. The immune tissue print assays are done directly on plant material and preserve and record the distribution of the pathogen with resolution of single infected phloem cells. Using this method, the pathogen has been readily detected in all tissues of infected sweet orange and lemon trees, a portion of the leaf midrib has been identified as ideal site for sampling. The tissue print assay also scales well to many samples. The rabbit polyclonal antibody has also been used to purify CaLas cells from plant extracts prior to PCR. Immune tissue prints and standard qPCR assays have been carried out on the same samples and are complementary. When both immune tissue prints and qPCR assays are used, the combined data show instances where each assay records a false negative, but together nearly all infected samples are detected.

Deployment of disease resistant or tolerant citrus rootstocks and scions

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Huanglongbing (HLB) is a devastating bacterial disease of citrus which causes yield loss, tree decline and reduces fruit and juice quality. HLB is caused by *Candidatus* Liberibacter asiaticus (*C*las) and transmitted by the Asian citrus psyllid (ACP, *Diaphorina citri*). Many horticultural management practices have evolved to manage HLB infection such as pest management practices, the establishment of Citrus Health Management Areas (CHMAs) and addition of bactericides. There has been much investment by many funding agencies in many research topics to find solutions to HLB, including plant breeding and biotechnology. Plant improvement to develop HLB resistant or tolerant rootstocks and scions through conventional or biotechnology is the most likely to provide sustainable solutions to HLB. The goal of Citrus Research Development Foundation (CRDF) is to support research and to deliver HLB solutions to growers as quickly as possible. There are several plant improvement research programs in the US using conventional and biotech methods. In Florida, there are researchers at the University of Florida and USDA-ARS research departments comprised of several programs and many researchers. Due to the diversity of programs, program objectives, approaches to research and evaluation, it is difficult to understand the scope and progress of post-HLB research and the current status of projects. CRDF is investing in efforts to work with researchers to develop a common development, evaluation, and deployment platform to accelerate commercial release of economically viable HLB resistant or tolerant rootstocks and scions.

Impact of *Candidatus* Liberibacter asiaticus infection on Asian citrus psyllid transcriptome

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The Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama is the insect vector of the fastidious plant pathogen bacterium *Candidatus* Liberibacter asiaticus (*C*Las), the causal agent of citrus greening disease, also known as Huanglongbing (HLB) which is the most significant and widespread threat to the citrus industry. To investigate gene expression profiles that associate with ACP- *C*Las interactions and identify genes in response to *C*Las infection, we constructed RNA-seq libraries from *C*Las-infected and *C*Las-free ACP samples of three different developmental stages (nymphal instars 1-3, nymphal instars 4-5, teneral and post-teneral adults). With 150 bp paired-end sequencing on the Illumina Hiseq2500, we generated 152 Gb of sequence data from 56 million reads per library/replicate, which was assembled into 34,122 contigs with 18,827 (55.2%) being annotated, which were then further analyzed for potential functional classification and potential roles in infection. The results suggested that gene expression in different developmental stages did not respond in the same manner to *C*Las infection. With more contigs being up or down- regulated, nymphal instars 4-5 showed a more sensitive response to *C*Las infection than nymphal instars 1-3 and adults. A comprehensive analysis of the transcriptomes revealed vector life stage differences and differential gene expression in response to *C*Las infection, and identified specific genes with roles in nutrition, development, immune response and transmission pathways.

Small scale system for testing antimicrobial compounds targeting '*Candidatus* Liberibacter asiaticus'

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In Florida approval was granted for field application to citrus trees of formulations of streptomycin and oxytetracycline to control infections by '*Ca*. Liberibacter asiaticus' and the subsequent development of the associated disease, huanglongbing (HLB). We developed a procedure for infecting individual trees by grafting a single symptomatic leaf to the tree. We wanted to determine if this procedure could serve as a small scale assay system for testing conventional antibiotics and other compounds for antimicrobial activity effective against '*Ca*. L. asiaticus'. The working hypothesis behind the assay was that the application of the candidate compound to the inoculum leaf before or after grafting would impede or prevent bacteria in the inoculum leaf from infecting the receptor tree. Aqueous solutions of oxytetracycline or streptomycin, containing either glycerol or the commercial compound Pentra-Bark to aid penetration were applied under 20 psi of pressure to both the adaxial and abaxial surfaces of individual symptomatic leaves before or after grafting the leaf to a receptor tree. Receptor trees were inspected periodically for symptoms and after five to seven months leaf samples were evaluated for infection using real time PCR to detect pathogen DNA. The cumulative results from six trials found 44/60 (73%), 46/75 (61%) and 53/80 (67%) of trees infected by grafted leaves treated with oxytetracycline, streptomycin or no treatment, respectively. The data suggest that under the conditions of this study, these two compounds failed to impede infection of the receptor trees relative to non-treated controls. The reasons for the lack of success in preventing infection are unknown but could be related to either the effectiveness of the compounds or the method of application, or both.

Biocuration: Deciphering the draft genome of Asian citrus psyllid one gene at a time

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The Asian citrus psyllid (*Diaphorina citri* Kuwayama) is the insect vector of the bacterium *Candidatus* Liberibacter asiaticus (CLas), the causal agent for the citrus greening or Huanglongbing disease which threatens citrus species worldwide. This vector is the primary target of approaches to stop the spread of the pathogen. Accurate structural and functional annotation of the psyllid's gene models and understanding its interactions with the pathogenic bacterium, CLas, is required for precise targeting using molecular methods. The draft genome was annotated with automated pipelines. Knowledge transfer from well-curated reference genomes like *Drosophila* to a newly sequenced insect is challenging due to the diversity and complexity among all insect genomes. We opted for manual curation of gene families that have key functional roles in *D. citri* biology and pathology. The community effort produced more than 450 manually curated gene models across developmental, RNAi regulatory, and immune-related pathways. Curators included undergraduate and graduate students from multiple institutions as well as experienced expert annotators from the i5k community. Here we report the official gene set (OGS v1.0) for the Asian citrus psyllid genome that includes manually curated genes involved in pathways of experimental interest for vector control strategies. All data is available on https://citrusgreening.org/.

Expression of *Diaphorina citri* hemocyanin protein correlates with color morphology and exposure to the HLB pathogen *Candidatus* Liberbacter asiaticus

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The Asian citrus psyllid, *Diaphorina citri* is the insect vector of *'Candidatus* Liberibacter asiaticus' (CLas), the causal agent of HLB. Proteomic characterization of *D. citri* identified hemocyanin as central to the insect's response to CLas-infected trees. Hemocyanin expression is highly induced psyllids reared on CLas-infected *Citrus medica* Linn. (Citron) and has been found to physically interact with a CLas vitamin A biosynthetic enzyme. Hemocyanins function as respiratory proteins of mollusks and arthropods with conserved histidine residues forming a coordination complex with copper ions binding oxygen for transport in the hemolymph. Hemocyanins have evolved from oxygen-binding tyrosinase enzymes, which participate in melanin biosynthesis and have documented roles in insect defense, including parasite encapsulation. This study evaluated the role that hemocyanin plays in the insect response to CLas infection. Specific dsRNA to hemocyanin in *D. citri* were designed and delivered via artificial diet feeding. Effects of hemocyanin suppression on CLas transmission were evaluated using detached leaf transmission assays. The levels of hemocyanin mRNA expression were highly correlated with the different *D. citri* color morphs and thus, we hypothesized that the blue abdominal color commonly observed in *D. citri* may be attributed to the oxygen-coordinating copper associated with hemocyanin in the insect. Insight into the function of *D. citri* hemocyanin may reveal novel strategies to specifically disrupt CLas transmission by the insect vector.

SmartTrap: Benefits of 3D printing for psyllid trap development and vector analysis methods

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The commonly employed technique for monitoring the Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayama) utilizes yellow cards covered with glue. These have several disadvantages: the sticky surface is messy and indiscriminate for other insects or debris trapped, it is difficult to remove intact specimens for complete identification, and insects are not well preserved for tissue analysis. An alternative design that can preferentially attract ACP while avoiding the sticky substance, while also preserving the specimens for complete analysis, is therefore desirable. This project enhances the mitigation of citrus greening through early detection of huanglongbing (HLB) through improved trapping technologies. Trapping of ACP, with "smart" traps that make it possible to accurately test for the presence and spread of the HLB pathogen, *Candidatus liberibacter* spp., facilitates the rapid site management response to enable delimitation and potential containment of citrus greening in regions where the disease is not widespread. The first attempt at psyllid trap design was a simple, color-attractive, yellow cylinder that eliminated the sticky glue, but the catch yield was consistently lower than that of the sticky trap, even when tested inside a psyllid rearing facility where the target population was high. Since making manual tweaks to our design quickly became both labor and time intensive, we decided to make the various physical modifications to our prototype by utilizing computer modeling software coupled to a 3D printer. This approach allows us to virtually model the design before testing, and then we can print viable traps within a single day, without the time needed to purchase and assemble the components derived from various sources; this removes a major impediment to rapid innovation. The modeling software can be used to make iterative tweaks to successive designs or to immediately create entirely new models, and the designs are limited only by the capabilities of the 3D printer. The printers deposit heated plastic into intricate designs, a method that is not possible using injection molding techniques, so the shapes of our traps can include projections and voids in strategic places to exploit the natural behaviors of citrus psyllids. Trap designs can easily be developed within a single day, allowing modifications and adjustments to be made in real time as new field data is acquired. Our improved "SmartTrap" designs can favor the capture of certain insects only, such that >50% of the insects collected would be psyllids. The trap base also contains a preservative liquid that allows those captured psyllids to be analyzed by standard molecular techniques. This enables researchers to better study the vector of citrus greening, therefore leading to improvements in control and prevention methods that may be more effective than the expensive, broad-spectrum pesticide sprays. The yellow trap color may be an adequate psyllid attractant for sticky traps, but our group is experimenting with slight color variations of different types of plastics and LED lights, incorporating the UV wavelengths that have been identified as psyllid-specific attractants. The concept of our latest design is to attract ACP to colored plastics and LEDs, where they will land on the primary cylindrical body of the trap. As they naturally crawl upward on rubberized plastic projections, designed to resemble the tender new growth of citrus stems, they will pass under the shade of an opaque overhang, where they will then crawl toward a view of sunlight that appears to be coming through the holes in the trap body, protected from the elements by a clear umbrella dome. Once inside the trap they will remain inside, preferring not to reenter the holes which appear dark from the interior perspective. They then fall down a slippery, fluon-lined funnel and into a preservative-filled test tube, which can be removed for inspection without dismantling the trap itself. Other researchers have identified potential chemical and auditory attractants for psyllids, and these features can easily be incorporated into a single trap design, made possible by 3D print technology. A number of our trap design features are currently being tested in the field. This presentation will show our latest designs and provide commentary on our observations. (1) The original smooth-cylinder trap model did not catch many psyllids, <10%, even in high density population exposure; (2) Psyllid trap efficacy also requires correct field positioning (compass direction, border and shade, controlled wind, etc.); (3) One design accidentally had a rougher texture on the outside, and it caught more psyllids than similar traps that were smooth; (4) Newer traps were then developed with intentional projections on the outside; (5) Our best trap design so far has yielded a catch rate of 30-40% ACP, compared to psyllid-capture on yellow sticky traps, as determined by a limited number of controlled field trials; (6) 3D-print designs can be modified rapidly from results of field testing, which is currently underway in FL, TX, and CA; (7) 3D-printed traps can also incorporate species cues for light, sound, and odor attractants to overcome field positioning effects.

Purification of the endosymbiont toxin Diaphorin from *Diaphorina citri* for structural and functional characterization

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The Asian citrus psyllid (ACP) is known to obtain nutritional and defensive benefits from its complement of endosymbiotic bacteria. The most abundant of its associated endosymbionts, '*Candidatus* Profftella armatura,' has been shown to devote approximately 15% of its predicted genome to polyketide biosynthesis. The interaction between the ACP and Profftella is an ideal target to combat the psyllid since this symbiont is found in all populations of the ACP surveyed thus far and in no other insect. A dual proteomic-metabolic analysis indicated that the complement of polyketides produced by Profftella plays a significant role in the insect host response to infection with '*Candidatus* Liberibacter asiaticus' (CLas), the bacterial pathogen responsible for Huanglongbing (HLB). The primary mechanism of this defense is via production of the cytotoxic polyketide diaphorin, estimated to be produced at 1.3 ± 0.3 mg per 1,000 adult insects. ACP populations reared on CLas-infected citrus trees show significant upregulation of Profftella proteins involved in polyketide biosynthesis as well as quantitative differences in abundance of diaphorin and a related polyketide compared to ACP reared on healthy trees. In this work, we aim to verify previous structural characterizations of diaphorin and probe its potential interactions with receptors in the ACP to gain a better understanding of the interplay between Profftella, diaphorin, and CLas within the insect. We extracted approximately 6,000 adult ACP and verified the presence of diaphorin in the extract using infusion mass spectrometry. Diaphorin is further enriched from the extract using preparative High Performance Liquid Chromatography (HPLC). Nuclear

magnetic resonance is used to evaluate the structure of diaphorin isolated within the fractions, and LC-high resolution mass spectrometry is used to confirm stability of the compound. Future plans include immobilization of purified diaphorin on a solid support resin for use in an affinity purification strategy to identify interacting insect proteins. This will allow a better understanding of the specific role and localization of diaphorin within the insect, as well as provide insight into the systemic response to CLas acquisition with an aim toward potential control strategies.

Evaluation of the control effect of antimicrobials against citrus huanglongbing via trunk injection

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Citrus Huanglongbing (HLB) or greening is a devastating bacterial disease that has destroyed millions of trees and is associated with phloem-residing '*Candidatus* Liberibacter asiaticus' (Las) in Florida. In this study, we evaluated the control effect of different antimicrobials including oxytetracycline, streptomycin, and plant defense inducers via trunk injection. Spatiotemporal dynamics of oxytetracycline in planta was evaluated. Las-infected 'Hamlin' sweet orange trees on 'Swingle' citrumelo rootstock at the early stage of decline were treated with oxytetracycline hydrochloride (OTC) using trunk injection with varying number of injection ports. Spatiotemporal distribution of OTC and dynamics of Las populations were monitored by HPLC method and qPCR assay, respectively. Uniform distribution of OTC throughout tree canopies and root systems was achieved 2 days post injection. High levels of OTC (>850 µg/kg) were maintained in leaf and root for at least 1 month and moderate OTC (>500 µg/kg) persisted for more than 9 months. Reduction of Las populations in root systems and leaves of OTC-treated trees were over 95% and 99% (i.e. 1.76 and 2.19 log reduction) between 2 and 28 DPI. Conditions of trees receiving antimicrobial treatment were improved, fruit yield was increased, and juice acidity was lowered than water-injected control even though their differences were not statistically significant during the test period. Our study demonstrated that trunk injection of OTC could be used as an effective measure for integrated management of citrus HLB.

Resources in the Citrus Genome Database that enable basic, translational, and applied research

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The Citrus Genome Database (CGD, www.citrusgenomedb.org) is being developed as a one-stop resource for citrus genetics, genomics, and breeding research. In this presentation we highlight CGD features that provide the citrus community with data and tools to combat Huanglongbing (HLB). The database has been redesigned and features a streamlined user interface that allows for quick access to data and tools and is also mobile friendly. It contains curated citrus genetic marker, map, and QTL data, genome data for clementine and sweet orange, as well as annotated reference transcriptomes (RefTrans) generated by analysis of published RNA-Seq and EST datasets. The database also has the most up-to-date version of CitrusCyc v3.0 which includes metabolic maps for the *C. clementina* and *C. sinensis* genomes. Tools such as BLAST for searches against the genome sequences, GBrowse/JBrowse for viewing genomes, and CMap/TripalMap for viewing and comparing genetic map data are also available on CGD. In addition to the tools to view and search published data in CGD, the Breeding Information Management System (BIMS) allows breeders to upload their data via the web, or directly from the Field Book app, to a private account. The private data than can be analyzed in conjunction with the public data. In addition to citrus data, the genomes of the different 'Candidatus Liberibacter' species and psyllid vector are available in CGD in JBrowse and the sequence data is searchable with the BLAST tool. As a community resource, CGD is being developed based on user feedback with the goal of providing the data and tools that will enable citrus crop improvement. CGD is supported by USDA-NRSP10, NSF-PGRP, and USDA-SCRI.

FANA and Morpholino treatments for targeting pathogens in citrus trees and psyllids

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First report of delivery of two synthesized oligo nucleotide products into plants. Subsequent feeding on treated plants by insects (Hemiptera and Coleoptera) resulted in ingestion and systemic movement of the two gene-based targeting products: FANA_ASO, (2'-deoxy-2'-fluoro-D-arabinonucleic acid)_(antisense oligonucleotides); and a PPMO (cell-penetrating peptide–morpholino oligonucleotide). Topical sprays onto citrus leaves, root absorption, and tree trunk injections, resulted in cell delivery of the molecules which were designed to: Asian citrus psyllid; Plant pathogenic bacteria of citrus, *Candidatus* Liberibacter asiaticus (CLas), Psyllid endosymbionts, and Diaprepes abbreviatus, weevil;. Treatments reduced CLas bacteria within infected citrus trees, reduced *Wolbachia* in cell cultures, the insects, while resulting in increased insect mortality. This is the first evidence for successful delivery of FANA_ASOs and PPMO into plants, and use of these molecules for plant delivered strategies to reduced plant pathogens in citrus, and to manage insect pests. Molecules were shown to move systemically through plant tissues as visualized with Confocal microscopy and spectrophotometry. Bacteria titers were reduced in treated plants on average by 50% (live/dead PMA bioassay) at three weeks post treatment. Adult insects showed systemic movement through hemolymph and organs: supra and sub esophageal ganglions, fatbodies, nerves, and alimentary tract. Results suggest a role for these products in the reduction of plant pathogens like CLas, associated with Citrus greening disease; and reduction of insect vectors of pathogens on citrus and other agricultural crops.

Absence of a functional glyoxalase system in *Candidatus* Liberibacter asiaticus confirms that the bacterium scavenges host cells for its energy requirements

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Methylglyoxal (MG) is a cytotoxic metabolite produced non-enzymatically from the glycolysis intermediates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. MG is a potent electrophile that readily glycates arginine residues resulting in carbonyl stress. Glyoxalase I and II (GloI and GloII) sequentially convert MG into D-lactic acid using glutathione (GSH) as a cofactor. Conserved across all domains of life, the glyoxalase system is essential for the mitigation of MG-induced carbonyl stress, preventing subsequent cell death, and recycling GSH for maintenance of cellular redox poise. *In silico* analysis surprisingly revealed that the uncultured, intracellular and lethal citrus pathogen *Ca.* Liberibacter asiaticus (Las) lacks GloI, the first enzyme of the MG detoxification system. Both GloI and GloII are, however, found in all cultured bacteria, including *Liberibacter crescens* strain BT-1 (Lcr). Comparative gene expression analyses in Lcr revealed significantly higher levels of expression of both *gloI* (B488_RS02175) and *gloII* (B488_RS05485) as compared to the housekeeping gene *prfA* during *in vitro* culture. In addition, the glycolysis pathway genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (B488_RS05930) and triosephosphate isomerase (TPI) (B488_RS02240) were also strongly expressed. In Las, however, expression of *gloII* (*gloI* is missing) and both glycolysis pathway genes GAPDH and TPI (CLIBASIA_00410 and CLIBASIA_02705, respectively) was negligible in both citrus as well as the insect hosts. Notably, Las has a functional ATP/ADP translocase gene (*nttA*, CLIBASIA_01040), an unusual feature of intracellular prokaryotes and plastids. Similar ATP/ADP translocase genes have been annotated in the genomes of all uncultured Liberibacters (that also lack MG detoxification pathway), except for Lcr. The loss of the MG detoxification pathway could be tolerated if Las

circumvents MG generation during glycolysis and directly imports ATP from the host cell. Additionally, Lcr cells in culture can import exogenous sucrose and a sugar transporter protein has been annotated in Lcr genome (B488_RS00965). Similar, sucrose transporter orthologs are absent in uncultured Liberibacters. Marker interruption of *gloI* in Lcr may be lethal, indicating that MG detoxification is a prerequisite for axenic growth *in vitro*. Experiments are underway to obtain *gloI* deletion mutants in Lcr in presence of a rescue clone carrying the complementing *gloI* gene from *Xanthomonas albilineans.*

***Wolbachia* protein suppresses holin promoter activity in *Ca.* Liberibacter asiaticus**

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Two circular prophage genomes (SC1 and SC2) have previously been described integrated in *Ca*. Liberibacter asiaticus (Las) strain UF506 genome, and all Florida Las strains have both prophages. Either one or rarely, both, prophages were reported absent in certain recently sequenced Chinese and Japanese Las strains. The SC1 lytic cycle, marked by upregulation of several late genes including a functionally lethal holin (SC1_gp110), is activated when Las is *in planta*, but not when infecting the Asian citrus psyllid (*Diaphorina citri* Kuwayama) host. The holin promoter was cloned into the wide host range vector pUFR071, structurally replacing the native *lacZ* promoter to drive the beta-glucuronidase (GUS) reporter gene. The holin promoter exhibited very strong and constitutive expression of the GUS reporter gene in *Liberibacter crescens* (Lcr), a culturable proxy for Las. We previously reported the presence of an unidentified, heat labile, protease-sensitive repressor in aqueous psyllid extracts that quenches the expression of the holin::GUS reporter. Tandem LCMSMS analyses were used to identify ca. 25 peptide fingerprints in the DNA-binding protein eluate captured by the holin promoter DNA immobilized on magnetic DynaBeads. A putative repressor protein was identified that was unique to the *Wolbachia* bacterial endosymbiont found in psyllids and absent in *Wolbachia* found in the fruit fly (*Drosphila*). This putative *Wolbachia* repressor gene was cloned in an expression vector and used for *in vitro* cell-free protein synthesis. The *in vitro* translated *Wolbachia* protein partially repressed the holin::GUS reporter in a dose-independent manner, as compared to the aqueous extract from the psyllid, thus indicating that complete suppression of holin promoter requires an additional partner. Heat-inactivated psyllid extract was unable to enhance the *Wolbachia* repressor- induced suppression of holin promoter activity, confirming the heat labile proteinaceous nature of the additional psyllid-sourced partner.

Chromosomally-encoded peroxiredoxins (CLIBASIA_00980 and CLIBASIA_00485) of *Ca.* Liberibacter asiaticus may be necessary for survival and colonization of citrus.

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A conspicuous feature of plant defense reactions in response to an attempted pathogen invasion is the engagement of an oxidative (primarily H_2O_2) burst. We have previously shown that an SC2 prophage-encoded peroxidase (SC2_gp095) in *Ca*. Liberibacter asiaticus (Las) strain UF506 represents an important lysogenic conversion gene whose expression may increase bacterial fitness and delay symptom development in the host plant. However, absence of either one or both prophages in certain recently sequenced Chinese and Japanese Las strains, prompted a review of H_2O_2-detoxification mechanisms conserved amongst Liberibacters. Two putative peroxiredoxin genes (PR2, CLIBASIA_00980 and BCP-like, CLIBASIA_00485) have been annotated on the Las chromosome, which are conserved and identical among all the sequenced Las strains. The BCP-like peroxiredoxin is predicted to be nonclassically secreted. The expression levels of Las PR2 in both citrus and psyllid were equivalent. Notably, however, the expression of the predicted secreted BCP-like peroxiredoxin was similar to that of PR2 in citrus but nearly undetectable in psyllids, reminiscent of the low level of SC2_gp095 peroxidase expression in psyllids. Despite carrying a native ortholog, Lcr transformed with the BCP-like peroxiredoxin showed a significant (200 fold) increase in tolerance to 100 µM H_2O_2, as compared to a marginal 4-5 fold tolerance provided by either the PR2 peroxiredoxin or SC2 peroxidase. Most importantly, BCP-like peroxiredoxin augmented Lcr tolerance to 50 µM *tert*-butyl hydroperoxide (*t*BOOH; an organic oxidizing agent) by nearly 1000 fold. We hypothesize that both the Las peroxiredoxins may be implicated in alleviating the oxidative burden originating from physiological electron transport reactions. However, the extracellular BCP-like peroxiredoxin may be essential for Las survival in an oxidative host cell environment through a) direct detoxification of host-generated H_2O_2, b) limiting the catastrophic chain reaction spread of a lipid peroxidation event initiated by H_2O_2 permeating the bacterial cell membrane, and c) most importantly, akin to the secreted SC2_gp095 peroxidase, dampening the systemic propagation of H_2O_2-mediated defense signaling in the host plant following an initial colonization event. Las BCP-like peroxiredoxin may represent a critical secreted effector conserved across all pathogenic Liberibacter species (even in the phageless Las strains) that functions to suppress host symptoms, a tactic used by most biotrophic plant pathogens.

Development of an improved internal control gene assay for real time PCR and digital droplet PCR detection of *Candidatus* Liberibacter asiaticus

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Detection of HLB-associated *Candidatus* Liberibacter asiaticus (*C*Las) by real time PCR (qPCR) has been widely used in citrus industry since its development a decade ago. However, the methodology is not adaptable to quantitation by digital droplet PCR (ddPCR) since it uses a multicopy mitochondrial gene (cytochrome oxidase; COX) as an internal control. Since there are hundreds of copies of COX gene per cell, inefficient amplification of the low copy target gene (16S RNA of *C*Las) can occur. In this study, we sequenced a single copy nuclear gene, malate dehydrogenase (MDH) from over 100 accessions including several citrus and citrus relatives in the subfamily Aurantioideae. The plant samples selected are known to be hosts of both HLB and the psyllid vector. Primers and probe were developed using conserved regions of MDH gene for Taqman® based qPCR and also for ddPCR analyses. We conducted qPCR assays using various dilutions of HLB infected plant samples to detect *C*Las in duplex reactions using two different internal controls - COX and MDH in separate reactions. ddPCR assays were conducted using a BioRad QX200 droplet digital PCR System. The advantages of using MDH as an internal control gene in both qPCR and ddPCR will be discussed. Since confirmatory tests to detect the presence *C*Las cannot be done with plant samples having low bacterial titers, additional confirmatory assays by ddPCR of other regions of *C*Las genome were also developed. Detection of *C*Las in samples with low titers is critical for both prevention and management of HLB, especially during early stages of the epidemic, and to slow down the spread of disease.

Development of tools to conduct field testing of HLB-associated Liberibacters for disease mitigation

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Testing of psyllid vectors for the presence of huanglongbing-associated Liberibacters is known to be an effective method for early detection of HLB in citrus groves. Presence of the pathogen in an area can be monitored many months or years ahead of disease detection in plant hosts. We are developing tools to: (a) Capture psyllids in the field in a preserved state which can then be tested for HLB pathogen weeks after trapping; Smart traps developed using 3D printers can be deployed in the field for year-round capture of testable psyllids; (b) Conduct on-site testing of psyllids for the presence of pathogens using a field-deployable

device based on loop-mediated amplification technology; (c) Develop assay methods capable of detecting all naturally occurring Liberibacter variants; conduct extension work to popularize and implement the novel methods developed in this USDA-NIFA funded project. The goal of the project is to familiarize citrus growers with tools that can be utilized for early detection to enable timely action aimed towards disease prevention and/or containment. Early detection of citrus HLB will lead to timely management practices resulting in disease mitigation.

Chemistry between *Candidatus* Liberibacter asiaticus and *Diaphorina citri* that disrupts citrus industries

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Citrus greening disease or Huanglongbing, putatively caused by the bacterium, *Candidatus* Liberibacter asiaticus (*C*Las) and transmitted by the Asian citrus psyllid *Diaphorina citri*, is the most serious threat to the U.S. citrus industry, which has citrus growing regions in Florida, California, Texas, and Arizona. In vector borne diseases, uncovering the tritrophic interactions between the pathogen, the vector and the host leads to understanding the mechanism of disease and identifying the weak link that we can be targeted to control the pathogen or to block the transmission by the vector. Metabolomics approaches are powerful tools to understanding the tritrophic interactions. We used GC-MS and LC-MS to study the chemical compositions of media surrounding the bacterial pathogen including vector haemolymph and citrus phloem sap. We also studied the effect of the infection with the pathogen on the metabolites of both host and vector. Information from this investigation will greatly help in understanding the pathogenicity mechanisms and will lead to designing an appropriate artificial medium to culture the bacteria which still unavailable in culture.

Establishing citrus rootstock traits using metabolomics to accelerate development of HLB tolerant rootstocks

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The rootstock is a major component of citrus production in Florida, and is of special importance in areas affected by diseases such as Phytophthora root rot, citrus tristeza virus, and HLB. This study is part of a larger project that seeks to discriminate rootstock cultivars with different tolerance to HLB using metabolomics. The ability to characterize rootstock sensitivity to HLB at an early seedling stage would greatly accelerate the development of new and HLB tolerant rootstocks without having to rely on long-term field trials. For this study, four rootstock cultivars: Cleopatra mandarin *(Citrus reticulata)*, Sour orange (*Citrus aurantium*), Ridge Pineapple sweet orange *(Citrus sinensis)* and Swingle citrumelo (*Citrus paradisi Macf. x P. trifoliata*), representing four particular different types of citrus germplasm, were grown as seedlings and were analyzed for their metabolite composition in leaves and roots via gas chromatography-time of flight mass spectrometry (GC-TOF-MS). Principal component analysis (PCA), hierarchical cluster analysis (HCA), and partial least-squares-discriminant analysis (PLS-DA) of the processed GC-TOF-MS data revealed clear differences between the tissue types and cultivars. Heat map and enrichment analysis reported that each cultivar has a unique profile consisting of specific and more abundant metabolites that may be associated with cultivar traits. The correlation between these more abundant compounds found for each cultivar and the cultivar-specific traits will be discussed. The information gained from this study will be used to initiate a database centered on understanding the different characteristics of rootstock cultivars. More studies are currently being conducted in our laboratory comprising other rootstocks cultivars grown as seedlings and as grafted plants, and will generate valuable information for citrus breeding programs, especially as they relate to HLB.

Prediction and computational analysis of Liberibacter virulence factors

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Computational analysis of the first sequences proteome from Candidatus Liberibacter asiaticus (Las) str. psy62, a vector borne bacterial pathogen of citrus, revealed potential secreted virulence factors as well as integrated prophage proteins that could contribute to disease. However with the sequencing of this single genome, the number of hypothetical proteins without assigned function remained relatively high (~30%). Since this study an additional seventeen Liberibacter genome assemblies from five species are publicly available, including five solanacearum strains that infect potato and two culturable *Liberibacter crescens* strains (Bt-1 and Bt-0). This increased availability of *Liberibacter* genus sequence data not only allows study of bacterial pathogen evolution in response to plant immunity, but also extends informative sequence that is useful for structure/function prediction of previously unknown gene products. Heatmaps depicting protein similarity distributions between the reference strain psy62 and new strains can reveal functional networks of similarly distributed proteins, potentially identifying virulence factors contributing to vector and/or plant specificity. Clustered heatmaps reveal many Las proteins as displaying a universal conservation that follows species distribution. These proteins tend to contribute to key cellular processes such translation, energy production, and transcription. In contrast, Las prophage regions, which include potential virulence determinants and an experimentally characterized effector (CLIBASIA_05590), display elevated variability. This prophage effector sequence divergence allows identification of a distantly related family (PHA00666, annotated as a putative protease) that is found in a number of Enterobacteria and Bordatella phage as well as a phage in the causative agent of citrus canker. Similarly variable Las genome regions encode previously unknown components of phage tail machinery and a phage outer membrane protein responsible for invasion, as well candidate virulence proteins with similar distributions as the prophage: a putative transmembrane protein (CLIBASIA_00915) similar to multidrug efflux proteins, a signal peptide protein (CLIBASIA_02180) similar to a TonB periplasmic component that helps to secrete bacterial toxins, and a previously unknown secreted protein (CLIBASIA_00070) with an identified Tad pilus assembly N-terminal domain and Von Willebrand factor type A (vWA) C-terminal domain. Finally, several conserved Las proteins have been lost in the potato infecting strains, suggesting that they might function in vector/plant specificity: quinone oxidoreductase (CLIBASIA_00875), formamidopyrimidine-DNA glycosylase (CLIBASIA_02560), putative flagellar motor switch/type III secretory protein (CLIBASIA_02875), F0F1 ATP synthase subunit B' (CLIBASIA_04940), and four hypothetical proteins of unknown function (CLIBASIA_00140, CLIBASIA_05030, CLIBASIA_04245, and CLIBASIA_05180).

Comparative proteomics and microscopy provide insights into transmission of *Candidatus* Liberibacter asiacticus by the Asian citrus psyllid

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Huanglongbing is associated with plant infection by the gram negative bacterium *Candidatus* Liberibacter asiaticus (CLas). CLas is transmitted in a circulative manner by the insect vector, *Diaphorina citri*, also known as the Asian citrus psyllid. Prior to transmission to a new host tree, CLas must cross insect tissues, including the gut barrier. We hypothesize that CLas-psyllid interactions at the gut are critical for bacterial invasion into the insect and by extension, the ability of the psyllid to transmit CLas. Previous work published by our lab showed that the psyllid midgut responds to CLas by inducing nuclear lysis and programmed cell death. To reveal the interplay between the insect and CLas at the molecular level, we used a parallel ‘omics approach: quantitative mass spectrometry analysis and RNA sequencing technology to compare the proteomes and transcriptomes of CLas-exposed and non-exposed psyllids, respectively. We determined that

exposure to CLas generates widespread changes in the psyllid gut at the molecular level, including drastic changes in actin cytoskeleton proteins, immune system proteins and proteins involved in iron transport and iron scavenging. The transcriptomics approach enabled us to discover novel long non-coding RNAs that are psyllid specific and induced by CLas. The proteomics approach revealed that the bacterial symbiont Wolbachia plays a complex and as of yet uncharacterized role in psyllid gut physiology and CLas interactions. Fluorescent and electron microscopy shows that Wolbachia co-localizes in the same gut cells as CLas, yet they do not overlap in their subcellular distribution, which suggests a cooperative relationship. Imaging studies also showed that CLas, but not Wolbachia, localizes in the psyllid midgut cell nucleus. Taken together, our results support a model whereby CLas mediates invasion across the gut barrier via directly interfering with nuclear processes and the actin cytoskeleton resulting in rapid midgut cell lysis in a cooperative manner with Wolbachia. Our talk will also highlight how we are using these molecular insights as the basis for the development of interdiction RNA and peptide molecules with affinity to specific gut proteins.

Introducing new and licensed huanglongbing tolerant citrus varieties into California. A case study – Florida.

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In the light of the current Huanglongbing (HLB) threat to the California (CA) citrus industry, and preliminary data indicating that some citrus varieties in Florida (FL) may possess some degree of tolerance to HLB, the CA citrus growers indicated a strong interest in proactively introducing new, licensed or public domain, citrus germplasm from FL in to CA. However, to this day there is no well-defined protocol for the introduction and commercialization of licensed citrus varieties in to CA. Projects supported by the industry (Citrus Research Board-CRB) and by the USDA HLB Multi-Agency Coordination (HLB-MAC) aim to introduce over 50 citrus scions and rootstocks from two FL breeding programs; University of Florida's Citrus Research and Education Center, Lake Alfred and USDA-ARS, Fort Pierce. The streamlined procedure includes over 130 pages of federal and state permits, HLB/Asian citrus psyllid movement mitigation protocols, and non-propagation and material transfer agreements. The introductory pathway includes the Bureau of Citrus Budwood Registration, FL and the Citrus Clonal Protection Program (CCPP) while as citrus material is released from quarantine and becomes available to the citrus centers of the National Clean Plant Network (NCPN), entities such as the Florida Foundation Seed Producers (FFSP) and the New Varieties Development and Management Corporation (NVDMC) will be invited to coordinate the use of citrus varieties. This project has already transformed the landscape of citrus germplasm exchange. The CCPP has received inquiries about this "new model" of citrus budwood movement and exchange from major citrus producing countries, such as Spain, due to the global threat of HLB and the need to have simple yet secure access to public and protected novel rootstock and scion citrus varieties. For real time updates on the introduced varieties visit www.ccpp.ucr.edu.

Exploring the mechanism of transmissible small nuclear RNA (TsnRNA) induced citrus dwarfing for huanglongbing management

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The term 'transmissible small nuclear ribonucleic acids' (TsnRNAs) describes well-characterized viroid RNA species that do not induce disease in specific citrus hosts, but act as regulatory genetic elements modifying tree performance. TsnRNA-IIIb (syn. citrus dwarfing viroid) reduced the canopy volume of Parent navel orange (PNO) trees on trifoliate rootstock (45% and 53.5% in standard [6m×6.7m] or high-density [3m×6.7m] plantings, respectively), increased yield per unit canopy volume, and concentrated the fruit in the best canopy area for efficient harvest, without affecting fruit quality. These findings raised questions regarding the mechanism of this plant-TsnRNA interaction and the potential value of such technology in Huanglongbing (HLB) management. To understand the plant mechanisms modulated by TsnRNAs resulting in citrus tree dwarfing, we initiated a physiological study in a 1997-planting of PNO trees on trifoliate rootstock with and without TsnRNA-IIIb. Our measurements showed that the TsnRNA-IIIb-induced decrease in PNO canopy volume resulted from reduced apical shoot development, suggesting possible phytohormone involvement. The results of a subsequent phytohormone profiling study will be discussed in relation to observed TsnRNA-IIIb-induced dwarfing and normal growth. The importance of elucidating the TsnRNA dwarfing mechanism lies in the potential to develop commercial applications that do not require a transmissible agent. Dwarfed trees are fundamental for high-density plantings that will be critical for meeting the challenges posed by water shortages, disease spread, farmland reduction, and labor costs, but primarily will advance HLB management by simplifying visual inspections, chemical control of the psyllid vector, and replanting at higher densities.

Mathematical Modeling of HLB Symptom Development

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In previous work we established experimentally that when CLas is transmitted locally at flush sites, the newly infected flush can become infectious within 15 days. Rather than a tree having to be symptomatic for transmission to occur, infected adult psyllids are capable of transmitting CLas to the next generation via nymphs feeding on infectious flush. We investigated the implications of this phenomenon through simulation and concluded that CLas could spread rapidly through a grove, essentially as quickly as the psyllid population migrated. In order to have a more complete model for HLB, we investigate how prevalence of CLas might lead to development of HLB symptoms. Recent research suggests that HLB symptoms begin in the root system. We propose a model for symptom development which focuses on the spread of CLas to the roots as the start of tree decline. In our model inoculum accumulates in new flush through the feeding of infected adult psyllids and psyllid nymphs. Upon maturation of a leaf, the accumulated inoculum has a probability of spreading to the roots and initiating tree decline. We consider the implications of this model for traditional spraying and potential RNAi treatment. We show that the model predicts a significant effect when RNAi is used in tandem with traditional sprays. However, this effect strongly depends on the psyllid invasion pattern. We will also discuss how the timing of an invasion relative to a flushing season influences the spread of HLB.

Resources for reaching out to growers and end-users on approaches to combating citrus greening disease

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Huanglongbing (HLB, or citrus greening) is a bacterial disease of citrus, which represents a crippling threat to the U.S. citrus industry. The responsible bacterium, *Ca*. Liberibacter asiaticus (CLas), is spread by the Asian citrus psyllid, *Diaphorina citri*. This invasive insect, first found in Florida in 1998, is now present in all U.S. citrus-producing states. In Florida, HLB infection affects most trees, reducing yields and producing off-tasting fruit and juice. California is second to Florida in total citrus acreage, but has a larger share of the higher value, fresh fruit market. CLas has now been identified in CA and thus it is imperative to inform growers and consumers about what research and approaches are being pursued to control the spread of the disease. Through both the

NIFA-sponsored NuPsyllid and RNAi-based strategy projects, we are developing extension and education programs for growers and end-users, which address the use of genetic tools to lessen the impact of HLB on citrus. We developed an extension outreach plan that enhances existing extension educator programming in citrus. We have three primary outreach approaches. (*i*) Development of Powerpoint Presentations, which can be tailored to specific venues and locations. Presentations will provide a general education on the genetic methods used to modify plants, viruses, and insects and cover state and national regulatory requirements and consumer attitudes. To bring the latest information on the various approaches to combat citrus greening, we will work directly with researchers involved in these efforts to obtain detailed information on precisely what and how they are being done and the current status of their efforts. (*ii*) Creation of Fact Sheets that cover general topics of classical and modern methods of genetic modification and how these are being used to modify various crop plants. Another will cover how genetic engineering and other mitigation approaches can be used to address the citrus problem. (*iii*) Citrus outreach website (http://ucanr.edu/sites/scienceforcitrushealth/), which is specifically designed to provide grower information on huanglongbing/citrus greening and genetic strategies for control and detection. It includes the following sections: About Us, Background, Research, Outreach, News and Links.

Comparative study of early detection techniques: TX2

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In December 2015 a TX double blind study was conducted to determine the accuracy of HLB early detection techniques in comparison to qPCR. A Wonderful citrus grove was used and trees randomly labeled by their staff. Samples were taken from 40 grove trees diagnosed positive, 30 grove trees diagnosed negative and 40 screen house trees diagnosed negative for HLB by qPCR. Three (Davis twister volatile organics) or four samples were taken from each tree- the initial sample taken from the diagnostic site and three equally spaced around the canopy. Leaf swabs were taken from 4 leaves to evaluate the microbiome (Leveau), needles were inserted into four other leaves to test for surface volatile organics (Applied Nanotechnologies), fifty petiole and midribs were harvested from each sampling site for freeze dried ground tissue for qPCR (CRB, Keremane, Kunta and McCollum), siRNAs (Jin) and metabolomics (Slupsky), and the stems taken for tissue blots (Ma). Samples were randomly relabeled by staff at the TAMU Kingsville Citrus Center before being shipped either to researchers or for freeze drying and grinding. Freeze ground samples were aliquoted for each lab by CRB lab staff and each aliquot individually relabeled by the Morse lab. Labs initially made disease determinations for each sample. All data was sent for tabulation to the Godfrey lab. Labs were then supplied with a key identifying which samples came from the same tree and determinations were made for each tree. Results were statistically analyzed (McRoberts) without any knowledge of the technique used for each data set. As expected, qPCR was accurate diagnosing HLB positive and negative samples based on tree evaluation, while accuracy of individual sample diagnosis was significantly reduced due to the uneven distribution of *C*Las. However, leaf microbiome profiling and metabolomics were superior to qPCR both for tree diagnoses and individual samples.

***Candidatus* Liberibacter asiaticus encodes a functional salicylic acid (SA) hydroxylase that degrades SA to suppress plant defenses**

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Salicylate (SA) is a plant hormone critical in plant defenses. SA hydroxylase is a flavoprotein monooxygenase able to enzymatically degrade SA in many bacteria. Here, we have demonstrated that *Candidatus* Liberibacter asiaticus (Las), the causal agent of the most devastating citrus HLB disease, evolves a clever strategy in suppressing plant defenses by degrading SA using a functional SA hydroxylase. This becomes possible since *Las* lives in the phloem and SA is transported in the phloem. Purified SA hydroxylase showed SA degrading activity. Overexpression of SA hydroxylase in planta affected plant defense responses. *Las* infection reduces citrus resistance against *Xanthomonas citri* (Xac), another severe bacterial pathogen. *Las* infected plants had reduced PR gene (PR1, PR2, and PR5) expression and SA accumulation in Duncan grapefruit and Valencia sweet orange in response to subsequent inoculation with $XacA^{w}$, which is nonpathogenic on both citrus varieties. *Las* also increased citrus susceptibility to subsequent infection by *X. citri*. To counteract this virulence mechanism of Las, foliar spraying of the SA analogs 2,6-dichloroisonicotinic acid (INA) and 2,1,3-benzothiadiazole (BTH) was conducted to control HLB. Both INA and BTH slowed down the increase of *Las* titers *in planta* and HLB disease severity compared to the negative control. This is first study that demonstrates pathogens suppress plant defenses by degrading SA.

Production of non-transgenic mutant plants via Agrobacterium-mediated transient CRISPR expression

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CRISPR/Cas may provide a powerful tool for development of HLB resistant citrus vsarieties. The most wide-used applications of CRISPR utilize stable integration of Cas9/sgRNA genes into host plant genomes and subsequently their expression in plant cells produces targeted mutations. For sexually-propagated annual crop plants, the Cas9/sgRNA transgenes can be eliminated from plant genomes following sexual reproduction. However, this strategy is not feasible for vegetatively propagated perennial plants, such as citrus, requiring years to reach sexual maturity or being heterozygous in genotype. To circumvent that problem, we have used Agrobacterium-mediated transient expression of Cas9/sgRNA genes to produce targeted mutations with no stable integration of transgenes into the host plant genome. We have also developed a highly efficient method based on DNA sequencing and high resolution DNA melt analyses to identify shoots with desirable mutations. The method we developed may be useful in developing non-transgenic HLB tolerant cultivars of citrus.

Development of genome-based anti-virulence therapeutics to control HLB

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Genomic sequence provides insight into genetic factors that are responsible for the disease. Due to un-cultural nature of HLB pathogen, conventional gene functional analysis cannot be applied. To circumvent this limitation, orthologous gene replacement technique has been developed to functionally characterize key virulence genes in "*Candidatus* Liberibacters asiaticus" (Clas). These results facilitate the development of antivirulence drugs that specifically target the functional domains of virulence genes/gene products to disarm pathogenicity or reduce virulence. This genome-based antivirulence treatment will provide a new approach to mitigate disease. The research progress towards the development of a novel therapeutic strategy for HLB management will be presented.

An update pattern for the successful plantation of Ponkan tangerine (*Citrus reticulata*) in HLB-infected region in China

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Huanglongbing (HLB), first reported in China in 1919, has been the most devastating citrus disease. Asian citrus psyllid (ACP, *Diaphorina citri*) was proved to be the vector of HLB in China in 1977. Today, ACP and HLB are widely distributed in 11 of the 18 citrus plantation provinces in China. Ponkan tangerine (*Citrus reticulata*) is one of the most HLB susceptible cultivars and over 80% of Ponkan orchards in Chinese Yongchun County have been ruined since HLB broke out in 2000. To develop a new system for the citrus sustainable plantation in HLB-infected areas becomes very important for citrus industry in China, as well as in the world. Here, a promising plantation pattern was developed for Ponkan tangerine in Yongchun County, which was summarized as following. (1) The orchard should be segregated by mountains or forest belt (its width >1000 m, its tree height >7 m, and the tree is not the ACP host). On the other hand, it is better to plant ACP-disliked trees, such as China fir *(Cunninghamia lanceolata*) around each planting plot. (2) Before replanting or planting citrus disease-free seedlings, all HLB positive or negative trees and other ACP host species (such as orange jessamine) should be removed thoroughly. Prior to removal, all trees should be treated with a foliar insecticide (such as Danitol, fenpropathrin) to kill all adult psyllids feeding on that tree. (3) The insect pest controlling system, such as insecticide mechanical spraying system should be installed in the orchard to ensure suppressing the ACP in a short time. (4) It is compulsory to use disease-free seedlings. Moreover, we recommend using big container-grown seedlings (the diameter at the graft union > 2 cm and the seedling height >1 m) and the planting density of 1.5~2 m×3~4m to accelerate the orchard producing profit. (5) The main task in the second year after planting is thinning flower in the spring, promoting tree growth in the summer and the early autumn, and stretching erected branches in the late autumn. (6) To clean the orchard in winter, suppress summer shoot and intensively promote autumn shoot plus spraying insecticide are the three key steps to control ACP spread. (7) Establish dynamic removal mechanism for HLB infected trees. Scouting for HLB infected trees should be done routinely so that infected trees can be removed in time and replaced with healthy trees (they were provisionally kept in the pot in the insect-free net house located in the edge of the orchard). By adopting this pattern, the 4-year-old tree has the height of 2.0~3.0 m and the average yield of 30 kg per tree; the percentage of the HLB infected tree is below 3%. Moreover, over one thousand hectares of Ponkan tangerine groves with the present pattern were added in Yongchun County. This plantation pattern shows a promising prospect for sustainable citrus plantation in HLB-infected region.

Additional evidence that the environment may be impacting HLB spread in Brazil

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In attempts to identify factors responsible for the irregular spread of HLB in Brazil several experiments have been carried out. Recently we showed that HLB-affected trees in regions of warmer and drier climates contained lower titers of *Candidatus* Liberibacter asiaticus (Las) in new shoots and lower percentages of shoots carrying Las (PD in press). Also, the lower the titer in the shoots the lower was the acquisition rate by adult psyllids caged on the shoots for 48 hours for acquisition access period (AAP). The data obtained would probably be more representative if nymphs rather than adults had been used for Las acquisition. This because in many places HLB and psyllid controls are deficient or null, with the diseased trees providing adequate sites for insect reproduction, potentially increasing the chances of Las acquisition even from low-titer shoots. In this work Las acquisition by adults and 3rd-instar nymphs was compared. They were caged on shoots that developed on HLB-affected trees in the field (FD), or on young plants kept in greenhouse (GH, 10.7 to 37.4°C) or growth chambers (GC1, 18 to 30°C; GC2, 24 to 38°C). AAPs were 48 h for nymphs and adults on detached shoots from FD trees, and 48 h for adults and variable time for nymphs (until becoming adults) on intact shoots from GH and GC plants. Shoots, nymphs, and adults were individually analyzed by qPCR. Las titers varied from avg. log 4.6±0.44 per g tissue in FD, 4.1±0.68 in GH, 6.1±0.06 in GC1, and 1.7±0.49 in GC2 shoots. Percentage of Las-positive adults and nymphs were 68.3% and 61.1% from FD, 32.3 and 61.9% from GH, 95 and 100% from GC1, and 0 and 4.4% from GC2. The low acquisition rates by adults and nymphs from low-titer shoots of the hot environment (GC2) indicate that the abandoned groves present in the distinct regions of SPS may not be impacting HLB spread at the same level as currently accepted.

Evaluation of sticky traps to capture *Diaphorina citri* under reduced population insect densities in Mexico

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During the last two years, the Mexican monitoring program of *Diaphorina citri*, the vector of the pathogenic bacteria associated with huanglongbing, has shown a marked reduction in presence of the insect in most of the citrus growing areas of the country. As management of the vector in Mexico, exhibits marked differences in respect to the performed in Brazil, or Florida, U.S.A., where there is an intensive use of insecticides, there are concerns that probably the official monitoring program is underestimating pest densities or that the traps have been failing to capture the insect, yielding optimistic results about abundance of *D. citri*. In order to contribute to discard the possible use of inefficient traps, the present study was conducted to evaluate these tools that have been used in the monitoring, as well as to obtain a better trap to be used under possibly scarce populations of the vector in Mexico, as a resultant future scenario from an effective pest control program. In this study, sticky traps in different designs, colors and color patterns were evaluated to determine the capacity to capture adults of the vector. During 2015-2016, we carried out 13 experiments in Nuevo León state, and two regional simultaneous tests in Michoacán, Nayarit, Nuevo León, Sinaloa, Tamaulipas, and Veracruz states. Several traps captured a noticeable number of *D. citri* adults. Notably, when the glue was standardized for all the traps by removing the own and applying one commercial, generic glue, the capture of insects in the specific ACP American trap was lower or similar than most commercial traps evaluated; while, in some of them such change in glue produced higher *D. citri* capture, suggesting effects from quality and quantity of glue used in its manufacture.

Optimization of PCR for reliable detection of viable *Candidatus* Liberibacter asiaticus (CLas) in citrus and estimation of viable CLas in symptomatic grapefruit leaves of different developmental stages during summer and fall

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Citrus Huanglongbing, associated with *Candidatus* Liberibacter asiaticus (CLas), is the most serious disease in the USA causing substantial economic losses. Accurate quantification of live CLas cells in the plant tissue is essential to devise effective disease management strategies and to test the efficacy of new disease control methods. Propidium monoazide (PMA), a DNA intercalating compound has been widely used in conjunction with qPCR, viability qPCR (V-qPCR), to estimate live bacteria. However, the V-qPCR assays used were not standardized and have the limitation that they cannot differentiate live and dead bacterial cells. We have evaluated several parameters associated with V-qPCR and have developed an improved protocol to detect live CLas from tissue samples. It is unclear if live CLas populations vary with age of the leaf and varying degrees of HLB disease symptom development. To address this question, we have quantified the live CLas populations in grapefruit leaves at different maturity stages and leaves with varying levels of symptoms collected from naturally infected field trees. V-qPCR results showed that lowest Las titers were found in immature leaves during the summer followed by a significant increase during the fall. During the summer, mature leaves harbored more live CLas than immature leaves. Leaves displaying corky veins harbored highest total Las titers compared to other blotchy mottling and nutrient deficiency like symptoms. However, live CLas populations were significantly lower.

CHMA design: Performance review, concerns, and a risk-based optimization for Treasure Coast areas in Florida

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Compared with the 48 officially recognized CHMAs in FL, the Treasure Coast area exhibited very poor control of ACP in 2014 where multiple ACP 'hot spots' (i.e. extremely high ACP counts) were recorded. The number of abandoned groves and uncoordinated spray patterns can lead to increasing ACP pressure. Without CHMA-wide control, HLB will spread quickly and the implications for new tree plantings may not be viable for production. A series discussion among Indian River Citrus League (IRCL) growers was held to assist in the development of CHMAs for Treasure Coast Citrus that integrate ACP control into new planting plans. Based on our risk models, many risk factors are considered in the Treasure Coast CHMA optimization including: ACP population dynamics, residential risk (e.g. proportion of human population in the landscape), abandoned groves, distance to transportation corridors, citrus nurseries, farmers markets, packing houses and green waste facilities. Other logistical concerns are also considered, such as commercial planting density, maximum/optimum number of growers per management area and elevation difference for movement of equipment. Following thousands of simulations and optimization, seven CHMAs are proposed for the Treasure Coast area. They are defined as Indian River North, Indian River South, 95 East, Okeechobee, St. Lucie West, East and South. The generated CHMA maps have been shared with growers via UF/IFAS extension (http://www.crec.ifas.ufl.edu/chma/) to monitor ACP control performance locally and statewide.

Calculating historical citrus reduction rate/pattern using aerial photographic and GIS techniques

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Ever since first detection in late 2005, HLB is now widely spread throughout most commercial citrus groves in Florida and caused a rapid decline of trees, as the disease continues to tighten its grip on Florida's citrus industry. However, there are limited studies that quantify the impact of HLB on citrus reduction rate over large spatial scales. Aerial photographs are most commonly used to measure historical changes since the advancement and availability of high-resolution, large-scale vertical aerial photography. This study presents a semi-automatic statistical classification method for annual citrus tree decline rate calculation and pattern recognition/analysis. High-resolution photography of Florida (~1 foot) from 2006 to 2015 can be obtained from LABINS (Land Boundary Information System), where citrus field imagery is converted to digital format and filtered out using user-defined citrus polygons in GIS. Subjected to selection bias, a number of commercial citrus fields are used to fully represent the spatial variability of the HLB situation. In order to estimate active citrus coverage accurately, we specified multiple training areas as the color-spectral properties of citrus canopy photography are apt to change for different times and locations. Also, efforts have been made to remove tree shadows by identifying neighboring points with similar color intensity. Transition from healthy to serious infected citrus grove results in potential significant changes in tree canopy size (e.g. most likely due to canopy decline/shrinkage and diseased tree removal). We compared annual tree coverage reduction rates across different landscapes, identified major patterns of tree loss within grove (i.e. edge effect), and quantified the relationship with HLB progress in Florida. Incorporating both spatio-temporal analysis and aerial photography has the benefit in determining the economic impact of HLB on actual citrus landscape. This proposed technique could be used as an enhancement tool in future HLB survey design and modeling.

Florida CHMA performance and implications on citrus production and sampling design

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Spatially interpolated Asian citrus psyllid (ACP) density population estimates were calculated from the Florida multi-pest survey (MPS). ACP density is summarized at 2km grid resolution across all commercial citrus plantations in Florida to provide an overview of ACP population change, i.e., dynamics. By investigating ACP changes through all cycles from 2011 to 2016, a new rating system has been designed for benchmarking and analysis of performance characteristics of each individual Citrus Health Management Area (CHMA). ACP density is classified into five different rating categories for each CHMA and cycle. A total rating value for each CHMA is calculated to rank its comparative efficacy of ACP control. Also, various relevant factors, such as grower participant rate, urban population size, abandoned grove acreage, ratio between commercial citrus and residential area, are analyzed to quantify their influence on CHMA performance. The relationship between citrus production (data collected from Florida Citrus Statistics) and CHMA performance is investigated, and this will help us understand the benefit of CHMA from an economic perspective. Accurate estimation of ACP population is also critical for future cost-effective pest management at the CHMA level. Related with CHMA performance scores, there are a number of CHMAs with high ACP uncertainty or 'vague' values. The amount of uncertainty is mainly influenced by the local variation of ACP observation in the field and the spatial pattern of ACP distribution within the CHMA. Bootstrap-based analysis has been implemented to address the suitable sample size for each CHMA, justifying realistically that the sample variance of the ACP population is not constant over time. The method is particularly useful for the MPS survey design as the project budget constrains the total number of citrus blocks that can be surveyed during a yearly time frame.

Agent-based model to predict/monitor the efficacy and cost of various ACP control strategies

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Citrus Huanglongbing (HLB), spread by the Asian citrus psyllid (ACP) vector, is a devastating disease threatening nearly every citrus producing area worldwide. Given the recent findings of ACP in California's Central Valley, an increase in ACP prevalence is inevitable which heightens the risk of HLB introduction into commercial citrus. This poses a major threat to the viability of the citrus industry emphasizing the urgent need for proactive regulatory intervention and disease control. Understanding the spread of ACP across different landscapes (i.e. residential, commercial, and mixed) is crucial in designing optimal survey methodologies and control strategies to mitigate the impact of HLB infection. We utilize an agent-based model approach to investigate the spread of ACP and HLB in a real-world setting. Instead of using 'toy' data, a 16 mile^2 area of Porterville, CA with residential and commercial citrus present is used as a case study for the simulation. The mathematical model incorporates ACP population dynamics as well as ACP and HLB spread by considering factors such as psyllid life span and mortality, net reproduction rate in the natural environment, psyllid dispersal distance, citrus host type and density, flush availability through new flush production or infected tree decline, and effects of different management schemes including biological control (via *Tamarixia*), large scale or infield survey, and chemical spray. We also conduct a cost-benefit analysis by assessing the productivity after different control methods are applied. This simulation model can quantify the influence of input epidemiological parameters on ACP/HLB development, and be utilized as a tool for HLB control planning.

Severity and incidence of HLB in orange trees grown on agroforestry system

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The development of symptoms of HLB in citrus plants is associated with the process of colonization by the bacteria *Candidatus* Liberibacter asiaticus and the increase in the production of reactive oxygen compounds (ROS). The formation of free radicals increases under conditions of high temperature and radiation, aggravating symptoms of HLB. The temperature of leaves of plants with symptoms is higher, probably due to lower rate of transpiration. Evidence of the benefit of

mitigating the symptoms with use of sunscreens was previously observed. The objective of this work was to verify the expression of symptoms of HLB in citrus growing in an agroforestry system, where solar radiation, temperature and humidity are modified. For this purpose, both the severity of symptoms and incidence of diseased plants were evaluated in a 4 years old orchard under organic cultivation of Valencia orange tree on the Rangpur lemon rootstock. The measurements were taken in the plants without shading (T1) and under two situations of shading, shadow of Gliricidia (T2) and Eucalyptus + Teak (T3). Citrus plants and forestry species were set at the same planting line. Row spacing between lines was 5 meters and between plants 2 meters. It was observed that plants with up to 70% shading around noon showed reduction in the development of symptoms of HLB with values between 5% affected canopy (T2) and 8% (T3), while in without shading (T1) presented 18% of the canopy with symptoms. The incidence of the disease was also lower in citrus plants in the shaded areas (42 %), while in the area without shading was 97%. The data were subjected to analysis of variance and, when detected significant difference, (n = 4) were compared by Duncan.

Leaf microbiota-based early detection of huanglongbing in citrus trees

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Huanglongbing (HLB) caused by the bacterium *Liberibacter asiaticus* (*Las*) is the most devastating disease of citrus in the United States today. Current methods for identifying infected trees include scouting for symptoms in the field and detection of *Las* by quantitative PCR (qPCR) in samples from suspected trees. However, because infected trees may appear asymptomatic and come up negative with qPCR, given the typically focal nature of the infection, there is a great and urgent need for additional detection methods. We describe here an indirect detection method based on *Las*-induced systemic changes in the microbiota (i.e. bacterial and fungal communities) of citrus trees following infection with Las but prior to symptom formation. More specifically, the method entails swabbing citrus leaves, extracting microbial DNA from the swabs, PCR amplifying the bacterial 16S and fungal ITS regions, sequencing using the Illumina platform, and analyzing the data using QIIME, a microbial community analysis pipeline. A combination of ordination techniques and taxon abundance rules are then used to call a tree positive, negative, or suspect. The method has been successfully used to distinguish infected trees from non-infected ones in greenhouse experiments at UC Davis and under field conditions in Texas. Our method is currently being optimized for deployment under California field conditions.

Impacts of host plants on the Asian citrus psyllid proteome and vectoring ability of *Candidatus* Liberibacter asiaticus, the pathogen associated with huanglongbing disease in citrus

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Little is known about plant-mediated effects on insects and the impact different host plant species may have on insect ability to vector plant pathogens, particularly in the huanglongbing pathosystem. It is thought that the pathogen associated with huanglongbing, '*Candidatus* Liberibacter asiaticus' (CLas), replicates within the insect vector, the Asian citrus psyllid (ACP). However, work from the Hall lab found that titers of CLas in ACP are lower when reared from infected *Murraya paniculata* than in psyllids reared on infected *Citrus sinensis*. We hypothesize that *Murraya*, a poor host of CLas, impacts ACP acquisition or pathogen infection, by either preventing the replication and/or accumulation of CLas that is normally thought to be seen in ACP reared on citrus, which are good hosts of CLas. To study these host-mediated impacts at the biochemical level, we reared populations of ACP on *Citrus sinensis*, *Murraya*, and also transferred groups between the two hosts for a period of 5 days. We performed a two-fold proteomic approach, analyzing the protein profiles of these ACP populations via 2-D difference gel electrophoresis (DIGE) and via high-resolution mass spectrometry. By comparing the proteomes of these ACP, we have found that a subset of the differentially expressed proteins in the host switch context are also altered by exposure to CLas-infected plants, showing that host switching has measurable effects on the biochemical pathways involved in CLas acquisition and transmission. Comparing proteins found in *Citrus*-reared ACP to *Murraya*-reared ACP by Fisher's Exact test, we found that reproductive, cell cycle and immune system-related proteins are among those significantly up-regulated in *Citrus*-reared ACP, whereas glucose dehydrogenase and a variety of muscle and cytoskeletal proteins are among those significantly up-regulated in *Murraya*-reared ACP. Expanding the analysis to include all four populations by ANOVA reveals a more complex picture of transient host-mediated metabolic reprogramming as the ACP adapts to a new host. These data provide new insights into the influence that host plants have on insect physiology and vector competency, as well as what proteins may be involved in successful acquisition of CLas by the ACP. Future plans include complimentary studies on ACP that harbor CLas, and studying the influence of host plants on transmission efficiencies.

Effects of citrus flush quality and psyllid population density on use of vibrational communication signals for *Diaphorina citri* management

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Diaphorina citri is an important pest of citrus because it vectors the devastating citrus greening bacterial disease. It is known that males locate potential mates on tree branches by use of vibrational communication. Research on this behavior may help combat the citrus-greening epidemic that has affected Florida and other regions, such as through trapping and the inhibition of psyllid mating. However, because this is a new area of study, many questions remain about the application of such technology in field environments. Potentially, vibrational communication behavior could be affected significantly by the quality of flush and/or by the density of *D. citri* on individual trees, both of which would affect the psyllid movement patterns that the vibrational communication attempts to pre-empt. Experiments were conducted to monitor calling and aggregation of *D. citri* males and females at different densities on feather flush and older flush of small citrus trees. Results of the study and implications for pest management are discussed.

Citrus huanglongbing (HLB) testing and diagnostic KIT

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Citrus huanglongbing (HLB), aka citrus greening, is the most important disease threating the citrus industry. The plants infected with HLB will die after several years, and healthy trees close to infested HLB citrus trees may become infected by HLB through the citrus psyllid insect vector. Therefore, the HLB diagnosis has become of key important for growers for HLB monitoring. Based on our studies, we have developed a diagnosis KIT, which can rapidly detect citrus huanglongbing and can be used by citrus grower themselves. The Kits consist of black plastic bag, test liquid A (de-staining agent), test liquid B (iodine liquid), test Tube, abrasive paper (or Sponge abrasive block), and forceps. The tests can be done as the followings: (a) *Dark treatment:* In the citrus grove, the leaves to be tested are covered by the black plastic bag, and are collected back home after 48 hours; (b) *Leaves decolonization:* First, the dark treated leaves collected from grove are rubbed with abrasive papers (or Sponge abrasive block), grind off the waxy layer on citrus leaves surface (both sides should be grinded). Then the grinded leaves are placed into test tube, and 15-30mL of the Testing-Liquid A (de-staining agent) are added. It will take about 4-6 hours, until the leaves turn a white color (decolorization). The tube can be shaken 2-3 times during in decolorization; (c) *Diagnosis:* Drain off Test-Liquid A after the leaves turn white, then add 3-5mL of Test-liquid B. The tube is again shaken, and kept for 2-5 minutes, before checking for leaf color change. The leaves can also be put in petri dish, then tested with 3-5mL iodine liquid; (d) *Results:* A blue or dark blue color change confirms HLB infection, if not, the leaves is healthy.

Individual-Based Modeling: Simulating the spatial dispersion of HLB and the effects of epidemic on orchard yield under different scenarios of psyllid's migration and sanitary management

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This work proposes a simulation model that evaluates the impacts on production and on the citrus plantations depending on the level of infection and the spread of huanglongbing (HLB). We used the Individual Based Modeling (IBM) methodology. In this model, we considered the invasion of psyllids from neighboring orchards and scenarios where the orchard performs the sanitary management, i.e. roguing symptomatic citrus plants and replanting healthy trees. The productivity of citrus trees was calculated through the model defined by Bassanezi RB and Bassanezi RC (2008. IRCHLB Proceedings. p. 301-304), which made possible to obtain the orchard yield depending on the incidence of HLB. The productivity and costs depend on the age of the plant, the sanitary management and the epidemiological states. To analyze the impact on orchard yield was made a simplified calculation of cash flow. The sanitary management is guided by the Normative Instruction of Brazilian Ministry of Agriculture, Livestock and Food Supply, which recommend to the citrus farmers to perform the inspection in an orchard every 3 months and roguing symptomatic plants. We also simulate the scenario with the replantation of healthy citrus with different ages (e.g. 12 months and 3 years old). It is known that the primary means of attraction and reproduction of psyllids is through the shoots of plants, in which the deposition of eggs is made only in young plant tissues. Older plants and young can have different levels of flush, what results in different levels of attraction to the vectors of diseases. The simulation results show an increase of the yield and a lower disease incidence with the sanitary management in long-term. Other contribution of this work is the easy way to change the many parameters and scenarios in the model.

The effect of HLB on the quality of cold pressed oils from Florida Hamlin and Valencia oranges

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Hamlin and Valencia oranges were each harvested twice per year for two growing years in Lake Alfred, FL and processed into cold pressed oils. During each harvest, both asymptomatic and symptomatic fruit were sampled. There was one sampling early in each orange's harvest season and a second sampling late in the harvest season. The oils underwent several quality tests including US Pharmacopeia mandated physiochemical tests (aldehyde content, UV absorbance, optical rotation, specific gravity, and refractive index); taste panels; aroma panels; and qualitative and quantitative gas chromatography. Overall results showed both symptomatic and asymptomatic Hamlin oils had aldehyde contents below the US Pharmacopeia minimum (1.2%) for both harvest years. For Hamlin oranges harvested in the early season harvest for the 2015-2016 season, there were significant differences between symptomatic and asymptomatic oils for aldehyde content, specific gravity, UV absorbance, and optical rotation. There were no significant differences between solutions made with Hamlin asymptomatic and symptomatic oils in taste panels. However, there were significant differences between early season Hamlin asymptomatic and symptomatic oils in aroma panels. Several differences were seen between asymptomatic and symptomatic Valencia oils for the US Pharmacopeia tests for both the 2014-2015 and 2015-2016 harvest seasons. Additionally, late season symptomatic Valencia oils exceeded the US Pharmacopeia maximum for specific gravity for both years. There were no significant differences between solutions made with Valencia asymptomatic and symptomatic oils in taste panels. However, there were significant differences between late season Valencia asymptomatic and symptomatic oils in aroma panels. Quantitative gas chromatography showed that multiple compounds important to orange aroma and flavor which are significantly different between asymptomatic and symptomatic samples for both Hamlin and Valencia oils. These compounds include decanal, linalool, citronellal, citronellol, neral, and geranial. The aroma panel results and decrease in concentration of these important orange aroma compounds show that HLB may have a negative effect on cold pressed Hamlin and Valencia oils.

After ten years of ACP, no HLB has been observed on citrus plants in Sonora, Mexico

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The Asian Citrus Psyllid (ACP) *Diaphorina citri* Kuwayama, was detected on March 2006, on citrus plants in the residential area of Ciudad Obregon Sonora, one year later it was observed in Guaymas and Hermosillo, Sonora. Since its discovery in Ciudad Obregon, surveys were carried out in order to determine its regional dispersion and migration to citrus commercial plantations. In 2010 an area-wide strategic management plan was established. The main objective was to keep ACP populations at the minimum level and lower the risk of HLB establishment in the state of Sonora. This plan continues until now and it has been successful in keeping ACP populations at very low levels and no HLB on citrus plants, have been detected. The plan is based in a coordinated effort and mandatory actions in commercial plantations including weekly surveys by yellow sticky traps and tap sampling, two area-wide insecticide applications are made one in the dormant stage (February-March) and other in the fall (September-October), besides 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. Citrus plants in rural and residential areas are also monitored and sprayed to reduce the ACP population at the regional level. Results of the area-wide management plan are considered satisfactory after ten years of the first detection of ACP in Sonora. Considerable reduction in ACP populations has been achieved and not HLB plants have been detected on the surveys realized. However, in the last two years HLB infected Psyllids were detected in rural areas of Southern Sonora and an aggressive action plan was implemented controlling ACP populations and eliminating citrus plants in the area were these infective Psyllids were observed. Further surveys and psyllid analysis for HLB have not shown any more infected insects or citrus plants in the area.

A conceptual framework for the evaluation of HLB surveillance activities

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Surveillance activities play an integral part in disease prevention and control, and underpin the three main stages of disease mitigation: the prevention of entry and establishment of exotic pathogens; the detailed investigation of more established pathogens; and the monitoring of disease control measures. As with any disease mitigation measure, it is important that surveillance activities are planned, implemented, and evaluated using scientifically valid approaches. This process of "survey validation" allows us to ensure that surveillance schemes are performing satisfactorily (from both a functional and an economic perspective), and allows appropriate adjustments to be made to improve survey performance and efficiency. Whilst survey validation is best achieved using a systematic methodology, the range of different surveillance aims in different settings means that this methodology must also be flexible. One way to achieve a suitable balance of structure and flexibility is to identify specific attributes of the surveillance aims as part of the validation process, which can then be used to determine which precise validation approaches are most appropriate. Although HLB is a threat to the citrus industry throughout the USA, its impact upon the industry to date at the state level has varied. In Florida, HLB is thought to be well established throughout all commercial orchards; in Texas, it has been reported in some orchards but at lower levels than in Florida; and in California, the disease has not yet been detected in commercial orchards. As a result of this variation, the surveillance activities in place differ between the three states. We use a recently developed survey validation

framework to evaluate these HLB surveillance activities. Since our approach allows the different epidemiological scenarios in each state to be explicitly accounted for within the context of a standardised framework, strengths and weaknesses of the different surveillance schemes can be better identified.

A method of determining how to balance survey effort for early detection of HLB across host and vector populations

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Many citrus production areas are currently threatened by HLB. Whilst minimising the risk of spread of the HLB bacterium (*Candidatus* Liberibacter asiaticus; Las) and insect vector (*Diaphorina citri*) are important strategies for reducing the risk of HLB entry, there will always be a potential risk of introduction. This means that effective early detection surveillance activities – focussed at detecting Las infection at an early stage – must be in place. Our previous work has demonstrated that the epidemiology of a pathogen (in particular, the rate of epidemic growth) should be considered when developing surveillance strategies. The presence of insect vectors as well as host plants in the HLB pathosystem adds another layer of complexity to the issue of surveillance, since it leads to the question of whether to sample from hosts, vectors, or both. Although this question has considerable implications for the design of the surveillance strategy, many surveillance schemes focus on sampling from host plants, with vectors sampled more opportunistically. To investigate this issue further, we have developed a statistical model of host and vector sampling and linked this to a mathematical model of pathogen transmission through the HLB pathosystem as a whole. The resultant model allows us to quantify the relative sampling efforts and/or costs required from hosts and vectors in order to detect a specified incidence of infection in either. From this, we demonstrate that the overall incidence at first detection is minimised when samples are exclusively collected from either hosts or vectors but not from both. As well as identifying whether hosts or vectors should be sampled, our method gives a numerical output which indicates how robust this decision is to changes in sampling costs. This has potential for use as a simple tool to determine where best to place sampling resources whilst accounting for both epidemiological issues and economic constraints.

Sensing and UAV technologies to assess citrus greening in Puerto Rico

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Citrus production is an important industry in Puerto Rico, with oranges (*Citrus sinensis* Macf.) accounting for 30% of the value of all fruits in 3,450 hectares planted. In the 2010-2011 season, 51.57 million fruits valued at $4.46 million were locally produced. Citrus greening identified in 2009 have reduced production approximately 50%. Presently, investigators make field visits to infected orchards and manually record plant parameters. This process is cumbersome and time consuming. This study used a color camera onboard an unmanned aerial vehicle (UAV) to acquire images of a citrus plants. The images of a citrus orchard at the Substation Fortuna were taken from the UAV at an altitude of 60 feet. Plant height, canopy coverage area and stem diameter were recorded. The observed plant parameters correlated well with the predicted plant parameters from the color images. Plant health indices are being investigated to identify severity of citrus greening and nutrient deficiency.

CLas population dynamics in citrus and ACP

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Relationships between CLas titer in ACP, CLas transmission by ACP to citrus, changes in CLas titer following infection, systemic movement of CLas in citrus and eventually expression of HLB symptoms, are all critical to HLB disease development. Incidence of CLas systemic infection (based on qPCR detection) was less than 50% one month after inoculation (MAI) for 171 citrus nursery trees graft inoculated with CLas. Four MAI 95% of the graft inoculated trees were systemically infected. CLas titer throughout the trees also increased with MAI. One MAI, most infected leaves had CLas titers of ca. 10^0 genomes/100˙mg^{-1} total nucleic acid (TNA), by 10 MAI ca. 50% of the plants had CLas titers between 10^5 and 10^6 genomes/100˙mg^{-1} TNA. CLas titers in the range of 10^2 to 10^3 genomes/ 100˙mg^{-1} TNA were less frequent than those lower or higher. At 10 MAI, 100% of plants with CLas titers in excess of 10^3 genomes/100˙mg^{-1} TNA were HLB symptomatic. Adult ACP infected with CLas were also used to inoculate citrus. Essentially 100% of ACP adults tested CLas positive, with titers in infected ACP adults most frequently ca. 10^3 copies per insect, although infrequently, CLas titer in some ACP reached 10^6 copies per individual insect. CLas was detected in citrus shoots ca. 21 days following exposure to CLas infected ACP; after an additional 15 day incubation the incidence of detection increased to nearly 100% along with CLas titer. HLB symptoms became visible ca. four months following exposure to CLas infected ACP. Our results indicate that development of systemic CLas infection and HLB symptoms in graft inoculated plants differ considerably from ACP inoculated plants. However, temporal changes in CLas titer over time were similar in graft and ACP inoculated trees. In no case were HLB symptoms observed when CLas titer was less than 10^3 genomes 100˙mg^{-1} TNA.

Enhanced "early" detection of CLas infections in citrus

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"Early" detection of CLas infection is essential to minimize the risk of HLB epidemics in areas where the pathogen has been recently introduced. Any delay in confirmation of CLas infection results in delays of regulatory and management actions, and increased spread of the pathogen even in areas with aggressive insecticidal ACP control. By default, to be considered "early", CLas infection must be confirmed prior to visible HLB symptoms when titer is very low. Currently, qPCR with CLas 16S rDNA primers is used to identify suspect infections in large scale surveys of citrus. We report here on results of experiments that were conducted to verify the sensitivity and consistency of qPCR-based CLas detection. Our results demonstrate that primers used to amplify an 88 bp fragment of CLas 16S rDNA target provide essentially 100% amplification efficiency (Log CN = 11.5 – 1/3.32Ct) indicating an appropriate endpoint (CN = 1) of Ct 38.3 These results were verified by analyses conducted among 4 laboratories. We demonstrate an inconsequential effect of the missing "G" nucleotide in the 16SrDNA primers used to amplify the CLas target, effects of threshold on CLas detection, and an approach to overcome a loss of sensitivity in multiplex qPCR. We found digital PCR to be equally sensitive to qPCR for CLas detection, but the method is not amenable to high through-put screening. Use of a nested PCR protocol can improve sensitivity of the conventional PCR used for confirmation of CLas infection. Using qPCR we found that CLas infections could be detected in citrus trees 21 days following exposure to CLas-infected ACP for 2 days. Our results support the sensitivity and robustness of qPCR for detection of CLas and demonstrate that qPCR is effective for "early" (i.e. pre-symptomatic) detection. However, although pPCR is extremely sensitive and selective for CLas detection, identification of appropriate diagnostic samples in non-symptomatic trees remains challenging.

Evaluation in vitro of novel antimicrobial compounds to control growth and biofilm formation of citrus bacterial pathogens

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Zinkicide™ and T-SOL are novel plant nutrient-based antimicrobial formulations developed for treating citrus bacterial pathogens including '*Candidatus* Liberibacter asiaticus' (Las) and *Xanthomonas citri* subsp. *citri* (Xac). As per design, Zinkicide™ and T-SOL are expected to enter phloem tissues of citrus plants when sprayed onto leaves or applied to soil to control phloem-limited pathogens such as Las. This study investigated the effects of Zinkicide™ and T-SOL *in vitro* on growth and biofilm formation of Las surrogate Xac in batch cultures and under flow conditions using microfluidic chambers. Xac was grown in 96-well and 24-well plates in Silva Buddenhagen (SB) medium and treated with serial dilutions of Zinkicide™ and T-SOL to test inhibition of growth and biofilm formation. Xac was also introduced to microfluidic chambers under constant media flow, and inhibitory action of Zinkicide™ and T-SOL on biofilm formation was recorded using time-lapse video imaging microscopy. The Zinkicide™ formulation had stronger antimicrobial properties compared to similar formulations with the same active ingredients, and the minimum inhibitory concentration of Zinkicide™ and T-SOL for Xac was calculated at 10ppm and 30ppm, respectively. Time-lapse video imaging showed that untreated Xac grown in microfluidic chambers formed biofilm whereas Xac treated with Zinkicide™ did not form any biofilm. Furthermore, Zinkicide™ inhibited growth of Xac biofilm when Zinkicide™ is applied to already formed Xac biofilm in microfluidic chambers. *In vitro* results confirmed that Zinkicide™ is effective against citrus bacterial pathogens and inhibits bacterial growth and biofilm formation under flow conditions.

Comparative transcriptional and anatomical analyses of tolerant irradiated sweet oranges and a susceptible sweet orange in response to '*Candidatus* Liberibacter asiaticus' infection

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Sweet oranges have little resistance to HLB, a disease presumably caused by *Canadidatus* Liberibacter asiaticus (*C*Las). As HLB has become widespread in Florida over ten years, we have very carefully followed the extensive collections of our breeding materials including mutants and germplasm accessions within the UF-CREC citrus breeding program. We have found that two 13 year-old Valencia clones (produced from irradiated budwood) and one Kansu orange are apparently tolerant to HLB. All multiple propagations of three clones have been rejuvenated by continual vigorous growth of new shoots with few foliar and fruit symptoms. In this study, we used comparative transcriptional and anatomical analyses to evaluate gene expression and anatomical differences between three tolerant Valencia types and a susceptible standard Valencia. Underlying tolerance mechanisms revealed by RNA-seq and anatomical analysis will be described. The results potentially lead to identification of key genes and the genetic mechanism in irradiated Valencia to restrain disease development.

Spray application of different kaolin formulations on sweet orange plants disrupt the settling and probing behavior of *Diaphorina citri*

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The psyllid *Diaphorina citri* is the vector of the bacteria associated with huanglongbing (HLB), which is the most destructive citrus disease worldwide. Chemical control is the primary tactic against this insect. However, alternative methods are important to achieve a more effective control in an integrated pest management programs. Thus, this research was carried out to assess the influence of different kaolin formulations on the settling and probing behavior of *D. citri*. In both studies, two wettable powder (WP) kaolin formulations (Tessenderlo Kerley, Inc./NovaSource) were sprayed three times at different concentrations on sweet orange plants. In the experiment to assess the settling behavior, three concentrations (3, 5 and 7% w/v) of both formulations were tested. A non-choice test was performed, where 16 adult psyllids were released in a cage with seedlings of the same treatment, and the number of psyllids/plant at different time intervals was counted. For the probing trial, the electrical penetration graph (EPG) technique was used. Adult psyllids were monitored for 6 h on nursery citrus trees treated with two kaolin formulations at 3 and 5% w/v. The two kaolin formulations have a repellent effect on *D. citri*, causing an overall reduction of 40% of psyllids settled on treated seedlings compared with untreated control. Moreover, both formulations disrupt *D. citri* probing behavior, with a significant reduction (60%) in the proportion of psyllids that reach the phloem compared with untreated nursery citrus trees. In general, there were no differences between the kaolin formulations and among the concentrations tested in both experiments (settling and probing). Then, both formulations could be used in an integrated *D. citri* management program. These findings reinforce the recommendation of kaolin application on young citrus planting as a useful strategy for HLB management, mainly on the edge of the farms.

pH and oxygen microenvironments in psyllid and citrus phloem

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One of the most crucial steps to find a long-term cure for Huanglongbing (HLB) is to culture the causative agent which is known as *Candidatus* Liberibacter asiaticus. In order to culture this bacterium *in vitro*, it is critical to know the physiochemical conditions in citrus phloem and psyllid organs where *C*Las present. The local microenvironments can be quantified inside the native host and vector using microelectrodes. Microelectrodes are needle-shaped sensors with tip size of less than 20 µm. The use of microelectrodes allows minimally-invasive measurement of the microscale gradients within native biological system. In this study, oxygen and pH microelectrodes were used to determine the local oxygen concentration and pH inside citrus phloem and psyllid including intestine and hemolymph. Measurements are done both in healthy and unhealthy psyllids and citrus plants. The results are compared together and with the previous available measurements done with chemical extract from those targets. Results of this study will benefit further design the suitable strategies to culture *Candidatus* Liberibacter asiaticus in vitro.

Mortality of *Tamarixia radiata* exposed to different insecticides used for the control of *Diaphorina citri*

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The Asian citrus psyllid (ACP; *Diaphorina citri*) is an important pest of citrus due to its role as a vector of the pathogens associated with huanglongbing (HLB). ACP was first discovered in California in 2008, and huanglongbing in 2012. Since 2011, the parasitoid *Tamarixia radiata* has been released into California for ACP control. This biocontrol agent is now widely established in residential areas of Southern California, and efforts are underway to establish it in commercial groves. Numerous constraints may exist on *T. radiata*'s activity in commercial citrus. One set of potential constraints includes non-target effects of various insecticides being used for ACP control. To better understand the potential role of *T. radiata* as part of an integrated pest management program, we evaluated the non-target effects on *T. radiata* adults and larvae from 20 commonly used conventional or organic insecticides via a pair of laboratory experiments. The first estimated mortality rates induced by different insecticides on parasitoid larvae within already parasitized *D. citri* nymphs. The second estimated the mortality of adult parasitoids exposed to insecticide-treated leaves 24, 48 and 72 hours after application. Mustang, Actara and Entrust had the strongest non-target effect on adult and larvae *T. radiata*. Minecto and oils had the weakest non-target effect on adult and larvae *T. radiata.* Minecto, a combination of thiamethoxam and cyantraniliprole, had the lowest impact on *T. radiata* of all insecticides evaluated, whereas VoliamFlexi with a similar combination and Actara with only thiamethoxam, had stronger non-target effect on adults and larvae of *T. radiata* - suggesting interactive effects of some active ingredients.

Molecular strategies to control hemipteran insect feeding (including the Asian citrus psyllid, *Diaphorina citri*) through degradation/ biosynthetic-inhibition of stylet sheath feeding structures

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Many phytophagous hemipteran insects feed by penetration of a stylet bundle into plant tissues to feed on phloem or xylem tissues. Among these are agriculturally important insect vectors of plant diseases which include (but are not limited to): whiteflies, aphids, mealybugs, scales, leafhoppers and psyllids, including the Asian citrus psyllid (*Diaphorina citri*-ACP)-the vector of the devastating citrus greening disease. A common trait of plant vascular feeding hemipterans is ability to form a 'stylet sheath' structure that encapsulates the stylet bundle while they penetrate into the plant tissues to feed. The exact function(s) of stylet sheaths in hemipteran feeding are not known; however, trait conservation across diverse phytophagous hemipterans implies a biological importance. It is speculated that sheaths may function to 'cloak' the insect feeding process to evade host plant defenses; it also may provide structural stability to the stylets during penetration. We have previously developed a method to isolate pure sheath structures and have used these sheaths to determine gross composition. The primary component of the sheaths is a polymeric glucosyl polysaccharide consisting primarily of "starch-like" alpha-1,4-glucosyl bonds but also a small portion of cellulose-like beta-1,4-glucosyl bonds. This was determined both by mass-spectrometry analysis and demonstration of degradation with amyloglucosidase and cellulase enzymes that are specific for starch and cellulose, respectively. Another major structural component was determined to be proteinaceous as demonstrated by the detection of proteins within purified sheaths and the ability to degrade polymerized sheaths using various proteases. We have further demonstrated that inhibitors of sheath formation can be topically applied to citrus leaves and that this results in inhibition of psyllid feeding on the citrus. This work is presented as a broadly applicable new concept in pest-insect control based on the use of molecules that block the insect's ability to establish a successful feeding site on the host plant.

Functional characterization of truncated and full length natural variants of a '*Ca.* Liberibacter asiaticus' chromosomal LC1-like repressor.

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All '*Candidatus* Liberacter asiaticus' (Las) strains analyzed to date carry a chromosomal gene predicted to encode a C1-like phage repressor (LC1; CLIBASIA_01645). This gene carries a polymorphic region with a variable number of tandem repeats (VNTRs) between the N and C-terminal domains that has been used as an epidemiological marker. Las strains carry either full length or truncated (and typically frame-shifted) versions of LC1 depending on the number of VNTRs. The bacteriophage λ C1-like repressor has been extensively studied and is described as a pivotal regulator of the λ lytic and lysogenic phases. Most, but not all Las genomes sequenced to date harbor two nearly identical bacteriophages, named SC1 and SC2. SC1 is maintained as a stable lysogen in psyllids but in periwinkle and citrus the lytic phase is activated and phage particles have been observed. The mechanism behind the activation of the lytic cycle *in planta* and its implied repression in psyllids is unknown. We hypothesize that LC1 could play an important role in repression of the late genes of SC1. Bioinformatics approaches revealed several putative binding sites for LC1 on SC1, including a bidirectional promoter region in SC1, between early and late genes SC1_gp125 and SC1_gp130. SC1_gp125 is predicted to encode a C2-like repressor, also implicated in phage late gene activation. Electrophoretic mobility shift assays demonstrated that LC1 binds to the SC1_gp125 / SC1_gp130 bidirectional promoter region, as well as to its own bidirectional promoter (flanking CLIBASIA_01645 and CLIBASIA_01640). To functionally evaluate the activity of LC1 and C2 promoters in each direction, various promoter regions were fused with a GFP reporter and these reporter constructs were transformed into *Liberibacter crescens* (Lcr) and *Escherichia coli*. The SC1_gp130, CLIBASIA_01640 and CLIBASIA_01645 promoters were not functional in *E. coli*, but were functional in Lcr. SC1_gp125 was functional in *E. coli* and also in Lcr, where its activity was significantly higher than any other Lcr promoters tested, and also higher than the (typically strong) *E. coli lacZ* promoter. These promoter::GFP reporter constructs were all individually cotransformed with plasmids expressing full-length LC1, truncated LC1 and C2-like repressors (all driven by the *LacZ* promoter) in Lcr. Both the truncated and full length versions of LC1 functionally repress the SC1_gp125 late gene/C2-like repressor promoter. In addition, the C2-like repressor self-represses its own promoter. Both repressors are therefore molecular targets for chemical control of Las, since interference with either should result in strong activation of SC1 lytic cycle late genes.

In vitro antimicrobial activity of Zinckicide™ against *Liberibacter crescens*, a surrogate of '*Candidatus* Liberibacter asiaticus'

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Zinkicide™ is a zinc-based nanoparticle with antimicrobial properties that was developed for treating citrus bacterial pathogens. The nano-scale size of this formulation allows systemic translocation of its active ingredients inside plants, which makes it more effective against phloem-limited pathogens such as '*Candidatus* Liberibacter asiaticus' (CLas). In this study we evaluated the effect of Zinkicide™ in vitro on planktonic growth and biofilm-like aggregate formation of the CLas surrogate *Liberibacter crescens*. Batch cultures assays were performed in 96 well plates with a concentration gradient from 0 to 40ppm of Zinkicide™. A microfluidic chamber system was also designed to further test the effectiveness of this formulation in a device that more closely resembles the plant vascular system, which is the habitat of CLas once host infection occurs. In this device, the preventive activity of the Zinkicide™ against planktonic and biofilm growth was assessed during 7 days of treatment exposure. Additionally, recovering capacity of *L. crescens* was tested with remaining cells in the microfluidic chamber after treatment exposure, by replacing Zinkicide™ by non-amended growth media and assessing bacterial growth during 10 additional days. The minimum inhibitory concentration of the compound in 96 well plate assays was 20ppm for both the planktonic and the biofilm/aggregate fractions. In the microfluidic chamber system, the Zinkicide™ totally inhibited planktonic growth and aggregate formation of *L. crescens* when applied at 30ppm. No evident cell growth was observed in the 10 additional days of culture after exchange of Zinkicide for non-amended media, suggesting a bactericide effect. Results are currently being confirmed.

Rapid lateral movement between sieve tubes by *Candidatus* Liberibacter asiaticus in split root trees

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Huanglongbing (HLB; syn. citrus greening), caused by the phloem-limited bacterium *Candidatus* Liberibacter asiaticus (Las), poses one of the largest threats to citrus production. In ten years, it has spread to most citrus trees in Florida. Citrus production during the 2015/16 crop year is down 54% from the pre-HLB era. After initial leaf infection by *Diaphorina citri* (Asian Citrus Psyllid), Las moves to, colonizes, and damages the entire root system, however Las and HLB symptoms remain sectored in the canopy. Canopy sectoring is thought to occur because of limited lateral connections of phloem sieve tubes. To investigate if lateral movement of Las between sieve tubes is distance or tissue specific, root systems of grafted and seedling trees were split to three different heights: below crown, above crown, and above graft union (or equivalent on seedlings). The trees were then graft inoculated at a consistent height above one side of the split root system. Infection of both halves of the root system was monitored weekly. Our results indicate the graft union and crown did not play a unique role in lateral movement. No difference was observed in infection between grafted and seedling trees. Greater variability in the time between Las detection

between the inoculated and opposite side in trees with the highest trunk split suggests that vertical distance is an important factor in lateral movement of the bacterium.

LotP, a novel Liberibacter asiaticus GroEL interacting protein

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CLIBASIA_03135 is a highly induced gene in CLas mRNA samples obtained from infected citrus plants. The expression of this gene is undetectable in samples obtained from CLas infected psyllids. The encoded protein could play an important role in helping the bacteria to thrive in the harsh conditions of the plant phloem. To evaluate the biochemical characteristics and its biological significance, we have cloned the gene and purified the encoded protein (re-named LotP). Co-immunoprecipitation assays allowed us to identify the GroEL chaperone as the main interacting protein. The specific interaction between LotP and GroEL was confirmed by using a two-hybrid system in *Escherichia coli*. LotP is a dimer in solution with a native molecular weight of 44 KDa and has ATPase activity *in vitro*. It displays remarkable structural homology, but no sequence conservation with the amino-terminal region of the *Bacillus subtilis* LON protease. LotP was systematically annotated as an ATP dependent LON protease, however, the *B. subtilis* LON protease is an ATP dependent aminopeptidase composed of 6 units each of which are 90 KDa. The results obtained in our analysis allowed us to define that LotP belongs to a different family of proteins involved in protein refolding. LotP is the first member of this large family yet to be biochemically characterized. Our results suggest these proteins modulate the activity of stress-response proteins by direct physical contact depending on the stress conditions present in the environment, hereby opening a possible way to combat HLB disease.

The molecular mechanism of LdtR, a global transcriptional regulator in the citrus pathogen Liberibacter asiaticus

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The expansion of huanglongbing (HLB) has resulted in a crisis in the citrus industry with no solutions in sight. We used a biochemical approach to identify new chemicals that targeted the transcriptional regulator LdtR from *Liberibacter asiaticus*. In the phylogenetically related *Sinorhizobium meliloti*, mutants in the *ldtR* homologue resulted in phenotypic changes, such as shortened rod-type phenotype and increased sensitivity to osmotic stress. The chemical inactivation of LdtR homologues in *S. meliloti* and *Liberibacter crescens*, resulted in a similar phenotype to the *ldtR* mutant. In this work, we identified and characterized the members of the LdtR regulon in *L. asiaticus*, and the impact of the identified small molecules on the regulatory activity of LdtR. *In vitro* DNA-binding assays and RNA-seq experiments confirmed that genes involved in processes such as cell division and cell wall biosynthesis are regulated by LdtR. The DNA binding sequence of LdtR target genes was predicted via *in silico* analyses and confirmed via DNase I footprinting, DNA gel shift assays, suggesting that LdtR might be involved in the regulation of more than 200 genes. These results provide new evidence about the regulatory network of *L. asiaticus* and offer molecular foundations for the design of therapeutics for the treatment of citrus greening disease.

Identification of ligand binding pocket of PrbP from Liberibacter asiaticus

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PrbP is a RNA polymerase interacting protein involved in global transcriptional regulation in *Liberibacter asiaticus* (Las). We found that $PrbP_{Las}$ interacts with the β-subunit of the RNA polymerase and binds to a specific sequence on the *rplK* promoter region, suggesting a regulatory role in genes expressions at transcription level. Genomic analyses revealed that Las transcription is controlled by a small set of transcription factors, leading to hypothesis that deactivating of $PrbP_{Las}$ is a potential therapeutic treatment. $PrbP_{Las}$ ligands were identified by differential scanning fluorimetry and verified by *in vitro* and *in vivo* experiments. In this study, we identified a $PrbP_{Las}$ ligand binding pocket and characterized its molecular interactions of binding. The structure of $PrbP_{Las}$ was modeled and the molecular interactions with the ligand were predicted using *in silico* docking. Site-directed mutagenesis of specific amino acids were followed by electrophoresis mobility shift assays, isothermal titration calorimetry, and *in vitro* assays to validate identified ligand-interacting amino acids. These results provide information on the binding mechanism of PrbP to a small inhibitory molecule and provide a starting scaffold for the identification and development of therapeutics targeting $PrbP_{Las}$ and other homologous proteins involved in pathogenicity.

Morphometric variation of *Diaphorina citri* adults is affected by temperature, photoperiod and rainfall

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Phenotypic plasticity provides a mechanism by which an organism can adapt to new or changing environments. Earlier studies have demonstrated the variability of *Diaphorina citri* Kuwayama (Asian citrus psyllid) population dynamics, but no analysis of morphological changes induced by seasonal or artificial laboratory induced conditions has yet been documented. Such morphometric variation has been found to correspond in dispersal capabilities in several insect taxa. In this study, the effects of temperature and photoperiod on morphometric variation of *D. citri* were examined through laboratory rearing of psyllids under controlled temperatures (20ºC, 28ºC and 30ºC) and under short (10.5 L: 13.5 D) and long (16 L: 8 D) day length photoperiods. Field-collected *D. citri* were collected monthly from three citrus groves in Fort Pierce, Gainesville and Immokalee, FL to evaluate potential field-associated environmental effects. Both traditional and geometric morphometric data were used to analyze the correlation between environmental and morphometric variation. A strong correlation was found between temperature and shape change, with larger and broader wings at colder temperatures in the laboratory. Short day length resulted in shorter and narrower wings as well. From the field, temperature, rainfall, and photoperiod were moderately associated with shape parameters. Adult *D. citri* with blue/green abdomens collected in the laboratory and field studies were larger in size and shape than those with brown/gray abdomens.

Long-term study of huanglongbing diagnosis using fibrous root tissue

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The biological agent associated with Huanglongbing (HLB) in citrus is a phloem-restricted Gram-negative alpha-proteobacteria that belongs to the genus '*Candidatus* Liberibacter'. Among three species of *Candidatus* Liberibacter, *Ca.* L. asiaticus (CLas) has recently invaded several citrus growing regions of the Americas, causing major economic losses. Early HLB detection is necessary in implementing HLB disease management strategies to mitigate the disease. The major source material for HLB qPCR diagnostic test is symptomatic leaves, but a combination of uneven distribution of the bacteria and the slow development of leaf symptoms which can resemble those induced by various biotic/abiotic stresses, reduces the probability of HLB early detection. We have previously shown that CLas is more evenly distributed in the root system of an HLB-positive tree than in the leaves, suggesting that root tissue may be an alternative source for qPCR diagnosis. We selected about 100 young (4-5 years) citrus trees located in an orchard with a low level of infection that is adjacent to an orchard with heavily infected trees from TX and FL, respectively. Leaf and fibrous root samples for HLB qPCR test were collected monthly from these trees since January, 2016. For the current study, we developed efficient DNA extraction and a new qPCR primers and probe system that target CLas 16s rDNA for root samples.

The HLB qPCR data showed that the number of HLB-positive trees confirmed with leaf tissue maintained the steady state during the course of the experiment while the number of HLB-positive trees detected by root tissue has started increasing drastically since May, 2016. The qPCR data showed that about 65% of TX and 80% of FL HLB positive trees were tested positive only with root samples while about 8% of TX and 6% of FL HLB-positive trees were tested positive only with leaf tissue. About 27% and 14% of HLB-positive trees from TX and FL, respectively, were tested positive both with root and leaf tissue. The survey results indicated that the HLB qPCR detected CLas among root tissue substantially more often than among leaf tissue.

Exogenous application of methyl jasmonate and salicylic acid on citrus foliage: Effects on foliar volatiles and aggregation behavior of Asian citrus psyllid (*Diaphorina citri*)

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Methyl jasmonate (MeJA) and salicylic acid (SA) are well-known activators of chemical defenses in plants. The SA pathway is involved in citrus response to infection by *Candidatus* Liberibacter asiaticus (*C*Las); less is known about the role of jasmonates in citrus defense response. We examined the effects of spray applications of MeJA and SA on the volatile profile of young citrus foliage, the site of Asian citrus psyllid (ACP) reproduction and development. MeJA and SA were mixed in a 0.1% aqueous Tween solution and sprayed onto potted infected (*C*Las+) and healthy (*C*Las-)Valencia sweet orange trees to the point of runoff. Control trees received 0.1% Tween solution alone. A purge and trap headspace system was used to collect volatiles for two consecutive days from the following treatments: *C*Las-/control; *C*Las-/MeJA or SA; *C*Las+/control; *C*Las+/MeJA or SA. All *C*Las+ trees had Ct values in the low-20's. The volatile profile of trees treated with SA was significantly altered; the primary effect being the emission of large amounts of methyl salicylate. Treatment with MeJA significantly altered foliar volatile emission in the following ways: 1) The total amounts of volatiles increased, *C*Las+ trees emitting the higher amounts than *C*Las- trees; 2) the proportions of limonene to (E)- β-ocimene and that of of alkyl aldehydes (C8-C12) to monoterpenes were reversed relative to control trees; 3) natural enemies attractants, such as indole and (E)-jasmone, were emitted by treated foliage; and, 4) higher amounts and a greater variety of sesquiterpenes were emitted by treated foliage. Additionally, the *C*Las+/control trees emitted greater amounts of methyl salicylate than *C*Las+/MeJA-treated trees, suggesting that MeJA may antagonize the SA pathway and biosynthesis of methyl salicylate. Citrus foliage infected with *C*Las emits methyl salicylate, which is a potent ACP attractant and has been implicated as the means by which the pathogen promotes its dispersal by the psyllid. Our results suggest that MeJA application may be useful for retarding production of methyl salicylate by infected trees. MeJA application also altered psyllid behavior. In laboratory assays, similar numbers of psyllids settled on untreated control and MeJA-treated citrus sprigs. However, the numbers of psyllid aggregations ($\geq$ 10 psyllids) were significantly higher on MeJA-treated than on control sprigs. Identification of the volatiles responsible for eliciting aggregation behavior could lead to the development of highly effective scent attractants for use in ACP detection and monitoring.

Las effectomics identifies a plant immune receptor for the development of durable HLB resistance in citrus

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Pathogens secret effector molecules to suppress host defense responses and facilitate the infection process. Plants, in turn, have evolved pathogen-specific resistance mechanisms, which are elicited upon recognition of these effectors and result in the activation of secondary defense responses. We identified 16 putative *Candidatus* Liberibacter asiaticus (Las) effectors via bioinformatics, and transiently expressed them in *Nicotiana benthamiana*. One of the 16 candidates, the mature protein sequence of Las5315mp was localized in the chloroplast and induced cell death in *N. benthamiana* at 3 days post inoculation (dpi). To characterize the effector domain(s) associated with the cell death phenotype in *N. benthamiana* we performed a functional screening based on transient expression of defined peptide sequence domains of Las5315mp effector. In this study, we identified interactions between Las5315mp and plant cell proteins, and unveiled a resistance genes (NBS-LRR gene) in the non-host *N. benthamiana*, which is involved in resistance response through cell death upon recognition of the pathogen effectors. This is the first report of a gene-for-gene resistance mechanism related to a Las effector that may be used to generate HLB-resistance upon expression of the corresponding resistance gene in citrus.

The accumulation of H_2O_2 and ATP in infected citrus plants reflect HLB progression and disease severity

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In response to a pathogen attack, multiple defense mechanisms are triggered in the host plants, including basal defense and gene-for-gene resistance. In particular, Las infection, causes extensive changes in gene expression for several major biological processes including stress responses, signal transduction, transport, cell organization and carbohydrate metabolism. Las deploys effectors that target mitochondria and chloroplasts, which are responsible for the synthesis of adenosine triphosphate (ATP) and have a critical role in in the creation of reactive oxygen species (ROS) for both pattern-triggered and effector-triggered immunity signaling in the plant. In this work we investigated HLB disease in relation to ROS and ATP regulation through the analysis of genes correlated to the production and detoxification of H_2O_2, since H_2O_2 is one of the major and most stable ROS regulating basic acclamatory, defense and developmental processes in plants. We found that Las infection increased the level of ATP and H_2O_2 in citrus leaves suppressed the H_2O_2 detoxification system, resulting in an overall increase in the level of H_2O_2, which becomes toxic for the plant and initiates damage to the tissues.

Effect of compromised abscission zone on taste quality of fruit affected with huanglongbing (HLB) and secondarily infected by *Lasiodiploida theobromae*

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Trees severely infected by huanglongbing (HLB) present increased fruit drop, and the harvested fruit are usually smaller and compromise juice quality by being sour, bitter and having off flavor. It was shown that fruit drop is a result of secondary infection at the calyx zone by the fungus *Lasiodiplodia theobromae*. The objective of this study was to identify whether those fruit that are ready to abscise have a different quality than fruit that do not have a developed abscission zone. 'Hamlin' and 'Valencia' orange trees, both healthy and HLB-affected, confirmed by qPCR analysis for *Candidatus* Liberibacter asiaticus (*C*Las), from two harvests (Hamlin) and one harvest (Valencia) were shaken, the dropped fruit collected, and the retained fruit harvested. The harvested fruit were washed, juiced using FMC commercial extractor, pasteurized and frozen for later chemical and sensory analyses. The juice was tasted by 55 untrained panelists in multiple paired-comparison tests where samples were presented as pairs of either "drop/retain", "drop/drop" or "retain/retain". Juice from healthy and HLB-affected trees were presented in separate pairs. Panelists could not differentiate juice from healthy fruit retained on the tree or dropped for all three harvests. However, for juice made with HLB-affected fruit, panelists could differentiate juice from fruit that had a developed abscission zone and dropped on the ground after shaking in comparison with fruit that was retained on the trees. The test was statistically significant for all three replications of Hamlin harvested in December, and in two of three replications of Hamlin harvested in January and Valencia harvested in April. A trained descriptive panel also found differences between juice made with fruit having different levels of abscission, with juice from HLB-infected dropped fruit having the most negative off flavor attributes, including sourness, bitterness, astringency, off flavor. Sensory data confirm chemical data and both sets of data were correlated, bringing further insight into off flavor induced by HLB in orange juice.

Estimating the economic efficiency of various HLB control strategies

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The implications of different control strategies is critical when developing viable disease management plans. Cost, efficacy, disease/pest prevalence, and perceived consequences/benefits, among many other factors, play a role in deciding what control methods to utilize. Additionally, these control methods tend to change over time, reacting or adapting to the current situation. As ACP and HLB continue to spread, the need to assess and implement economically sustainable control options is paramount. We can investigate and compare the production benefits against the fiscal costs of various control strategies through mathematical modeling. We extend a spatially-explicit, stochastic, individual-based compartmental model (SECIR) to incorporate different combinations of control measures such as survey, insecticides/pest control, tree removal, and tree replacement. For surveying, we can implement different survey patterns and probabilities of disease detection. Insecticide spraying can be routine (e.g. annual spray schedule) as well as reactive (e.g. initiate an applicable spray depending on disease presence to reduce pest pressure). Further, infected tree removal or localized culling (removal of all hosts within a certain distance of detected infection) can be invoked to reduce the local inoculum, and replanting can be initiated. Under different combinations of these control measures, we can calculate the costs, model the spread of HLB, and determine the yield/production benefit.

Different scenario-based simulations of ACP & HLB dynamics in Central Valley, CA for consideration of disease management

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A better understanding of the transmission of HLB between the psyllids and citrus hosts in natural landscape is crucial to formulating effective control strategies. A spatially-explicit agent-based model, which simulates the actions and interactions of autonomous agents within the epidemiological system, has been developed to investigate how ACP and HLB spread in the Central Valley of California, an intermixed landscape of residential and commercial citrus. This study is a practical extension of the mathematical model with purposes to quantify the influence of input epidemiologic parameters on disease progress under sensitivity analysis, and investigate the efficiency of ACP/HLB management strategies by running scenario-based simulations. Although there are numerous hypothetical management strategies for testing, we focus more on survey design (i.e. disease detection level), and biological and chemical control strategies (i.e. frequency, timing, efficacy and coordination). In addition, we incorporated these management strategies into disease modelling with consideration of the social-economic perspective of citrus growers. Growers' awareness of ACP/HLB and their attitude toward control strategies will change along with disease development, and we evaluate the set-points that yield optimal operation cost and sustainable control using a cost-benefit analysis. Our results indicate that climate change can have a large effect on the performance and spatiotemporal distribution of ACP and biological control agent populations in central California. ACP spread occurs more frequently and faster within commercial citrus clusters, but comparatively slower for low density or well separated residential areas. For 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.

The effect of citrus fruits price fluctuation on the occurrence situation of HLB: Based on the empirical study of China

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The occurrence of citrus fruits HLB has caused great losses to the citrus fruits production, but there is no effective way to cure HLB at present. For the occurrence of HLB, there is natural inducement as well as economic and human factors. This paper aims to explore the impact of economic factors on the occurrence of citrus fruits HLB. From the perspective of economic, the paper analyzed the influence of citrus price fluctuation on the farmers' behavior of orchard management and control. To carry out accurate analysis on farmers group of citrus fruit production, this paper further discussed the relationship between citrus fruit price fluctuation and farmers behavior of orchard management and control in different operation scale. The results and conclusions will help to prevent the occurrence of citrus fruits HLB and adopt scientific comprehensive measures for the citrus fruits HLB prevention and control.

Organic and conventional programs for management of Asian citrus psyllid (*Diaphorina citri*) vector of huanglongbing pathogens

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The Asian citrus psyllid (ACP) vectors causal pathogens of huanglongbing (HLB) or citrus greening disease. Its control is critical in all habitats including organic citrus grown in the United States and other regions of the world. Three separate organic programs, organic insecticides applied alone (Program 1) or with horticultural mineral oil (HMO) (Program 2) and insecticidal soap (Program 3) were compared with one conventional program for impact on ACP and beneficial insects including release of nymphal parasitoid *Tamarixia radiata* in bearing citrus in southwest Florida. During the dormant winter season, Pyganic applied alone or with 435 oil or M-pede applied in November, December and January and Danitol applied in November and January all provided significant reduction in ACP with residual lasting longer for Danitol. Pyganic applied with 435 oil or M-pede provided better control than Pyganic alone. Organic programs 2 and 3 rotated organic insecticides with 435 oil or M-pede resulting in 50% reduction in use of insecticides while providing better ACP control than program 1 with organic insecticides only. However, ACP was reduced more in the conventional program. *Tamarixia radiata*, ectoparasitoid of ACP nymphs was released in all programs but recovered more from ACP nymphs in the organic program compared to the conventional program. Lacewings, spiders, ants and ladybeetles were observed in all programs that also may have contributed to ACP reduction. Best yields were obtained in programs using organic insecticides with 435 oil (HMO) or conventional insecticides. Monthly applications of Pyganic with 435 oil during dormant winter period and rotation of organic insecticides with oil during growing season appear to be reasonable options for ACP management in organic citrus. Organic insecticides will also be suitable for conventional citrus growers as selective options to avoid excessive use of non-selective insecticides, to limit pesticide resistance and harm to beneficial insects.

Speed of *Candidatus* Liberibacter asiaticus movement in citrus plants

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Candidatus Liberibacter asiaticus (Las) colonizes the citrus phloem and causes the most destructive citrus disease. Understanding how the bacterium interacts with citrus is important to improve or devise new methods of disease control. The objective of this work was to assess Las movement in citrus plants, from the initial infection site to the root, which is the part of the tree that apparently first suffers after pathogen infection. Experiments were carried out using 208 three-year-old, 60-cm high, potted Valencia/Volkamer lemon. The plants were grafted at the stem 60 cm above the substrate surface with 3- to 4-cm-long buds from Las-positive branches, and assessed over time at different distances from the inoculation site to determine the presence of the bacterium. Two evaluation procedures were used in three experiments carried out in two time periods. In the first, the stem of the inoculated plants were pruned sequentially at 10, 20, 30, and 40 cm below the inoculation site at 14, 21, 28, 35, and 42 DPI. The new leaves that developed at the top of the remaining stems were assessed regularly for HLB symptoms and through qPCR at six months post inoculation. This procedure was used in two experiments carried out from August 2015 to January 2016 (E1) and from March to August 2016 (E2). In the second procedure, fibrous

roots (0.3 g) and one-centimeter long bark rings were removed just below the inoculation site and, at 10-cm intervals from it, up to near the soil level. The evaluations were made at 7, 14, 21, 28, 35, 42, and 49 days post inoculation (DPI), with the samples processed and analyzed through qPCR. This procedure was used in just one experiment (E3) carried out from March to April 2016. The fastest Las speed were 1.14, 1.43, and 2.02 cm per day for E1, E2, and E3, respectively. The frequencies of the evaluated sites positive for Las, at all evaluation dates, were then subjected to regression analysis. The logistic model better described Las distribution in the plants over time. The probability of detecting Las at any portion of the plant was a function of time and distance from the inoculum. The longer the time between the inoculation and sampling dates and the shorter the distance from the inoculum, at any given time, the higher the probability of testing positive. Extrapolations made using the regression model allowed estimations of minimum 51 and 131 days for Las to reach the root of 2- and 7-m high citrus trees from a single inoculation site at the top of the tree. This fast movement explains the difficulties of curing an HLB affected tree and reinforces the importance of the implementation of areawide preventive actions to control the disease in citrus orchards.

Developing HLB resistance

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One of the long-term solutions for cultivation of citrus in presence of HLB may be development of disease resistant cultivars. In the genus *Citrus*, resistance to HLB is not known although some tolerant varieties have been reported. We have identified resistance and significant field tolerance in many citrus relative genera native to Australia. Field and greenhouse studies indicated that many naturally occurring hybrids of citrus and *Eremocitrus*/*Microcitrus* are resistant to HLB. Since the HLB resistance in the Australian types appears to be heritable, we have generated hundreds of citrus hybrids by crossing *Citrus* and *Poncirus* with disease resistant/tolerant, sexually compatible Australian citrus relatives. The hybrid plants are challenged by exposure to psyllids carrying HLB-associated Liberibacter pathogen. Preliminary evaluations of the hybrids in green house experiments conducted in Fort Pierce, FL indicate that a significant number of the novel hybrids appear to be disease resistant. Further testing to confirm HLB resistance is in progress. The promising hybrids will be useful in understanding the basis of HLB resistance and in developing methods to impart resistance to commercial citrus cultivars.

Protein interaction networks at the host-microbe interface in *Diaphorina citri*, the insect vector of the citrus greening pathogen

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The Asian citrus psyllid (*Diaphorina citri)* is the insect vector responsible for the worldwide spread of '*Candidatus* Liberibacter asiaticus' (CLas), the bacterial pathogen associated with citrus greening disease. Developmental changes in the insect vector impact pathogen transmission, such that *D. citri* transmission of CLas is more efficient when bacteria are acquired by nymphs as compared to adults. We hypothesize that expression changes in the *D. citri* immune system, including the insect's commensal microbiota, occur during development and regulate vector competency. In support of this hypothesis, more proteins, with greater fold changes, were differentially expressed in response to CLas in adults as compared to nymphs, including insect proteins involved in bacterial adhesion and immunity. Discovery of protein interaction networks has broad applicability in the study of host-microbe relationships. Using Protein Interaction Reporter (PIR) technology, we show how protein interaction networks in *D. citri* are regulated during development and in response to CLas-infected citrus trees. Notably, a hemocyanin protein highly upregulated in response to CLas was found to physically interact with the CLas coenzyme A (CoA) biosynthesis enzyme phosphopantothenoylcysteine synthetase/decarboxylase. In addition, hemocyanin was found to physically interact with several other *D. citri* signaling and stress response proteins. Co-evolved protein interaction networks at the host-microbe interface are highly specific targets for controlling the insect vector responsible for the spread of citrus greening.

***Candidatus* Liberibacter crescens detected in citrus**

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Huanglongbing (HLB), also known as citrus greening, is one of the most destructive citrus diseases worldwide and is seen as a major threat to the multimillion dollar citrus industry in California. The vector of two of the bacterial species associated with this disease, *Candidatus* Liberibacter asiaticus (CLas) and *Ca.* L. americanus (CLam), is the Asian citrus psyllid (ACP), *Diaphorina citri*. In August 2008, the first ACP in California was found in a trap located in San Diego County. The detection triggered an ongoing state wide risk-based survey and HLB testing program for ACP and HLB host plants in California. Since 2008, a total of 135,000 citrus trees and 252,000 ACP samples have been tested so far by the California Department of Food and Agriculture using the United States Department of Agriculture HLB Work Instruction which utilizes a TaqMan real-time PCR assay based on 16S rDNA primers and probe developed by Li et al. (2006. J Microbiol Methods. 66:104). After eight years of challenging Li's 16S HLB primers with hundreds of thousands of environmental samples, we have found that Li's primers are highly specific but on rare occasions will cross amplify other closely related bacteria such as *Ca*. L. crescens (Lcr). Lcr has never been reported in citrus before, and has only been reported in Babaco papaya during a survey for Papaya Bunchytop Disease in Puerto Rico. Currently there are six recognized species of *Candidatus* Liberibacter, four of which are pathogenic and two that have not been shown to cause symptoms in plants. Lcr falls in the latter category and is also the only member of this group that has successfully been cultured in artificial media. To date, Lcr has been confirmed in six citrus samples collected in three different counties in California and one from Hawaii. The detections were made during routine HLB testing in which the samples produced Fam Ct values between 33-36. A 1,149 bp fragment of the 16S ribosomal RNA gene was amplified from the DNA extracts using conventional PCR with primers Ol1 and OI2c. The sequence showed 99% identity with the corresponding regions of *Ca*. L. crescens (NR 102476.1) strain BT-1. All samples had the same two single nucleotide polymorphisms (SNPs) differing with positions characteristic to Lcr. This is the first report of Lcr in citrus. Follow up sampling and testing of the CA trees confirmed all were negative for the HLB associated bacterium. Only one tree was retested for Lcr and was confirmed positive. Plans to retest the other trees for Lcr are underway.

Genome re-sequencing and transcriptome analyses of the constitutive disease resistance (*CDR*) gene family in *Poncirus trifoliata* and its hybrids

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Huanglongbing (HLB) is the most devastating disease of citrus. *Poncirus trifoliata* and its hybrids with *Citrus* have shown some level of resistance/tolerance to HLB. Although no specific genes have been identified in *P. trifoliata* for its resistance/tolerance to HLB, recent gene expression studies showed that constitutive disease resistance (*CDR*) genes might have potential roles in *Poncirus* against HLB. Based on Hidden Markov Model and BLAST searches, we have identified a total of 17 copies of CDR genes in two citrus genomes databases (*CcCDR1-CcCDR9* in *C. clementina* and *CsCDR1-CsCDR8* in *C. sinensis* databases), deduced their structures, and investigated their phylogenetic

relationships. Through genome re-sequencing and transcriptome sequencing, we identified eight *CDR* genes in the *Poncirus* genome (*PtCDR1-PtCDR8*). *PtCDR2* and *PtCDR8* were found in high abundance in *Poncirus* leaf transcriptomes. Real-time PCR revealed that expression of *PtCDR2* and *PtCDR8* were consistently higher in HLB-tolerant genotypes than in HLB-susceptible genotypes, and these two genes appear to be strong candidate genes for future studies of their role in citrus-*Candidatus* Liberibacter asiaticus (*C*Las) interactions.

NB-LRR resistance gene mining and variant analysis in *Poncirus* using whole genome re-sequencing

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Huanglongbing (HLB) is the most serious recent threat to the U.S. citrus industry. *Poncirus trifoliata* and some of its hybrids have shown resistance or tolerance to HLB, but the *Poncirus* genes for HLB resistance are to be identified and characterized. Disease resistance genes in plant mostly belong to the nucleotide-binding site leucine-rich repeat (NB-LRR) class. The aim of the present study was to identify and characterize NB-LRR genes in *Poncirus*. Approximately 1.3 million of paired end DNA sequence reads for two *Poncirus* genotypes (DPI-50-7 and 'Flying Dragon') and two *Poncirus* hybrids (US-897 and US-812) were mapped to nucleotide sequences of recently characterized NB-LRR genes from the *Citrus clementina* genome using TopHat and BWA, respectively. RNA-Seq data revealed that 53 and 60 *Poncirus* NB-LRR genes expressed in DPI 50-7 and 'Flying Dragon' leaves, respectively, when grown in the field under natural HLB disease pressure or grown in the greenhouse without HLB. SNPEff annotation of the variants identified 12,774 SNP and 1,293 indels in *Poncirus* NB-LRR genes. A small percentage of the sequence variants in the coding regions (about 2.5%) could be considered as high-effect variants. Mining of *Poncirus* NB-LRR genes will accelerate future efforts to determine the roles of these genes in *Poncirus*' resistance or tolerance to HLB.

Detection of '*Candidatus* Liberibacter species' from citrus in Eastern Africa

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'*Candidatus* Liberibacter asiaticus' (Las), the causal agent of citrus huanglongbing (HLB), was reported from Uganda in 2015. Following this report, surveys were conducted in south-eastern Uganda, southern Kenya and eastern Tanzania of citrus showing mottling symptoms, typical of both HLB and African Greening. Total DNA was extracted from these samples and assessed for the presence of Liberibacter species by real-time PCR using a generic Liberibacter test. Liberibacter positive samples were further characterised to species level by conventional PCR targeting three conserved genes within the Liberibacter genome and the amplification products sequenced. The genes assessed were the 16S rRNA, ribosomal protein J gene (*rplJ)* and the outer membrane protein gene *(omp).* Phylogenetic analyses of the sequences demonstrated that a number of samples from both Uganda and Tanzania were infected with '*Candidatus* Liberibacter africanus' (Laf), the bacterial agent associated with African Greening. Additionally, samples from Uganda, Kenya and Tanzania were shown to contain Liberibacter sequences characteristic of '*Candidatus* Liberibacter africanus subsp. clausenae' (LafCl) which was first described from indigenous *Clausena anisata* (Horsewood) trees in South Africa. This is the first report of natural infection of citrus with LafCl. We were not able to verify the presence of Las in Uganda, nor from samples collected from Kenya or Tanzania. It however remains of utmost importance for the South African citrus industry to continuously monitor the spread of HLB in Africa, which was confirmed to be present in Ethiopia.

A natural variant of *X. citri*, as potential biocontrol of pathogens in citrus

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Xanthomonas citri ssp. *citri* (*X. citri*) strain A is the causative agent of Asiatic citrus canker, a disease that seriously affects most commercially important *Citrus* spp. worldwide. We have previously identified a natural variant, *X. citri* A^T, that triggers a host-specific defense response in *Citrus limon*. Characterization of this response shown that *X. citri* A^T triggers a hypersensitive response (HR) associated with the interference on biofilm development and the activation of autophagy-related vacuolar processes. Moreover, this plant response involves an extensive transcriptional reprogramming setting in motion cell wall reinforcement, oxidative burst and accumulation of salicylic acid (SA) and phenolic compounds. Interestingly, we demonstrate that this defense response protects *C. limon* plants from disease upon subsequent challenges by pathogenic *X. citri*. Further studies are required to explore if this defense response decreases susceptibility to other citrus pathogens, including Huanglongbing (HLB).

A microbiome approach to citrus huanglongbing

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The aim of our project is to uncover correlations between tree health and endophytic or epiphytic microorganisms associated with citrus trees in the context of Huanglongbing (HLB). We hypothesize that a descriptive and quantitative appreciation for these associations will reveal new methods of early disease detection and the identification of microorganisms with practical potential to prevent or mitigate establishment of the HLB pathogen. We aim to establish principles of what constitutes a healthy microbiome of citrus trees, how *Candidatus* Liberibacter asiaticus (*C*Las) infection disrupts the microbiome, and how an unhealthy microbiome may be remedied. We have sampled both HLB-diseased and –survivor trees from orchards located in Florida. Survivor trees are defined as trees under high disease pressure that expressed few HLB symptoms. Roots, leaves, and stems samples were collected from about one hundred trees for culture dependent and culture independent analyses. Bacterial and fungal ribosomal DNA was sequenced from cultures and citrus tissues using Illumina MiSeq. We have catalogued the culturable and non-culturable bacteria and fungi associated with roots, leaves, and budwood of citrus trees. In addition, we have identified positive and negative correlations between CLas titer and the abundance of specific fungal and bacterial groups. Our results provide a foundation for future research that will hopefully lead to identification of novel management strategies for HLB.

Using long reads, optical maps and long-range scaffolding to improve the *Diaphorina citri* genome

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The current *Diaphorina citri* draft genome assembly (NCBI-DIACI_1.1) was sequenced with Illumina paired-end and mate-pair reads. Low coverage Pacbio was used for scaffolding and the scaffold N50 for this 486Mb assembly (163,023 scaffolds) is 109.8kb. A BUSCO analysis

shows a significant number of conserved single-copy markers either missing (26%) or fragmented (22%) with only 51% present in full-length copies. A community-driven genome annotation has also identified a number of misassemblies and missing genes in the current genome. This is, in-part, due to the complexity introduced when assembling a heterogeneous sample containing DNA from multiple psyllids and potentially exacerbated by the use of short reads. We have generated 36.2Gb of Pacbio long reads from 41 SMRT cells with a coverage of 80X for the 400-450Mb psyllid genome. The Canu assembler was used to create an interim assembly with a contig N50 of 115.8kb and 8300 contigs. This will be polished with Pacbio and Illumina paired-end reads followed by scaffolding with Illumina mate-pair reads. Chicago libraries utilize chromatin crosslinks to associate sequences originating from the same large DNA fragment and will also be used for scaffolding. BioNano optical maps generated using high molecular weight DNA from adult psyllid tissue will be used to provide long-range scaffolding. This will be the first time all these methods have been applied to resolve an insect genome from a highly heterogeneous sample. The new assembly will be available on https://citrusgreening.org/.

Incidence and distribution of *Diaphorina citri* carrying *Candidatus* Liberibacter asiaticus

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We evaluated the percentage of psyllid population with Las (Las+) and its correlation with insect population captured in sticky cards and the HLB management in the property over a year. Assessment was carried out in four citrus regions in São Paulo state according to the Fundecitrus Phytosanitary Alert System. Cards were installed, read and replaced every two weeks on the period of February 2014 to February 2015 in the regions of Avaré, Santa Cruz do Rio Pardo (southwest), Araraquara (center) and Bebedouro (north). In each reading, up to 50 psyllids per regions were collected and detection of insects Las+ was made by qPCR. Bi-monthly averages of the percentage of psyllids Las+ were compared. Santa Cruz do Rio Pardo region had 71% of Las+ samples and differed from Bebedouro region that had 56%. Avaré and Araraquara regions had 68 and 66%, respectively. The number of psyllids increased gradually between July and August in Avaré and Santa Cruz regions, from September to October in Araraquara region and intermittently from October in Bebedouro region. Potentially infective psyllid was higher in early spring in Avaré and Santa Cruz, with the largest population; Araraquara were higher starting in October, peaked in December (spring to beginning of summer season); Bebedouro region reaches the highest values in January and February (summer). Percentage of Las+ psyllids collected in properties without HLB management were higher and statistically different from the averages on the properties that adopted psyllid control. The relationship between the percentage of psyllids Las+ and the number of captured psyllids in the region in a given time denotes the most critical time of year that psyllids Las+ intake in the orchards can occur. The region of Araraquara has the largest insect Las+ population in summer, while Bebedouro has the smallest, during the whole year.

Nutrition plays a key role in mitigating disease severity and defense towards CLas in huanglongbing infected citrus

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In absence of a definitive cure, long term strategies that combine Huanglongbing (HLB) tolerant citrus germplasm with improved nutritional programs can potentially result in tree survival and increased plant productivity under endemic HLB conditions. This study evaluated the responses of HLB-infected 'Valencia' grafted either onto Swingle or an HLB tolerant rootstock developed in our breeding program. Trees were fertilized at a 6 monthly interval with a POLYON® controlled release nursery mix (NM) or controlled release fertilizer (CRF; supplemented with 4.5% Ca and increased levels of several micronutrients) containing 4-5 biological replicates in each combination. Responses were measured in terms of plant phenotype, nutrient content, and RT-qPCR based differential gene expression (DGE) in 'Valencia' leaves. DGE analyses were conducted on samples collected 13, 25 and 37 weeks after bud-sprouting (WAS) for several nutrient transporters and systemic acquired resistance (SAR) related genes. DGE analysis at 37 WAS revealed that SAR inducing gene(s) and several nutrient transporter genes were upregulated under CRF supply compared to NM supply in 'Valencia'/improved rootstock grafts. 'Valencia' trees grafted onto Swingle and under CRF supply demonstrated lower SAR gene expression - when compared to 'Valencia' grafted onto our improved rootstock. In addition, several nutrient transporter genes were upregulated but at lower levels than that observed on 'Valencia' grafted onto our improved rootstock. The DGE results could be phenotypically correlated with visual appearance of the plants in each graft combination. Leaves of 'Valencia' grafted onto our improved rootstock visually exhibited less HLB symptoms when compared to the 'Valencia'/Swingle grafts under both nutrition treatments. Our results suggest that the newly developed HLB tolerant rootstock can achieve homeostasis for nutrient transport under NM, and can enhance defense and the nutrient transport under CRF supply following HLB infection. We demonstrate that a combination of improved nutrition on the HLB tolerant rootstock can help to mitigate HLB severity by improving plant health and potentially increase production under disease pressure. Further research will validate these results on field trees grown under NM or CRF treatments.

Characterization of the outer membrane protein, OmpA, in *Liberibacter* species and its role as phage receptor

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Liberibacter crescens (*Lcr*) is the closest cultivable species to "*Candidatus* Liberibacter asiaticus*"* (*C*Las). It was isolated from diseased Babaco papaya (*Carica stipulata* x *C. pubescens*) in 1995 in Puerto Rico; however it has not been determined to cause any plant disease. Due to the homology of this non-pathogenic species to *C*Las and as it is the sole cultivable strain belonging to this group, *Lcr* is the best candidate to study the genetics and physiology of *Liberibacter*. Our main goal is to find a phage-based mechanism for engineering this uncultivated pathogen in order to render it more amenable to culture, and to understand the complex interactions participating in the plant-vector-pathogen system. In addition these bacteriophages will be developed as potential anti-*C*Las control agents. Initially, we are using *Lcr* as trap species to isolate *Liberibacter*-infecting bacteriophages. The expression of putative *C*Las phage receptors in *Lcr* will permit a more *C*Las specific interaction and a more pathogen lead search. The essential porin RopA1 from *Sinorhizobium meliloti* is a phage receptor. This protein is homologous to the OmpA protein from *E. coli* which also participates in pathogenesis and bacterial adhesion. Small alterations in the external surface-exposed residues of *S. meliloti* OmpA inhibited the adsorption by certain phages confirming the role of this porin in phage specificity. Engineering the outer membrane protein porin (OmpA) from *Lcr* BT-1 to resemble *C*Las OmpA is expected to facilitate isolation of bacteriophages able to bind to and infect *C*Las. It will also permit characterization of OmpA-dependent phenotypes in *C.* Liberibacter species and other *Rhizobiaceae*.

A novel attract-and-kill device for strengthening the management of Asian citrus psyllid

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Control strategies that exploit the behavior of Asian citrus psyllid will lead to sustainable management practices of this economically important pest. ACP is a diurnal insect that strongly responds to visual cues. We developed an attract-and-kill device (AK) mimicking a citrus flush shoot that is lime-green in color and impregnated with a potent pyrethroid. Our strategy is to lure adult psyllids onto AK devices and to quickly kill individuals that contact these devices. In laboratory bioassays, AK devices induced >95% adult psyllid mortality after 5 to 10 sec exposure time. The AK devices remained potent for up to 12 weeks after exposure

to weathering in citrus tree canopy. In greenhouse studies, a caged-potted citrus tree baited with one AK device resulted in 70% reduction of psyllid populations. Similarly, deployment of 20 to 200 AK devices per residential citrus tree led to 5-76% decrease in psyllid population relative to untreated trees. However, there was no density-dependent response in reduction of psyllid population relative to AK deployment densities. We determined that 20-50 AK devices per tree was the optimal deployment density. We are testing different deployment strategies of these AK devices at the grove and landscape levels to strengthen psyllid area-wide management strategies.

Identification of gut epithelium binding peptides that reduce systemic movement of '*Candidatus*' Liberibacter asiaticus within the Asian citrus psyllid vector

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The Asian citrus psyllid is the only known vector of the bacterium, '*Candidatus*' Liberibacter asiaticus (CLas), that causes huanglongbing or citrus greening disease. This insect acquires CLas from an infected citrus tree while feeding as a nymph. Transmission to uninfected trees occurs when infected adults emerge and fly to, and feed on, uninfected trees. Our current understanding of the CLas-psyllid interaction suggests that adults become competent for transmission only after the bacterium moves from the insect gut into the hemolymph and eventually to the salivary glands. We hypothesize that specific molecular interactions between the bacterium and gut epithelial cell membranes are necessary to initiate the movement of the bacterium throughout the psyllid's body. Furthermore, we hypothesize that if we could block these specific molecular interactions, we may block the bacterium from reaching the psyllid's salivary glands and therefore block the psyllid's ability to transmit this bacterium to healthy trees. We tested these hypotheses by first developing an assay to screen and identify psyllid gut membrane binding peptides from a combinatorial peptide library and then feeding selected peptides to psyllids and determining their ability to reduce movement of CLas from the gut to the salivary glands. A total of 7 peptides were identified that bind psyllid gut preparations that demonstrated gut-binding after oral uptake. These peptides were detected bound to gut epithelial cells in adults that had emerged from nymphs that were fed on the peptides 4 days earlier. Using qPCR assays, we demonstrated that oral delivery of a combination of three of these peptides apparently inhibited movement of the bacterium into the psyllid's salivary glands. These results support our above stated hypotheses and demonstrate a potentially new strategy for preventing the spread of citrus greening disease.

Development of a screening bioassay for in planta evaluation of bactericidal compounds and it use in discovery of new bactericidal compounds active against '*Candidatus*' Liberibacter asiaticus within citrus

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Research on development of solutions to citrus greening has been hampered by the inability to culture '*Candidatus*' Liberibacter asiaticus (CLas), the bacterium associated with this disease. Further complicating the matter, this bacterium resides within the phloem of the plant and targeting it with bactericides requires penetration into the plant so that the bactericide can reach the bacterium. To overcome these obstacles we developed a rapid screen for both bactericidal activity against CLas and phloem mobility within the citrus phloem In this assay, single CLas+ leaves from Valencia are removed from CLas+ infected citrus, indexed for CLas titer by Q-PCR and Q-RT-PCR of petiole clippings. The leaves are then placed in liquid solutions for 6 days, after which, the CLas titer is determined in both the petiole and leaf midrib. Using this assay we have demonstrated that streptomycin and penicillin are active against the CLas bacterium but that streptomycin bactericidal activity moves more readily into the leaf phloem. We have also used this assay to identify two new antimicrobial molecules more active at reducing leaf midrib CLas than the standard antibiotics when compared at the same concentration (2.5 mM). Using both Q-PCR and Q-RT-PCR we demonstrated that reductions in CLas16S rRNA abundance was more pronounced than reductions in the cognate DNA sequence. Therefore analysis of 16S rRNA is a more sensitive analysis of perturbations in CLas status in the plant. This assay allows concomitant analysis of bactericide activity and plant mobility and is currently being used to screen potential antimicrobial molecules for use in fighting citrus greening.

Regulation of gene expression through root application of double-stranded RNA in citrus

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RNA interference (RNAi)**,** mediated by double-stranded RNA (dsRNA) is a gene suppression method that triggers sequence specific mRNA degradation. In citrus, transgenic expression of antisense RNA or hairpin RNA can be used to generate plants with specific genes silenced. However, this technique involves lengthy procedures because of slow growth and long juvenility of citrus trees. Here we set out to develop a quick and convenient method to silence genes in established trees. In one test, commercially synthetized dsRNA was applied weekly to potted Hamlin sweet oranges grown in artificial rooting medium. A 170 base pair dsRNA homologous to citrus *phytoene desaturase* (*CiPDS*) was able to significantly reduce the expression of the target transcript, at a dose of 200 µg per plant. Photo-bleaching and reduced chlorophyll content, from suppression of *CiPDS*, were observed in 1 tree at a dose of 200 µg and 2 trees at a dose of 2 mg per plant. In another test, citrus *Terminal Flower 1* (*CiTFL1*) that negatively regulates flower initiation was targeted in an attempt to induce early flowering. Persian lime plants grown in soil had dsRNA applied as drench weekly, in combination with cold and drought treatment to induce flowering. After 11 treatments, flowers were observed from two individual plants treated with 200 µg dsRNA-*CiTFL1*, but not from the control treatment (water). In summary, initial results indicated root application of dsRNA is capable of triggering gene silencing in citrus. We hypothesize that the magnitude and consistency of the silencing effect can be further improved through optimized application protocols. With appropriate targets identified, we hope that RNAi may be used in citrus plantings to alter physiological/developmental plant responses to improve tolerance to *Candidatus* Liberibacter, or mitigate negative effects caused by infection.

Huanglongbing tolerance-associated genes are identified by comparative transcriptomics using bacterial flagellin 22 as a proxy to challenge citrus

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Plant defense elicited by pathogen-associated molecular patterns (PAMPs) is an important component of disease resistance. Canker resistance in citrus correlates with responsiveness to *Xcc*-flg22, the 22 amino acid active region from the flagellin of *Xanthomonas citri* ssp. *citri*, the causal agent of citrus canker. Using RNA-seq we compared the transcriptome responses in moderately HLB and canker-tolerant 'Sun Chu Sha' mandarin and HLB and canker-susceptible 'Duncan' grapefruit to *Xcc*-flg22 and the flg22 from *Candidatus* Liberibacter asiaticus (*C*Las-flg22), the causal agent of Huanglongbing (HLB). Our results showed that 86 genes were differentially regulated by *C*Las-flg22 only in 'Sun Chu Sha' mandarin but not by *Xcc*-flg22, or in 'Duncan' grapefruit by either flg22. The 16 genes with highest differential expression were selected for RT-qPCR validation, and 10 genes were consistent with the RNA-seq results. To evaluate if these genes were associated with HLB tolerance, 'Cleopatra' mandarin (similar to 'Sun Chu Sha') and 'Duncan' grapefruit plants were inoculated with *C*Las using psyllid infestation. *C*Las titer and gene expression were monitored biweekly for 10 weeks after inoculation. High bacterial titer (C_t<30) was observed at 2 weeks in 'Duncan' but not until 6 weeks in 'Cleopatra'. RT-qPCR results indicated that 5 of the studied genes were differentially

expressed between the 'Cleopatra' HLB-infected and the uninfected control plants, but not in 'Duncan'. It is worth noting that the induction of these genes was detected before bacterial infection was detected. Although not fully annotated in the citrus genomic databases, the function of some of these genes include a peroxidase, gibberellin 2-beta-dioxygenase, glucan endo-1,3-beta-D-glucosidase and an F-box domain containing protein. We will continue to characterize the expression of these genes and their association to HLB tolerance in other citrus genotypes, and determine if they may serve as marker genes for selection of tolerant material.

Chitin induces pathogen-associated molecular pattern-triggered immunity that affects Asian citrus psyllid feeding behavior in 'Sun Chu Sha' mandarin

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Pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) is an important component of the inducible defense system of plants. Citrus species possess varying levels of responsiveness to PAMP flg22 derived from the flagellum of *Xanthomonas citri* ssp. *citri* (*Xcc*-flg22), the bacterial causal agent of citrus canker. Higher sensitivity to *Xcc*-flg22 is mediated by robust expression of the plant cell surface receptor gene FLS2 and confers an increased level of canker resistance. In this study, we explored the induction of PTI by chitin, another PAMP that is a structural component of insect stylets and exoskeleton, and studied its effect on the feeding behavior of Asian citrus psyllid (ACP, *Diaphorina citri*), the vector of Huanglongbing. The results showed that infiltration of chitin solution into 'Sun Chu Sha' mandarin leaves induced expression after 1h of defense genes including *WRKY22*, *GST1*, *SGT1*, *RAR1*, *PAL1*, *NDR1* and *EDS1*, a set of genes previously shown to be induced by *Xcc*-flg22 in the canker-resistant 'Sun Chu Sha', but not in canker-susceptible varieties. However, the chitin-induced gene expression was not detected at 18h after infiltration. ACP feeding behavior was monitored using electrical penetration graph (EPG) to study how induction of PTI by chitin modifies the number and duration of xylem and phloem feeding bouts. When adult ACP started feeding at 1h after infiltration, reduced xylem and phloem feeding were observed in chitin-treated leaves compared with untreated controls. However, this difference was smaller when psyllids started feeding at 18h after infiltration. In summary, our study shows that chitin treatment induced a transient PTI that can affect ACP phloem ingestion, and this inducible defense will be further studied for its utilization on selection and generation of resistant citrus materials.

Molecular characterization of *Candidatus* Liberibacter asiaticus population from Brazil.

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Huanglongbing was first detected in Brazil in 2004, with the presence of *Ca*. L. asiaticus and *Ca*. L. americanus. *Ca*. L. asiaticus (Las) is the predominant species found in affected trees today and HLB has spread unevenly in São Paulo state. Although molecular characterization of Las isolates from Brazil is reported, we aimed a broader analysis in number of samples to cover the population structure of *Ca*. L. asiaticus. Besides, a subset of greenhouse kept isolates, infected with Las, was included in the study. Samples were collected in fifty municipalities, from sweet orange varieties Hamlin, Natal, Pera, Rubi, Valencia, Valencia Americana and Westin, from orchards established from 1985 to 2013, with or without irrigation. Las positive DNA samples were analyzed with markers and for the presence of prophages SC1 and SC2. About three quarter of the population presented the same pattern for the presence of selected genes, while the remaining of the samples had a diverse set of patterns, including some pattern not reported before. The current population structure of Las is being compared with previous data to figure out if variation in time has been observed.

Is area-wide pest management useful against HLB? Evidence from Florida

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Huanglongbing (HLB) currently poses a severe threat to citrus production worldwide. No cure is yet available for growers to deal with the disease. While working on developing short- and long-term treatments, scientists recommend controlling the vector of the disease. In this regard, area-wide pest management has been proposed as a superior alternative to individual pest management. We analyze a unique dataset of farm-level yields in Florida that allowed us to test such hypothesis, and quantify the differential economic benefit in two areas with different implicit level of participation. Our findings provide evidence on the efficiency of a well-performing CHMA to deal with HLB. In addition, we present survey data that provide insights about producers' preferences and opinions regarding area-wide pest management. Despite the relatively high benefit we found CHMAs can provide, the strategic uncertainty involved in relying on neighbors seems to impose too high of a cost for most growers, who end up not coordinating sprays.

The roll of bactericides in huanglongbing management

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The Citrus Research and Development Foundation has implemented field trials to evaluate bactericides and delivery systems for use in citrus against *Candidatus* Liberibacter asiaticus (*C*Las). These trials evaluate changes in the bacterial titer, as well as the manifestation of Huanglongbing (HLB), based on tree measurements and observations. To test materials that are not field-ready, an *in vitro* assay using *Liberibacter crescens* as a surrogate for *C*Las, and a greenhouse assay have been developed. More than one thousand potential bactericides have been tested in the *in vitro* assay, the *in planta* assay has a lower throughput and therefor fewer samples have been tested. The impact of bactericides in HLB management programs has not yet been determined, including bactericides presently in use. CRDF has invested in research on the efficacy and use-pattern of the bactericides oxytetracycline and streptomycin, leading to the present availability for use in citrus. A better understanding of oxytetracycline and streptomycin is needed; to this end, the multi-year research trials funded by CRDF continue and new studies are in place to evaluate the impact on tree health and yield in individual grower programs. Previously, bactericides have not been used to treat bacterial diseases in plant agriculture, and historic research on this disease has not changed this paradigm, but new bactericides, changes in bactericide formulations, more effective adjuvants, and new application technologies may shift this paradigm. The results of assays and field trials and the challenges of bringing new bactericides to market will be discussed in this presentation.

Seasonal and interannual variability in the density of the Asian citrus psyllid (*Diaphorina citri*; ACP) and natural enemies in Southern California

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The Asian citrus psyllid (*Diaphorina citri*; ACP), an invasive citrus pest and vector of the bacterium (*Candidatus* Liberibacter asiaticus) agent of the citrus greening disease, Huanglongbing (HLB). ACP was first detected in Southern California in 2008 and has since spread throughout the region. *Diaphorina citri* is one of several invasive insects being monitored in Temecula Valley, Riverside County, California. The goal of this study was to quantify ACP adult populations and natural enemies (e.g. lady beetles and lacewings) over time in groves of different citrus varieties. Additionally, we assessed the relationship between ACP and natural enemies to determine if predators track ACP dynamics in the field. Currently, there are 134 yellow sticky traps deployed in citrus groves throughout Temecula Valley, which are inspected year round on a biweekly to monthly basis. The mean number of ACP adults per trap was higher in 2016 (18.6 adults/trap) compared to previous years (2014=3.8, 2015=4.6 adults/trap). Seasonal ACP patterns also differed among years, with primarily a Fall peak in 2014, Fall and Spring peaks in 2015, and primarily a Spring peak in 2016. Preliminary analysis of the data also suggest that predator dynamics may follow the seasonal pattern of ACP.

UV reflective polyethylene mulch to protect young trees from Asian citrus psyllid and HLB

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Citrus production has plummeted in Florida by more than 50% in the last 10 years, although many Florida growers still maintain yields in spite of huanglongbing by increasing inputs of insecticides and nutrients. Consequently, focus is shifting to health and profitability of the next generation of trees. Area wide Asian citrus psyllid (ACP) management and new planting systems are needed to maintain tree health and bring new blocks into rapid production. Systemic insecticides applied to the soil supplemented by foliar sprays provide some protection against ACP but have proven insufficient to adequately control HLB infection during critical the first 3 years after planting. Planting into a bed covered with metalized polyethylene film offers an additional layer of protection by warding off incoming ACP adults through flight disruption. Lack of similar effects with white mulch reflecting only visible light indicates that reflected light in the UV range is required. Use of plastic mulch is a novel practice in citrus production requiring delivery of water, nutrients and systemic pesticides by drip irrigation. Therefore, adoption of this practice to commercial production is necessarily a gradual process. Nevertheless, some large scale trials have been conducted on commercial farms and interest in growing as results come in and technology improves. The metalized mulch must be of sufficient thickness and protected with a clear coat to last 3 years. Ground must be well prepared and film mechanically installed as a tight 125-145 cm wide strip, higher in the center to shed water and sprays. Drip irrigation can be provided by one or two relatively inexpensive drip tubes placed under the mulch or punch-in emitters at the base of each tree. Trees are typically planted through 15 cm holes cut in the plastic using a post-hole digger. Oil containing sprays should be avoided to protect the polyethylene from undo degradation. Metalized mulch consistently reduces ACP populations and HLB incidence compared to whiteface mulch or bare ground. In addition, the mulch plus drip irrigation system increases soil moisture, reduces weed pressure, and accelerates growth. Chemigation has proved more efficient than soil drenches for delivering systemic insecticide once the root system is established. Yield after 3.5 years in a large scale replicated experiment was improved by 44% from trees planted on UV reflective mulch and treated with insecticides compared to insecticides alone and brix improved by 5%. Thus, metalized mulch can improve growing conditions and augment current insecticide based ACP control measures for young trees. The potential to more than compensate for additional costs by savings of water, fertilizer and herbicides, shortening time to crop profitability and increasing yields has inspired interest in using this technology to protect young citrus plantings threatened by HLB.

Resistance and tolerance to huanglongbing in citrus

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Huanglongbing (HLB) is severely impacting Florida citrus. The sweet oranges and grapefruit dominating Florida production appear to be among the most HLB-susceptible citrus cultivars. Transition to resistant or tolerant cultivars will be essential to sustain the Florida citrus industry, with solutions needed in the short and long term. Evidence mounts that useful resistance/tolerance to HLB is present in cultivated citrus and greater resistance may be drawn from more distant members of the gene pool, and both approaches are targeted by the USDA citrus breeding program. Potentially useful HLB- tolerance is apparent in several mandarin hybrids. Evaluation of existing cultivars (including sweet orange and grapefruit) was conducted using available nursery trees. After six years of growth following almost immediate infection, ‘SugarBelle/Sour Orange’ and ‘Tango/Kuharske’ had the largest trunk diameters, good canopy density, and were progressing into fairly normal bearing. In a planting of seedlings from 85 diverse gene bank accessions there is a strong association between tolerance and pedigrees including citron. Hybridization is ongoing to combine diverse sources of tolerance. There is evidence of resistance to HLB in several *Citrus* cross-compatible genera. In a study of diverse scions on both unifoliate and trifoliate rootstocks, rootstock had little effect but scions with *Poncirus* in their pedigrees had significantly lower populations of the HLB bacterium. Strong resistance and even immunity should be possible using genetic engineering and USDA efforts are also underway to produce HLB-resistant scions using biotechnology.

Engineering mobile RNA to enhance citrus defense responses 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, and some triggers, such as Reactive Oxygen Species (ROS), can activate or prime plant defenses over long distances. There are also some anti-apoptosis proteins in plants which dampen plant defense responses. By suppressing expression of these anti-apoptosis proteins, PCD should occur in a more rapid manner and with augmented defense responses upon pathogen infection. Huanglongbing (HLB), caused by *Ca.* Liberibacter asiaticus (Las), threatens citrus production worldwide. Las grows strictly inside living citrus cells. Citrus cells detect Las, but fail to trigger adequate native defense systems or PCD until the bacteria have multiplied to very high levels. To help accomplish this defense suppression, Las secretes both a functional peroxiredoxin and a peroxidase directly into the citrus cell cytoplasm that significantly dampen both the ROS response and signaling pathway. We have developed a citrus gene silencing strategy to suppress a brake on the natural citrus defense reaction to Las. Five silencing constructs were used to transform Carrizo rootstocks. Average silencing efficiencies of the transformed rootstocks ranged from 77.30% to 82.35%. Using a Las flagellin protein fragment (flg22) applied at 10 µM as a proxy for Las infection, expression levels of three citrus defense response genes NDR1, PR1 and EDS1 were significantly higher in silenced lines than in NT controls. Challenge inoculations with Las were then performed by approach grafting selected transgenic Carrizo lines representing all 5 silencing constructs to mature Las-infected non-transgenic citrus scions. Eight different grafting events were monitored over 16 months and 7 of 8 Carrizos either never got infected or became Las negative after Las infection. Following release from the Las infected citrus source rootstock, three of the Las infected, NT mature scions, still attached to the transgenic rootstock, became Las negative. These results indicate that silencing signals can move from transgenic rootstock to NT mature citrus scion and enhance the citrus defense reaction sufficiently to cure Las from an infected NT branch.

Soil microbial product interactions with HLB in Valencia/Swingle trees over three seasons at three contrasting sites in Florida

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The purpose of these field trials was to test the hypothesis that soil-applied microbial products can mitigate the effects of HLB on tree health and yield. Five soil amendments: BioFlourish, Ecofriendly, Serenade, Quantum, and Aliette were applied to Valencia/Swingle trees over three seasons (2014-2016) at recommended rates at a Ridge site, an East coast site, and a Southwest Florida site and compared to untreated control trees. A subset of trees within each treatment at each site was also mulched annually with mature cow manure. At the Ridge site with 19-year-old trees, Bioflourish treated trees were the largest whereas Quantum treated and untreated control trees were the smallest. There were no treatment effects on Yield, Fruit quality, visual Disease Index score (DI) or HLB status (as measured by PCR CT values). At the East coast site with 6-year-old trees, all treatments had a lower DI score (looked better) than the untreated control trees. There were no treatment effects however, on tree canopy size, yield, fruit quality or HLB status. At the SW Florida site with 11-year-old trees which also had the lowest overall HLB status of the three sites, Bioflourish, Alliette, Quantum and Ecofriendly treated trees were significantly larger and had greater yield than the Serenade and Untreated control trees. Quantum treated trees had the lowest DI score but there was no effect on HLB status. Overall, root density was not affected by treatments. The mulch treatment had very little effect on the measured variables and treatment effects on leaf mineral nutrition were not remarkable. Thus, there were no meaningful effects of these soil microbial amendments on tree health and yield of HLB affected trees.

Canopy and root response of HLB-affected citrus trees to steam-generated thermotherapy

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No effective control measures have been identified for citrus huanglongbing (HLB), which has threatened the citrus industry of Florida for a decade. The phloem-limited bacterium, *Candidatus* Liberibacter asiaticus (*Las*), is the causal agent of HLB and infects both the canopy and root system. Blotchy mottle on leaves, small and lop-sided fruit, and premature fruit drop are the major symptoms of HLB. Based on the hypothesis that heat treatment (thermotherapy) can reduce *Las* titer in the infected trees, the responses of canopy and root system are being studied, along with the movement of bacteria within the tree, post-treatment. This study explores steam as a heat source for rapid treatment to extend the productivity of HLB-affected citrus trees in the field. Optimum temperature-time duration is important to determine the heat needed to reduce *Las* titer while minimizing the effect on tree productivity, but this may not be consistent at all times of year. A split-plot randomized complete block design field trial was laid out with the main plots at 3-month intervals to determine optimum time-of-year to reduce *Las* titer and the subplots at 6 temperature-time combinations (55°C/0s; 55°C/60s; 55°C/90s; 55°C/120s; 60°C/30s; and untreated control, UTC) with 3 blocks per time of year. Four leaves/tree from 10 trees/treatment were collected every month and tested for *Las* titer via qPCR. Roots were assessed for *Las* titer on the same trees as roots are an untreated reservoir. Results from the summer showed an overall reduction of *Las* titer in the root and canopy for all treatments when compared to the UTC. There was significant difference *($P \leq 0.0001$)* among the treatments for the root samples but not the leaves. Preliminary data from the winter treatment showed significantly lower ($P \leq 0.0001$) *Las* titer in the canopy than UTC after 2-months post treatment, for all the temperature-time combinations. In the roots, *Las* titer decreased significantly ($P \leq 0.0001$) for 55°C/0s, 55°C/90s and 60°C/30s, after 3 months of treatment compared to the UTC, suggesting *Las* movement from root to shoot along with the stored carbohydrates. The spring treatment resulted in a decrease in bacterial titer one-month post-treatment, but the titer in leaves increased in the second month. The 60°C/30s treatment significantly reduced ($P \leq 0.001$) *Las* titer compared to other treatments two months after thermotherapy. For the roots, all the treatments, except 55°C/0s, showed significantly lower ($P \leq 0.0001$) *Las* titers in the 1st and 2nd months, post-treatment. For each season, there was a significant ($P \leq 0.001$) interaction between the treatments and time after thermotherapy for the roots. Variations in *Las* titer within UTC were observed, regardless the season which indicates an influence of natural physiological and environmental factors in bacterial movement in a tree. Seasonality and physiological state of the tree seem to play critical role for *Las* distribution and movement within a particular tree. Physical damage to the canopy from heat stress may alter phloem flow and bacterial movement between roots and the treated canopy.

Genome sequence and genetic diversity of the huanglongbing pathogen *Cadidatus* Liberibacter asiaticus

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Huanglongbing (HLB) is one of the most destructive citrus diseases and has caused tremendous damage to the citrus industry worldwide. It is caused by the Gram-negative bacterial pathogen *Candidatus* Liberibacter asiaticus (CLas). In the United States, HLB has been detected in Florida, Texas, and California. CLas is psyllid transmitted and resides in the phloem. In order to gain greater insight into CLas biology and genetic diversity, we have initiated genome sequencing and comparative analyses of CLas from different geographical regions. CLas strains from Texas, Florida, California, and China have been sequenced. CLas contains the Sec secretion system, through which a variety of sec delivered effectors (SDEs) may be secreted into the phloem. We have mined genome sequences to identify core SDEs. Data will be presented on the identity and conservation of the CLas effector repertoire as well as phylogenetic relationships between different strains. Genomic analyses of CLas strains from different geographical regions will facilitate a greater understanding of CLas population structures, virulence components, and will aid in the development of tools for molecular diagnosis.

Residual impact of field-weathered insecticides on Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae) nymphs

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The effective management of the Asian citrus psyllid, *Diaphorna citri*, currently relies on insecticides as the primary method to suppress ACP/HLB in commercial California citrus groves. Most insecticides kill ACP if they directly contact the insect. However, nymphs are often tucked inside curled leaves, which limits insecticide contact. Therefore, insecticide residual activity is an important element of ACP control. This study estimated the residual efficacy of nineteen different insecticides against *D. citri* nymphs placed on field treated and field-weathered leaves. Thiamethoxam and the mixtures imidacloprid+beta-cyfluthrin, chlorantraniliprole +thiamethoxam, abamectin + thiamethoxam were found to have longer residual control (45-54 days of greater than 50% mortality) compared to cyfluthrin, fenpropathrin, beta-cyfluthrin, zeta cypermethrin and spinetoram, flupyradifurone, spirotetramat, abamectin, cyantraniliprole, diflubenzuron, fenpyroximate, and abamectin + yantraniliprole. The pyrethroid insecticides tested had no statistically detectable activity 25 days after application. Of the organic insecticides, spinosad + oil showed greater residual control of *D. citri* compared to pyrethrins + oil, but the effects of organic insecticides were short lived; by ten days post treatment there was no statistically detectable residual control of nymphs.

Effect of four strobirulin-based fungicides and salicylic acid on the vascular system plugging and bacterial titer of citrus trees infected with huanglongbing (HLB) in Jamaica

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The axial parenchyma of the phloem of plants is the tissue composed of living cells that communicate with the sieve elements and companion cells via plasmodesmata allowing short distance transport of starch. Anatomical studies on HLB found massive accumulations of starch in leaf samples that plugged the vascular system of infected plants. We studied the effect of different treatments on the vascular system plugging and bacterial titer of citrus trees infected with huanglongbing (HLB) at Block 54 of New Works Farm of Trade Winds Citrus in Jamaica. The trees were Valencia/Swingle planted on 2000 infected with HLB for over 8 years and treated with different strobirulin-based fungicides, salicylic acid and a carboxamid/strobirulin fungicide (six treatments with four repetitions of ten trees distributed completely randomized within the grove) on the three main flushing events between 2014 and 2015. Six flushes of each of the 10 trees of each repetition were marked after each application. Two of them were sampled three months after the application, other two six months after and the remaining two nine months after. For the Vascular Plugging evaluation, less than 1 mm thick longitudinal petiole cuts from the two older leafs of every flush were dyed with a 2% iodine solution and placed in the microscope to measure the % of axial parenchyma plugged inside a determine visual field. For the qPCR analysis, we sampled the next four leaves of each flush together with tissue of the other flush in that same tree, getting one consolidated sample per tree. Results evidenced no correlation between bacterial titer and vascular plugging. On the other hand, we were able to observe significant reductions of the vascular plugging as an effect of the treatments that was related to the specific treatment, number of applications and flush age. Based on these results we concluded that foliar applications of one Strobirulin-based fungicide and Salicylic acid reduce the undesired impact of HLB on the vascular system of highly infected citrus trees and could become a necessary tool to overcome the deleterious effects of the disease.

In-field thermotherapy for combating citrus greening (huanglongbing)

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Citrus greening disease (Huanglongbing, HLB) is a destructive bacterial infection of citrus which clogs vascular tissues and results in the death of the tree. Lacking any clear biological cure, citrus growers have begun to explore the use of thermotherapy as a method of impeding the spread and diminishing the effects of citrus greening in commercial groves. Thermotherapy, also called heat treatment, is the use of heat to eliminate disease from plants and produce and has been used at smaller scales for pathogen elimination since the early 20th century. The goal of the thermotherapy process for treating HLB is to heat a citrus tree to a temperature at which the citrus greening bacteria is killed, but the tree itself is left unharmed. However, the practical and technological challenges of using thermotherapy on mature citrus trees are unique and unperfected, especially considering that to-date, only a handful of commercial-scale citrus thermotherapy machines exist, all of which are located in Florida. Some of the problems associated to applications of thermotherapy are engineering-related and others are related to our lack of understanding of how exactly thermotherapy works. An example of an engineering problem is how to uniformly distribute and maintain the right amount of temperature within tree canopies of different shapes and different leaf densities. An example of a scientific unknown is what is the best time of year to apply thermotherapy to the trees. Using practical experiences from researchers and citrus growers in Florida, current thermotherapy methods and technologies are detailed and initial lessons learned from application of thermotherapy to over 8,000 trees in Central Florida are discussed.

Genetic solutions for biological control of disease spread by insects – application to huanglongbing disease of citrus

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For the past 4 years the Citrus Research and Development Foundation has led a diverse group of scientists in an ambitious effort to create an option to replace existing psyllid populations with a population of insects incapable of spreading HLB disease. "*Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease of Citrus"* is the first nationally coordinated program grant from the National Institutes of Food and Agriculture with this type of technology mission. The origin of this team proposal, accomplishments to date and future outlook for the nuPsyllid project will be reviewed.

Mineral nutrient uptake in huanglongbing-affected sweet orange plants

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Florida Citrus industry is struggling due to the endemic of Huanglongbing (HLB). The current estimated citrus production is 70 million boxes for 2016-2017, which is approximately 70% lowers than citrus production in 2002-2003 (pre-HLB conditions). In past few years several reports of use of nutritional products to extend the vigor and productivity of the HLB-affected trees by commercial citrus growers have been documented. Anecdotal evidences from grower's trials suggest positive effect of such application on tree health and yield. Additionally, scientific reports published on root health of HLB-affected plants suggest that HLB-affected plants have reduced, diminished, and weak root system. Therefore, a constant, enhanced, and balanced supply of mineral nutrients has become a standard grove management practice. Hence, to develop a better understanding of mineral nutrition physiology and nutrient uptake in HLB-affected plants this study was conducted. The overall goal of this study was to investigate the mineral nutrient uptake in HLB-affected plants. This study was conducted in a green-house, healthy and HLB-affected plants were grown in hydroponic system in Hoagland solution. Our results indicate that HLB-affected plants had approximately 25% and 40 % less root and shoot system, respectively. The overall nutrient uptake was slower in HLB-affected plants as compared healthy plants. A slight increase in pH of growing media was observed in HLB-affected plants as compared to healthy plants. When nutrient uptake was corrected per gram of root tissue we found that nutrient uptake efficiency for all the nutrients except for nitrogen and manganese for HLB-affected plants was lower than healthy plants. Root anatomical study and gene expression analysis is still underway. Our results suggest that HLB infection alters the nutrient uptake physiology. HLB-affected plants show qualitative changes in mineral nutrient uptake in addition to quantitative changes. Therefore, fertilization program for HLB-affected plants should be carefully designed.

Development of disease resistant/tolerant citrus varieties using CRISPR technology

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Citrus Huanglongbing (HLB) is currently the most destructive disease on citrus worldwide. The causal agent for HLB in USA is *Candidatus* Liberibacter asiaticus (Las), a phloem-limited fastidious α-proteobacterium, which is transmitted by Asian citrus psyllids (ACP, *Diaphorina citri*). Interestingly, Las contains a complete General Secretory Pathway (GSP/Sec-translocon), which can be a potent system for the transportation of Las proteins including the virulence factors into the extracytoplasmic milieu. In this study we characterize the Sec-translocon dependent, signal peptide containing extracytoplasmic proteins of Las. A total of 150 proteins of Las_psy62 strain were predicted to contain signal peptides targeting them out of the cell cytoplasm via the Sec-translocon. For experimental validation of the predicted extracytoplasmic proteins, *Escherichia coli* based alkaline phosphatase (PhoA) gene fusion assays were conducted. A total of 89 out of the 150 predicted Las proteins were experimentally validated to contain signal peptides. We hypothesized that some of the Sec-dependent extracytoplasmic proteins are putative effectors. Those putative effectors were transgenically expressed in citrus to identify their putative targets for HLB development. The targets of the putative effectors have been identified using yeast two hybrid, pull-down, and Bimolecular fluorescence complementation assays. To develop correspondent control strategy, we have adapted Cas9/sgRNA to modify the citrus genome to modify effector targets. We aim to generate HLB resistant or tolerant plants by disrupting the interaction between Sec-dependent effectors (SDEs) and their targets in citrus.

Combined UPLC-MS/MS and MALDI-MSI analyses identify metabolic differences between *C*Lso-infected and uninfected psyllids

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The potato/tomato psyllid, *Bactericera cockerelli*, is the vector of the bacterium *Candidatus* Liberibacter solanacearum (*C*Lso), also known as *Ca.* L. psyllaurous, which causes zebra chip and leaf yellowing diseases in solanaceous crops. In order to find potential metabolic markers for quickly tracking the presence of *C*Lso in *B. cockerelli*, we applied both nontargeted ultra-performance liquid chromatography tandem mass spectrometry (UPLC MS/MS) and matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) to samples of infected and uninfected psyllids. In the UPLC-MS/MS analysis, 963 metabolites were determined to have differential abundance levels between the comparison groups, including 491 being up regulated and 475 being down regulated in the infected psyllids relative to the uninfected control. MALDI-IMS was used to investigate spatial differences in metabolite accumulation. Several highly abundant metabolites were found to have obviously different expression patterns in infected relative to control psyllids. For example, a compound with *m/z* = 756.553 (determined to be a phosphatidyl choline) was highly expressed in the abdomen of infected psyllids, but was down regulated in uninfected insects. These results suggest that infection by *C*Lso imparts significant metabolic changes to the insect, which may provide clues regarding host-pathogen interaction and infection mechanisms.

Selection, molecular and genetic analysis of HLB-tolerant/resistant variants: Re-sequencing of HLB-tolerant and susceptible *Citrus* plants

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Because of the extensive spread and a lack of control measures for Citrus Huanglongbing (HLB), Florida's citrus industry has lost billions of dollars and is fighting for its survival. Fortunately, some tolerance/resistance to HLB within citrus and citrus relatives has been observed in Florida citrus groves, providing evidence that there are variations regarding HLB susceptibility within the citrus gene pool. Working with citrus growers, we have begun the process of confirming and characterizing the HLB tolerance/resistance of some volunteer seedlings that showed apparent HLB tolerance/resistance in preliminary screening. We recently re-sequenced one of HLB-resistant Duncan tree (GM6) and one HLB-susceptible Duncan tree as our control. We mapped illumina reads to *C. clementina* reference genome (v.1.0) using Burrows-Wheeler Aligner (BWA) v0.7.12 software and identified sequence variants using SAMtools. SNPEff annotation showed that most of the variants are SNPs. The numbers of high/moderate impact variants are 677 and 2175 in GM6 and the control (Duncon), respectively. The numbers of genes carrying high/moderate variants are 88 and 142 in GM6 and the control tree, respectively. Among them, 73 genes are common in both the GM6 and control tree. Only 15 genes with high/moderate impact variants are specific to GM6. This preliminary data showed that the number of variants that lead to HLB-resistance may be limited and the resequencing of the HLB-resistant citrus trees may allow us to identify the genes related to HLB tolerance/resistance or susceptibility.

Adoption and validation of Ribonucleotide Reductase (RNR)-based real-time assays for detection of HLB '*Candidatus* Liberibacter asiaticus' (*C*Las)

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Huanglongbing (HLB), aka Citrus Greening, is a well-known destructive disease that threatens the multi-billion dollar citrus industry in the United States and citrus production in other countries around the world. HLB '*Candidatus* Liberibacter asiaticus' (*C*Las) of Asian origin is the only species currently found in the U.S., and sensitive methods for detection are needed to help prevent the spread of the disease. Partial sequence of the *ribonucleotide reductase* (RNR) gene is conserved and present in five-copies per *C*Las genome, presumptively making it a more sensitive target for detection than the 3-copy 16S sequence used in the HLBaspr assay. We recently adopted a RNR-based real-time PCR assay and validated the ability to detect HLB *C*Las by testing a total of 223 diverse citrus samples. The RNR assay showed improvement in analytical sensitivity and specificity over the 16S HLBaspr method. The testing results for both the 56 HLB *C*Las positive isolates and 167 HLB *C*Las negative samples were 100% match with the HLB determinations made by APHIS HLB confirmatory methods (i.e. *CLas* 16S sequencing, Heat Shock Protein (HSP) and Chaperonin (CPR) based real-time PCRs). Serial dilutions of 4 HLB *C*Las positive plant DNA samples from CA, TX, FL and India were tested to compare efficiencies and limits of detection, and RNR Ct values dropped around 1 to 2.5 in a side-by-side comparison with the 16S HLBaspr method. The data suggests that the RNR based real-time PCR could be a more sensitive and specific method for detection of HLB *C*Las than the 16S HLBaspr assay and warrants further studies. Converting this assay into isothermal technology for potential detection of field samples is also being further investigated.

An integrated approach to understand host response and create new citrus cultivars that defy HLB disease

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Commercial citrus varieties have little resistance to Huanglongbing (HLB), a disease presumably caused by *Candidatus* Liberibacter asiaticus (*C*Las). As HLB has become widespread in Florida over ten years, we have very carefully followed the extensive collection of existing hybrid families, somaclonal variants, induced mutants, and germplasm accessions within the UF-CREC citrus breeding program. We have observed very substantial differences in the speed with which over 8000 individuals become affected by HLB, as well as the severity of symptoms in selected six different field trails. In total 343 accessions have been identified that showed a very healthy appearance. Transcriptome profiling using RNA-seq data collected from *C*Las- and mock-inoculated tolerant and susceptible cultivars reveals critical involvement of reprogramming of signaling pathway in early response to *C*Las. Time series analysis of transcriptomes reveals the strong quantitative nature of defense signaling in tolerant varieties. In addition, a comparative proteomic analysis of different rootstocks affecting fruit quality shows that there are significant changes in several metabolic and signaling pathways, as well as vesicle trafficking. A mandarin breeding population (n=192) was genotyped using Axion citrus 56K SNP Array. The linkage disequilibrium and utility of genomic selection for breeding HLB tolerant cultivars were investigated. Overall, our studies indicated that HLB tolerance is complex trait, and could be exploited by using an advanced breeding tools such as genomic selection.

Mitigating citrus huanglongbing of new plantings in Florida with integrated management

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Currently, the Florida 9 billion dollar citrus industry is fighting for its survival due to severe citrus HLB epidemics. Because HLB is a systemic disease, effective elimination of Las bacteria from the entire citrus tree is essential for curing the disease. To aim this goal, we carried out an integrated management of chemotherapy, thermotherapy and extra-nutrition in three field trials using a randomized split-plot experimental design. Ten effective antimicrobials selected from our graft assay and in greenhouse test were applied to 16 tree-replicates as the major-block in each location (grove). Nutritional and heat treatments were applied to 92 trees in each grove as sub-split plot. The thermotherapy was performed by steam treatment at 125~128 °F for 180 seconds. Trees were 3-4 years old with 4-8 feet heights. Following to the Field Trial Tree Evaluation Methods developed by CRDF, we investigated tree canopy, tree health, fruit drop and fruit quality and Las bacterial titers by real-time PCR. The eighteen-month results showed that penicillin, aliette, carvacol and silver products (silver phosphite and silver nitrate) significantly reduced Las titers and improved tree health compared to untreated controls. However, heat-treatment and extra-nutrition were not effective to eliminate the Las bacterium, but to promote the citrus growth or vigor. Based on the results of tree disease severity, the treated trees fruited better and looked more healthy after one-year of treatment, especially in the most severe grove B with tree disease severity (DS) reduced from 3.19 (May, 2015) to 2.13 (May, 2016). As a general observation, trees in the trial groves that were visually less affected by HLB at the initiation of the trial responded better than those in poorer health. The integrated practices (antimicrobial treatment coupled with heat treatment and nutrition fertilization) decreased the fruit drop by 10~20 %, and increased the fruit and juice weight by 3~13 %, while decreasing the ratio of brix to acid by 0.2~5.0 %. Pen and PCY increased the Brix when coupled with additional nutrition. Based on these results, it is concluded that chemotherapy coupled with additional nutrition should be helpful in mitigating HLB in Florida grapefruit production and hopefully other citrus.

Antimicrobial effect of non-antibiotic compounds against '*Candidatus* Liberibacter asiaticus' in HLB-affected citrus plants

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Citrus huanglongbing (HLB), caused by three species of fastidious, phloem-limited '*Candidatus* Liberibacter', is one of the most destructive citrus diseases worldwide. As a potential and urgent control strategy for citrus HLB, 46 antimicrobials (non-antibiotics) were screened for

effectiveness and phytotoxicity using the optimized graft-based screening system with '*Candidatus* Liberibacter asiaticus' (Las) - infected citrus scions, whereas ampicillin (antibiotics) was used as positive control and water as negative control. Results of principal component (PCA) and hierarchical clustering analyses (HCA) demonstrated that 46 antimicrobials were clustered into 3 groups: effective, partly effective, and not effective. In spite of different modes of actions, 15 antimicrobials, such as Alliette, Carvacrol and P-Cymene, were all effective in eliminating or suppressing Las with both the lowest Las infection rate and titers of the treated scions and inoculated rootstock. The non-effective group contained 14 compounds and water control, such as 2-amino-5-chlorobenzoxazole, DL-2-aminobutyric acid, and did not eliminate or suppress Las in the tested concentrations, resulting in plants with increased titers of Las. The other 11 antimicrobials, including berberine chloride, benzyl isothiocyanate, partly eliminated or suppressed Las in the treated and graft-inoculated plants. The effective antimicrobials identified in this study are potential candidates for control of citrus HLB, either for the rescue of infected citrus germplasm or for restricted field application.

Editing *DMR6* orthologs in citrus via a CRISPR/Cas9 system

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Plant immune responses typically involve the activation of inducible defenses and are usually under tight control of negative regulators. *Arabidopsis* DMR6 (down mildew resistance 6) is a 2OG Fe (II)-dependent oxygenase that acts as a suppressor of plant immunity and its gene expression is upregulated during pathogen infection. Mutation of *Arabidopsis DMR6* and its orthologs in potato and tomato results in elevated salicylic acid levels and conferred broad-spectrum disease resistance. Huanglongbing (HLB) is a devastating disease in citrus worldwide with no highly effective control methods identified. Previously, we found that *Arabidopsis DMR6*-like genes were downregulated in more tolerant 'Jackson' grapefruit (*Citrus paradisi* hybrid) compared with those of the susceptible 'Marsh' grapefruit (*Citrus paradisi*). Here, we conducted a gene expression survey of the *Arabidopsis DMR6* orthologs in 'Hamlin' sweet orange (*Citrus sinensis*), 'Clementine' mandarin (*Citrus clementina*), 'Carrizo' citrange (*Citrus sinensis* × *Poncirus trifoliata*.), rough lemon (*Citrus jambhiri*), sour orange (*Citrus* × *aurantium*) and citron (*Citrus medica*). The expression levels of citrus *DMR6* genes (Locus IDs: orange1.1g019665m.g and Ciclev10005149m.g) were significantly higher in all Las-infected trees compared with healthy trees in each citrus genotype. A CRISPR/Cas9 system was used to target this gene in 'Hamlin' and 'Carrizo'. Four single guide RNAs (sgRNAs) were designed based on the coding sequences of citrus *DMR6* genes with 100% identity between 'Hamlin', 'Carrizo' and 'Clementine' mandarin. Three sgRNAs were verified as effective in cleavage of DNA templates *in vitro* and were used for developing CRISPR constructs. Agrobacterium-mediated transformation of 'Hamlin' and 'Carrizo' using these constructs is underway. Our goal is to generate *DMR6* mutated citrus and determine resistance of transgenic plants against HLB.

Investigation of Hexanoic acid as an inducer of huanglongbing resistance

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Huanglongbing (HLB), caused by *Candidatus* Liberibacter asiaticus (CLas), is arguably the most damaging disease on citrus and threatens citrus production worldwide. Plant defense inducers including β-aminobutyric acid (BABA), 2,1,3-benzothiadiazole (BTH), and 2,6-dichloroisonicotinic acid (INA) provided a modest reduction of HLB progression in the field. Hexanoic acid (HX) is a monocarboxylic acid that can induce early broad-spectrum defenses in *Arabidopsis* and tomato by inducing callose deposition and activing salicylic acid and jasmonic acid pathways. Moreover, HX was shown to enhance citrus immunity against citrus brown spot and citrus canker and also exhibited antimicrobial activity on the causal agents, *Alternaria alternata* and *Xanthomonas citri* subsp.*citri*, respectively (Llorens et al., 2013; Llorens et al., 2015). In this work, we used Liberibacter crescens (Lcr) as a CLas-proxy to test the bacteriostatic effect of HX. Significant growth inhibition (94.2%) of Lcr was observed at a concentration of 3.2 mM (highest concentration tested). HX was applied as a foliar spray (2.5 mM) on 12 month-old 'Hamlin' seedlings one day before challenging the plant with CLas infected Asian citrus psyllids (ACP). Electrical penetration graph analysis revealed that the feeding behaviors of ACP on HX-treated plants were not affected. ACP inoculated trees tested CLas-positive at 10 weeks after inoculation but no significant differences in CLas titers were observed between HX-treated and control plants at this stage. Continuing HX treatment and monitoring of plant CLas titers are underway.

Huanglongbing impairs the rhizosphere to rhizoplane enrichment process of the citrus root-associated microbiome

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Roots are the primary site for plant-microbe interactions. Among the three root-associated layers (i.e., rhizosphere, rhizoplane and endosphere), rhizoplane is a key component serving as a critical gating role that controls microbial entry into plant roots. The microbial communities colonizing the three layers are believed to be gradually enriched from the bulk soil inoculum; however, how this enrichment process, particularly the rhizosphere to rhizoplane step, is affected by biotic stresses, such as disease, remains elusive. Here, we address this question using the citrus root-associated microbiome as a model. We identified the rhizosphere to rhizoplane-enriched taxonomic and functional properties of the citrus root-associated microbiome and determined how they were affected by Huanglongbing (HLB), a severe systemic disease caused by *Candidatus* Liberibacter asiaticus, using metagenomic and metatranscriptomic approaches. Multiple rhizoplane-enriched genera were identified, with *Bradyrhizobium* and *Burkholderia* being the most dominant. Plant-derived carbon source is an important driving force for the enrichment process. The enrichment of functional attributes, such as motility, chemotaxis, secretion systems and lipopolysaccharides (LPS) synthesis, demonstrated more active microbe-plant interactions on the rhizoplane compared with rhizosphere. We found that HLB impaired the rhizosphere to rhizoplane enrichment process of the citrus root-associated microbiome in three ways: 1) decreasing the relative abundance of most of the rhizoplane enriched genera; 2) reducing the relative abundance and/or expression activity of the functional attributes involved in microbe-plant interactions; and 3) recruiting more functional features involved in autotrophic life cycle adoption, such as carbon fixation and nitrogen nitrification in the HLB rhizoplane microbiome. Finally, our data showed that inoculation of *Burkholderia* strains isolated from healthy citrus root-associated microbiome could trigger the expression of genes involved in induced systemic resistance of the inoculated plants.

RNA-sequencing analysis of the abscission-related transcriptome in the citrus calyx abscission zone of huanglongbing-affected sweet orange

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Citrus greening or huanglongbing (HLB) disease is associated with an increase in pre-harvest fruit drop, for which the molecular mechanisms remain unknown. Recent studies have implicated a secondary infection by fungus *Lasiodiplodia theobromae* in enhancing fruit drop from HLB-affected trees. In order to understand the molecular basis of the HLB-associated fruit abscission, transcriptomes in citrus calyx abscission zones were analyzed and compared among healthy fruit (H) from healthy (*C*Las negative) trees, and fruit dropped (D) or retained (R) from HLB-affected (*C*Las positive) trees upon shaking the trees, by means of RNA-Sequencing analysis (RNA-Seq). Results showed up-regulated biotic stress, lipid and secondary metabolism, whereas genes related to carbohydrate metabolism and cell wall were down-regulated. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of differentially expressed genes (DEGs) indicated that the most significant GO term was "response to chitin" (p= 9.70E-09), while "response to jasmonic acid" and

"phenylpropanoid biosynthesis" were also significant. Among KEGG pathways, "alpha-linolenic acid metabolism" was the most significant ($p < 0.005$), which produces jasmonates (JA). In addition, "plant hormone signal transduction" and "phenylpropanoid biosynthesis" were also significant pathways ($p < 0.05$). Hierarchical clustering analysis indicated genes related to synthesis and signaling of ethylene (ET) and JA were consistently up-regulated, while abscisic acid, auxin, brassinosteroid, cytokinin, and gibberellin were generally down-regulated in D versus R and H fruit. In agreement with the hormone expression profiles, substantial numbers of downstream JA/ET-responsive defense (the hallmark of anti-fungal defense) related genes were up-regulated in D fruit as well. Quantitative RT-PCR confirmed RNA-Seq results for 30 representative genes covering each of the DEG categories. In conclusion, HLB-associated fruit abscission is mediated by JA/ET signaling, likely in response to a secondary fungal infection.

Whole genome sequence analyses revealed that strains of "Candidatus Liberibacter asiaticus" recently found in two California locations were different

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Candidatus Liberibacter asiaticus (CLas), an α-proteobacterium, is associated with citrus Huanglongbing (HLB; yellow shoot disease). In California, CLas was first detected in the residential neighborhoods in Los Angeles County of Hacienda Heights (HH) in 2012 and in San Gabriel (SG) in 2015. Although all infected trees were destroyed in compliance with a state mandate, citrus industry stakeholder concerns about HLB in California remain high. Little is known about the biology of CLas, particularly the California strains, hindering effective HLB management efforts. In this study, next generation sequencing (NGS) technology (Illumina MiSeq) was employed to characterize the HH and SG strains of CLas. Data sets containing >4 billion (Giga) bp of sequence were generated from each sample. Two prophages (P-HHCA1-2 and P-SGCA5-1) were identified by the consensus of mapping the MiSeq reads onto the two known Florida CLas prophage sequences, SC1 and SC2. P-HHCA1-2 was a SC2-like (100% coverage and 97% identity), or Type 2, prophage 38,920 bp in size. P-SGCA5-1 was a SC1-like (94% coverage and 96% identity), or Type 1, prophage 37,487 bp in size spanning two contigs. Phylogenetic analysis revealed that P-HHCA1-2 was part of an Asiatic lineage within the Type 2 prophage group. Similarly, P-SGCA5-1 was part of an Asiatic lineage within Type 1 prophage group. The Asiatic relatedness of both P-HHCA1-2 and P-SGCA5-1 was further supported by the analysis of single nucleotide polymorphisms (SNPs) in the prophage terminase gene that has been established for CLas strain differentiation. The presence of different prophages suggests that the two California CLas strains could have been introduced from different sources. An alternative explanation is that there is a mixed CLas population containing the two types of prophages, and limited sampling in alternate geographic regions may not accurately depict the true CLas diversity. More accurate pathway analysis may be achieved by genomic analysis of multiple strains per geographic region.

Evaluation of *nrdB* for improvement of detection in "*Candidatus* Liberibacter asiaticus"

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"Candidatus Liberibacter asiaticus*"* (CLas), an α-proteobacterium, is associated with citrus Huanglongbing (HLB, yellow shoot disease), is currently threatening citrus production worldwide. Research in CLas biology is challenging because the bacterium cannot be cultivated *in vitro*. Detection of CLas is a critical step in HLB diagnosis. In the current standard procedure, CLas is detected by PCR based on unique sequence characters in the bacterial 16S rRNA gene. 16S rRNA gene is shared by all bacteria and used for bacterial classification. Yet, the high similarity of 16S rRNA genes among bacteria could also cause false positive results in PCR detection of CLas, particularly at the presence of CLas related bacteria and when CLas is in low titer. We recently identified a unique CLas gene *nrdB*, encoding β-subunit of ribonucleotide reductase (RNR), a critical enzyme involving bacterial proliferation. CLas has five copies of *nrdB*, contrasting to the three copies of 16S rRNA gene. Therefore, PCR using *nrdB*-based primer set was three times more sensitive than the 16S rRNA gene-based primer set. Phylogenetic analyses based on *nrdB* nucleotide and amino acid sequences showed a distinct monophyletic lineage of CLas in eubacteria, similar to that based on 16S rRNA gene sequences. However, significantly more sequence variations or single nucleotide polymorphisms (SNPs) were found in *nrdB* sequences between CLas and the closely related species, "*Candidatus* Liberibacter africanus", another HLB associated bacterium found in South Africa, than in 16S rRNA gene sequences. This indicates that *nrdB* sequence is more discriminative and more specific than 16S rRNA gene. The high discriminative, along with high sensitive capacity, of *nrdB*-based PCR provide a new and probably better choice for application in early detection of CLas.