Session 1:
International and National Citrus Industries, Regulation, and Grower Experiences

1.1

Preliminary Evaluation of the Single-Tree, Huanglongbing Find in California

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Huanglongbing (HLB, citrus greening) associated with '*Candidatus* Liberibacter' species is a widespread devastating citrus disease not previously reported in California (CA). In March 2012, '*C.* Liberibacter asiaticus' (CLas) was detected from an Asian citrus psyllid (ACP, *Diaphorina citri*) sample from Los Angeles, CA at the Jerry Dimitman Laboratory of the Citrus Research Board. Subsequent citrus plant surveys within a 400m area of the CLas-positive ACP sample performed by the California Department of Food and Agriculture identified an infected multi-grafted citrus tree at a residence in Los Angeles, CA. The CLas-positive tree was removed and nucleic acids from different plant tissues (i.e. roots, trunk, stems, and leaves) were extracted and distributed to several federal, state, and university laboratories nationwide for preliminary evaluation. Labs attempted to identify the species and graft types of the infected citrus, study the genetic characteristics and genome diversity of the detected bacterium, as well as test for other graft-transmissible citrus pathogens (GTCP). Preliminary data suggested that one type of lemon was the original rootstock that received over 20 citrus grafts. CLas DNA population analysis suggested a possible single Asian origin. Preliminary tests indicated the possible presence of other GTCP. Further evaluations on the CA CLas-positive find are ongoing.

1.2

Response of Government and the Citrus Industry to the Discovery of Asian Citrus Psyllid in Arizona

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In October, 2009, about three months after the first find of Asian Citrus Psyllid (ACP) in San Luis Rio Colorado Sonora, a colony of ACP was found just across the border in San Luis Arizona. Since then, twelve additional sites have been found in Arizona, all except two in Yuma County. Less than 50 individual ACP have been found since 2009 and all have been eradicated. No ACP found in Arizona has yet tested positive for HLB. As of now, much of southwest Arizona is under federal quarantine for ACP and trees and fruit that move out of the quarantine area require special treatment. The response of the Arizona Department of Agriculture to the discovery of ACP has been to increase trapping and eradication activities using funds received from the Federal and State Government. The University of Arizona and the citrus industry have responded by establishing screenhouses to produce trees that can provide disease-free budwood. The industry has also developed a plan to establish an area-wide spray program if eradication efforts are not successful. Extension and outreach efforts have been directed toward the industry and the homeowner. The location of ACP finds in Arizona suggests that both Mexican ACP populations near the border and the transport of citrus fruit from the interior of Mexico lead to the establishment of the insect in Arizona. The small numbers of ACP found in Arizona, in contrast with populations found in coastal California, suggest that ACP populations may be adversely affected by the arid climate of the region, and that timely detection and eradication efforts are the keys to controlling spread of the ACP in arid regions.

1.3

First Detection of Huanglongbing and Implementation of its Mitigation Efforts in Texas

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After six years of intensive statewide surveys, Huanglongbing (HLB) was first detected in two adjacent commercial groves in Texas in 2012, and *Candidatus* Liberibacter asiaticus (CLas) was found associated with the disease. Assessment of affected trees within the two infected groves was made by visual inspection of foliage and PCR-testing of symptomatic tissue. HLB-infected trees exhibited an aggregated distribution and a strong perimeter effect in the two groves. While in one grove (sweet orange), more qPCR positive trees were observed in the south-eastern side of the grove, in the other (grapefruit) more HLB-infected were found on the western border, adjacent to the sweet orange grove suggesting movement of infected psyllids between the two groves. The Asian psyllid vector, *Diaphorina citri* Kuwayama, known vector of CLas, reported for the first time in 2001, is widespread in Texas. In an effort to reduce the risk of HLB in Texas, a proactive area-wide psyllid control program was initiated in 2010 which has contributed to significant reduction of psyllid populations in Texas. The detection of HLB has led to an intensification of psyllid control measures in groves within a mile radius of the HLB-positive sites, both in commercial and residential settings. All known infected trees have been removed and a five mile-radius quarantine has been put in place to regulate movement of all Rutaceae plants and to ensure safe harvesting of citrus.

Setamou, M., J. da Graca and R. Prewett. 2012. HLB in Texas: Steps and challenges to curb this threat. *Citrograph* 3(5): 32-38.

1.4

HLB in Argentina: a New Disease Outbreak

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Huanglongbing (HLB) caused by Candidatus Liberibacter asiaticus was reported in 2004 in São Paulo, Brazil and three years later in the southern state of Paraná, 300 km away from Argentina's Northeastern border. In 2009 the Argentine citrus industry (AFINOA and CECNEA) and the institutions MAGyP, SENASA, INTA, INASE, and Obispo Colombres set up a task force to develop quarantine guidelines for prevention of introduction of HLB into Argentine citrus areas. This program is based on measures including 1) border inspections, 2) citrus nursery certification, 3) control on the production, transit and trade of citrus fruit, 4) field survey and diagnosis for the early detection of the disease in trees or the vector *Diaphorina citri* in citrus groves, 5) development of research and technology capacity, and 6) communication about the quarantine program and the disease. Diagnostic laboratories were set up in each citrus region and more than 100 inspectors were deployed in different citrus areas for 1) survey of *Diaphorina citri* by yellow sticky traps, 2) visual inspection of *Murraya paniculata*, 3) inspection of all citrus nursery production under aphid mesh screen according to Resolution 930/09, and 4) survey of 100 % of the citrus area (150,000 ha) with at least 10 surveys of the highest risk area; There are 52,000 inspection locations for 13,160 tree or Diaphorina samples. In June 2012, a positive detection of HLB was confirmed in a backyard tree in the Northern Misiones Province across the border from Brazil. Since then, 5 surveys were carried out in the area surrounding the focus with the detection of 15 positive trees, all in backyards. The HLB positive trees include 12 tangerines, and 3 Rangpur limes from 7 to more than 10 years old. In all cases, the trees were eradicated by the owner. The psyllid population in this area is very low and all PCR samples of the vector are negative. At present, HLB has not been detected in commercial groves.

1.5

After the science is finished, the work begins – Navigating the legal and regulatory processes for the deregulation of genetically-enhanced HLB-resistant citrus

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Since the discovery of citrus Huanglongbing (HLB) in Florida in 2005, research efforts to develop and identify germplasm resistant to HLB have intensified greatly. Many research groups in Florida and elsewhere are screening existing citrus varieties and members of the Rutaceae in an attempt to identify useful sources of resistance that can be used in traditional breeding programs to produce commercial scions and rootstocks resistant to HLB. Although progress has been made, it is generally accepted that although some level of tolerance and resistance have been identified, it is not likely that these will be sufficient to confer commercially acceptable levels of resistance in the short term. Similarly, it is widely accepted that genetic modification using a biotechnology approach is likely to be the only way to achieve acceptable levels of resistance in commercial varieties in the near term. Progress has been made by many groups to produce and screen plants with a wide variety of genes and approaches, and more than one group is starting the process to collect the data necessary for deregulation. However, the deregulation process is daunting and full of hurdles and the science may actually be the easiest and the cheapest part of the project. The process as it applies to one project will be presented to demonstrate what is involved as the industry moves forward with this technology.

1.6 P

Huanglongbing Surveillance Program Actions in the State of Bahia, Brazil.

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Huanglongbing (HLB) is found in South/Southeastern states of Brazil, but citrus is grown all over the country. For that reason, surveillance procedures should be carried out frequently and contingency plans developed. This study reports the actions of the State Bureau of Agricultural Defense of Bahia (ADAB), for a commercial orchard in Bom Jesus da Lapa (state of Bahia, Northeastern Brazil) suspected of having HLB symptomatic plants. Besides having a Contingency Plan, a protocol that establishes actions to detect and eradicate the disease, ADAB is also a partner of the collaborative network HLB BioMath. In August 2012, ADAB team inspected 22 hectares of three year old orchards of both Pêra sweet orange and Ponkan mandarin. Scouting was performed on total area, and 4.5% of the plants were marked as having HLB-like symptoms. Samples were tested with polymerase chain reaction (PCR) by the Phytopathological Clinic of Sylvio Moreira Citrus Center. The results were negative for HLB bacterium, but a Phytoplasma of 16SrIX Group was found. All symptomatic plants will be eliminated and the surveillance area will be extended. Despite this finding, Bom Jesus da Lapa is 700km away from the most important citrus regions in Bahia: Reconcavo and Litoral Norte. In those regions no suspected plant was ever reported.

Index terms: *Candidatus* Liberibacter, Free Area, Agricultural Defense.

1.7 P

Incidence of Huanglongbing in commercial orchards in northwest Paraná, Brazil.

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In the Parana state, Brazil, the Huanglongbing has been advancing on the most production important areas. In this study the objective was monitoring the disease incidence in sweet orange varieties Pera, Valencia and Folha Murcha (leaf wilt) in commercial orchards in the northwest of the Parana state. The plants were grouped by age and management of the insect vector, Diaphorina citri, in 35 commercial orchards. Every three months a full assessment of the orchard was performed, totaling six evaluations in each orchard. This monitoring consisted of the walk in the street, or with platform, in throughout the orchard, observing and noting the plants symptomatic for the disease. Orchards of Pera variety had a higher incidence of the disease when the plants were aged 0-5 years and in orchards over the age of 11 years and chemical management of psyllid was made only when their presence. In orchards Pera variety of ages 6 to 10 years and above 11 years, when the chemical management of insect occurred only by new shoots and without assessing the presence of the insect, also showed a higher incidence. The Folha Murcha was the variety with the lowest disease incidence, except when chemical management occurred without assessing the presence of the psyllid in orchards aged 6 to 10 years and above 11 years, when insecticide applications were made every 20 days, without any evaluation of the presence of the vector.

Support: CNPq, Capes, Araucaria Foundation

Topic Categories: Epidemiology

1.8 P

The Citrus Sanitation Center of the Estación Experimental Agroindustrial "Obispo Colombres", Tucumán, Argentina

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Argentina is the largest lemon producing country in the world and Tucumán province leads in lemon production. Knowing that disease free citrus propagating materials prevent the spread of diseases and are the basis of a profitable industry, the Estación Experimental Agroindustrial Obispo Colombres of Tucumán (EEAOC) established the Citrus Sanitation Center (CSC) in 2004. The goal of the CSC is to provide the important citrus varieties and rootstocks true to type and free of graft-transmissible pathogens as primary sources of propagating material for citrus growers and researchers in northwest Argentina. A national citrus certification program became mandatory in 2010 and enclosure of all commercial nurseries in January 2011. At present, most of the main citrus varieties and rootstocks of commercial interest have been recovered through the standard procedure of shoot-tip grafting *in vitro*. Mother trees and increase blocks are maintained in insect proof greenhouses to supply of budwood for rapid nursery multiplication. Regular testing of mother trees by biological, serological and molecular methods is performed for different virus, viroids, CVC, citrus canker and now HLB. Current surveys report that Tucuman is free of the vector, Diaphorina citri, and the HLB pathogen. The CSC will also perform HLB testing during quarantine entry of imported varieties to provide a program for safe accession of citrus germplasm and provision of clean budwood.

Session 2:

Detection and Diagnosis

2.1

Detection of Liberibacter asiaticus in a single infected Asian citrus psyllid adult or nymph: Impact of dilution with clean Asian citrus psyllids (Diaphorina citri) during extraction.

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Now that the presence of Huanglongbing (HLB has been confirmed in California, protecting the state's citrus industry through early detection of disease is essential in curtailing its spread. Because '*Candidatus* Liberibacter asiaticus' (CLas), the putative causal agent of HLB, reproduces in its vector, the Asian citrus psyllid (ACP), *Diaphorina citri*, Kuwayama, accurate testing of the insect is vital. While the current method of testing, quantitative polymerase chain reaction (QPCR) targeting a region of the CLas 16S ribosomal gene, is highly sensitive, because insect secondary metabolites interfere with downstream applications, there is concern about the number of insects pooled in DNA extractions without compromising CLas detection. We are determining the pooling limit through experiments using CLas infected ACP from a colony at USDA-ARS in Fort Pierce, Florida and a clean UC Riverside, California quarantine colony. Individual insect DNA extracts from the infected colonies are evaluated by QPCR to identify positives and the DNA pooled. DNA equivalent to a single positive psyllid is added to 4, 9, 14, 19 or 24 intact clean psyllids and extracted and the presence of Liberibacter determined by QPCR. While the CRB-CPDPP laboratory is performing the single psyllid extractions and providing the pooled positive psyllid DNA samples, multiple laboratories are participating in the extraction and evaluation portion of the project. In addition, *Candidatus* Liberibacter solanacearum (CLsol) infected and clean potato psyllids, *Bactericerca cockerelli*, from colonies at the USDA-ARS Repository in Riverside will be studied as a control. Potato psyllids generally acquire CLsol at rates of 100%. Therefore, in addition to experiments using pooled potato psyllid DNA equivalent to a single CLsol positive psyllid, single intact potato psyllids from the CLsol positive colony will be extracted with 4, 9, 14, 19 or 24 clean potato psyllids and CLsol determined to verify the validity of using pooled positive DNA.

Allen ML, O'Brochta DA, Atkinson PW, LeVesque CS. 2001. Stable, Germ-Line Transformation of *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Ent. 38(5):701-710.

Bash J, Bulluck R, da Graça J, French JV, Gottwald T, Halbert S, Hall D, Levy L, Michalak PS, Polek ML, Shubert T. 2012. USDA New Pest Response Guidelines Citrus Greening Disease. USDA-APHIS-PPQ.

Bextine B, Blua M, Harshman D, Miller T. 2005. A SYBR Green-Based Real-Time Polymerase Chain Reaction Protocol and Novel DNA Extraction Technique to Detect *Xylella fastidiosa* in *Homalodisca coagulata*. J. Econ. Ent. 98(3):667-672.

Bové J M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. 88:7-37.

Hall D G, Shatters RG, Carpenter JE, Shapiro JP. 2010. Progress toward an artificial diet for adult Asian citrus psyllid. Ann. Entomol. Soc. Am. 103: 611-617

Hung T-H, Hung S-C, Chen C-N, Hsu M-H, Su H-J. 2004. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector–pathogen relationships. Plant Path. 53(1):96-102.

Li W, Hartung SJ, Levy L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. J Microbiol. Methods. 66:105-115.

Manjunath KL, Halbert SE, Ramadugu C, Webb S, Lee RF. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and Its Importance in the Management of Citrus Huanglongbing in Florida. Phytopath. 98(4):387-396.

2.2

Single chain antibodies against '*Ca*. Liberibacter asiaticus'

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Antibodies are widely used as microbiological reagents, but antibodies that recognize '*Ca*. Liberibacter asiaticus' are generally lacking. We have developed and applied immunization and affinity screening methods to create a primary library of recombinant single chain variable fragment (scFv) antibodies in an M13 vector, pKM19. The antibody population is enriched for antibodies that bind antigens of 'Ca. Liberibacter asiaticus' and *Diaphorina citri*. The primary library has more than 10^7 unique antibodies and the genes that encode them. We have screened this library of antibodies for antibodies that bind to specifically chosen proteins that are present on the surface of '*Ca*. Liberibacter asiaticus'. These proteins were used as 'bait' for affinity-based selection of scFvs that bind to the major outer membrane protein, OmpA; the polysaccharide capsule expressing protein KpsF; a protein component of the type IV pilus (CapF); and two flagellar proteins FlhA and FlgI. These scFvs have been used in ELISA and dot blot assays against purified protein antigens and '*Ca*. Liberibacter asiaticus' infected plant extracts. We also have isolated scFv that bind to surface exposed portions of the TolC proteins and of a protein called InvA. These proteins may have critical roles in pathogenicity. Thus far, screening of these scFvs is more efficient when using phage bound, rather than soluble scFvs. We have demonstrated a technology to produce antibodies and select at will and against any protein target encoded by 'Ca. Liberibacter asiaticus'. Future applications will include advanced diagnostic methods for huanglongbing and the development of immune labeling reagents for in planta applications.

2.3

Portable Chemical Sensors for Monitoring Infection-Specific Volatiles in Asymptomatic Citrus

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Volatile organic compounds (VOCs) are emitted from all plants, and there is mounting evidence these VOCs reflect internal health status and change in response to pathogen infection and other cues. Our group has developed a portable chemical sensing platform that can monitor for VOC emission changes that result from citrus bacterial and viral infections. To date, our VOC library includes putative signal fingerprints for Huanglongbing (HLB), citrus tristeza virus (CTV) and citrus variegated chlorosis (CVC). Our mobile platform is robust and capable of operating in field conditions. We have also developed customized data analysis methods to compare data from unknown samples to our database and to determine the probability of infection for a newly sampled tree.

2.4

Repertoire of novel sequence signatures for the detection of *Candidatus* Liberibacter asiaticus by quantitative real time-PCR

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Huanglongbing (HLB) or citrus greening is a devastating disease of citrus [1]. Circumstantial evidence indicates that HLB is caused by *Ca.* Liberibacter asiaticus in the United States as well as in Asia [1, 2]. Accurate detection of Las in infected trees and psyllids plays important role in regulation and serves as one important control measurement in citrus producing areas without HLB to prevent it from being endemic [3]. Among the diagnosis tools, quantitative real time-PCR (qRT-PCR) based on selective candidate genes/regions like 16S rDNA, β operon, or *nusG-rplK* regions have been developed and most widely used [4,5,6,7,8]. Generally those sequences are highly homologous across closely related bacterial species, therefore, prone to be less specific to Las and misdiagnosis. In order to specifically detect Las by qRT-PCR, we exploited the known genome sequence of Las and performed an exhaustive sequence search for all the unique genomic regions. By designing the qRT-PCR primers specific to the identified 34 unique genes, we specifically detected the Las with no cross reactivity to the closely related species e.g. *Ca.* L. americanus and *Ca*. L. africanus. The sensitivity of most of our primer sets is comparable or better than 16S rDNA based primers. In conclusion, we have identified and experimentally validated the repertoire of novel sequence signatures that can facilitate the detection of Las from the infected plant by qPCR thereby aid in controlling the disease.

Citation

1. Bové, JM. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. 2006. Journal of Plant Pathology, 88 (1), 7-37.
2. Lopes SA, Frare GF, Bertolini E, Cambra M, Fernandes NG, Ayres AJ, Marin DR, and Bové JM. 2009. Liberibacters Associated with Citrus Huanglongbing in Brazil: 'Candidatus Liberibacter asiaticus' Is Heat Tolerant, 'Ca. L. americanus' Is Heat Sensitive. Plant Disease. 93: 257-262.
3. Gottwald, TR, da Graça, JV, and Bassanezi, RB. 2007. Citrus Huanglongbing: The pathogen and its impact. Online. Plant Health Progress doi:10.1094/PHP-2007-0906-01-RV.
4. Li W, Hartung JS, Levy L. Quantitative real-time PCR for detection and identification of

Candidatus Liberibacter species associated with citrus huanglongbing. J Microbiol Methods 2006;66:104e15.

5. Hocquellet A, Toorawa P, Bové JM, Garnier M. 1999. Detection and identification of the two Candidatus Liberobacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the beta operon.Mol Cell Probes. Oct;13(5):373-379.
6. Morgan JK, Zhou L, Li W, Shatters RG, Keremane M, Duan YP.2012. Improved real-time PCR detection of 'Candidatus Liberibacter asiaticus' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes. Mol Cell Probes. Apr;26(2):90-98.
7. Okuda M, Matsumoto M, Tanaka Y, Subandiyah S, Iwanami T. 2005. Characterization of the tufB-secE-nusG-rplKSJL-ropB gene cluster of the citrus greening organism and detection by loop-mediated isothermal amplification. Plant Dis 89:705e11.
8. Fujikawa T, Iwanami T. 2012. Sensitive and robust detection of citrus greening (huanglongbing) bacterium “Candidatus Liberibacter asiaticus” by DNA amplification with new 16S rDNA-specific primers. Mol Cell Probes. Oct;26(5):194-197.

2.5

Tree-side Molecular Testing for DNA from the HLB Bacterium *Candidatus* Liberibacter asiaticus

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HLB affects all citrus cultivars and causes tree decline resulting in greatly reduced citrus production in Asia, Africa, the Indian subcontinent, the Arabian Peninsula, Brazil, and the United States. To enable integrated pest management strategies for combating HLB, we have developed a sensitive nucleic acid-based HLB diagnostic test based on Mesa Tech International, Inc.'s (MTI's) new point-of-use diagnostic platform, MTIDx, and targeting hyvI/hyvII genes of *Candidatus* Liberibacter asiaticus (Las) (Zhou et al., 2011). The MTIDx platform integrates sample preparation, rapid nucleic acid amplification and sequence-specific hybridization-based detection. The simplicity of the test may offer end users with little or no specialized training the opportunity to obtain molecular test results comparable to laboratory-based PCR methods without costly instrumentation. Here, we will report on the efficacy of MTI's nucleic acid testing platform for the detection of *Candidatus* Liberibacter asiaticus bacteria in greenhouse and field collected citrus tissue and in the insect vector of the pathogen (*Diaphorina citri* Kuwayama). Comparative studies evaluating the MTIDx platform's performance relative to widely accepted laboratory PCR testing will be presented. Results from these studies will be discussed in the context of their implications for HLB management.

2.6

Relationship between Ct values, HLB symptoms and CLas titer

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We determined the frequency of Ct values for 20,000 HLB diagnostic samples collected by field scouts in Florida. The Ct frequency distribution revealed that the vast majority of samples produced no amplicon (Ct values > 40) and were therefore considered negative for CLas, indicating that scouts were commonly sampling leaves that exhibiting symptoms that mimicked HLB, but were not actually HLB. However, these samples were collected fairly early in the Florida HLB epidemic when symptoms were more ambiguous than is the current situation. Of the samples that did produce amplicons, Ct = 22 was by far the most frequent value and was the peak of a perfectly symmetrical bell shaped curve with a leading edge of Ct=28 and a tailing edge of Ct=18. Because scouts collect samples based on visual HLB symptoms we can conclude that Ct values between 28 and 18 represent the range of Ct values most typical of symptomatic leaves. Conversion of Ct values to CLas genomes$\cdot g^{-1}$ fwt based on a standard curve reveals that: 1) the majority of samples were CLas-negative (Ct > than 38); 2) CLas-infected, asymptomatic leaves have CLas titers of 10^2 to 10^4 genomes $g\cdot^{-1}$ fwt (Ct 38-30); 3) the titer of CLas in HLB-symptomatic fwt is 10^6 to 10^7 genomes$\cdot$ g^{-1} fwt (Ct 24-19) and 4) no samples exceed 10^8 CLas genomes $g\cdot^{-1}$ fwt (Ct < 18). These results provide insights into the relationship between Ct values, CLas titers and HLB symptoms.

2.7

Visualization of '*Candidatus* Liberibacter asiaticus' Cells in Citrus Seed Coats with Fluorescence In Situ Hybridization and Transmission Electron Microscopy

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'*Candidatus* Liberibacter asiaticus' is bacterium implicated as the causal agent of the economically damaging disease of citrus called huanglongbing (HLB). The bacterium is spread by movement of infected citrus propagation material and by the Asian citrus psyllid, *Diaphorina citri* Kuwayama. Seed transmission is a possible additional route of dissemination for the pathogen. Some published studies on seed transmission found abundant bacterial DNA in seed coats but no infections of germinated seedlings. No direct observations of bacteria in seed coats were made in these studies. In vascular bundles from citrus seed coats, we confirmed the presence of bacterial cells with transmission electron microscopy (TEM) and Fluorescence In Situ Hybridization (FISH). The physical measurements and the morphology of individual bacterial cells revealed by TEM and FISH were consistent with those described in the literature for '*Ca*. Liberibacter asiaticus'. No bacterial cells were observed in seed coats from non-infected trees. A library of clones of prokaryote 16S rRNA gene sequences amplified from a non-infected tree contained no '*Ca*. Liberibacter asiaticus' sequences, whereas 95% of the sequences in a similar library prepared from an infected tree were '*Ca*. Liberibacter asiaticus', providing additional data that the bacterial cells observed by TEM and FISH in seed coats from infected trees were '*Ca*. Liberibacter asiaticus'.

2.8 P

Guidelines for Selection of Tissues for Electron Microscopy Confirmation of *Candidatus* Liberibacter spp. in Huanglongbing-affected Citrus

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Polymerase chain reaction (PCR) with the pathogen-specific primers and electron microscopy are the two techniques of choice that have been used for detection and identification of *Candidatus* Liberibacter spp. in the Huanglongbing (HLB)-affected citrus. Due to the low population and uneven distribution of Liberibacter in the diseased citrus trees finding the bacteria with transmission electron microscopy has been a challenge. Work with samples from HLB-affected citrus during the past 5 years has resulted in certain guidelines for the selection of plant tissues that have led to success in visualization of the bacteria. With PCR-positive field citrus trees, the best source is phloem tissue from seed coats of developing seeds in young fruit supported by leaves with blotchy mottle symptoms. These half mature seeds have been shown to contain large numbers of intact bacteria. However, when working with young potted trees there are usually no fruit to sample. In these trees we had some success in finding bacteria in petioles or midveins of PCR-positive, young developing leaves (2/3 to fully expanded, but not hardened) sampled above leaves showing blotchy mottle symptoms. However, we have found larger numbers of bacteria in roots of the potted diseased trees. We have experienced the most success in sampling root tissue in areas where the phloem has just completed differentiation, in PCR-positive small pioneer and fibrous roots showing primary and secondary growth.

2.9 P

Comparison of optical sensing techniques for detecting citrus diseases.

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Citrus diseases such as Huanglongbing (HLB) and citrus canker result in major production and economic losses in Florida. Therefore, there is an urgent need for a sensing technique that can be used to detect these diseases and apply suitable management practices. An ideal sensing technique should be rapid, accurate and reliable to provide economic, production and agricultural benefits. Optical sensing techniques such as visible-near infrared spectroscopy offer rapid sensing of plant diseases. Although optical sensing methods have few limitations, they offer unique benefits that can greatly aid in citrus disease detection. We have worked on several spectroscopic and imaging techniques to detect citrus diseases, especially HLB. This work presents the comparative performance of these techniques and discusses the benefits and limitations of each of these methods. The spectral techniques that will be discussed in this paper are: visible-near infrared spectroscopy, mid-infrared spectroscopy, fluorescence spectroscopy, multiband imaging, and thermal imaging. We collected spectral data representing healthy and diseased leaves from the citrus trees of different cultivar, followed by pre- and post-processing offsite using mathematical models. The classification studies using Naïve-Bayes classifier, Bagged Decision trees and Support Vector Machine were performed before or after principal component analysis, depending on the dataset. Our results indicated that most of these techniques showed a classification accuracy of about 90% and higher. However, the suitability of a technique would depend on its application. This paper summarizes the major findings from different spectroscopic techniques and compares their performance in relation to their applications.

2.10 P

Advances in HLB Detection Using Agdia's Isothermal AmplifyRP™ Platform

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Huanglongbing (HLB) disease is found throughout Asia, in Brazil, Mexico, the USA, and parts of Africa and has seriously affected citrus production in many regions. The three species of the Candidatus Liberibacter which have been identified are *Candidatus Liberibacter asiaticus*, *Candidatus. L. americanus*, and *Candidatus L. africanus*.

We discuss here improvements in the AmplifyRP™ platform which allow for easy, accurate, and specific detection and identification of the three causative species of HLB. The single-component test systems allow for the use of either purified nucleic acid preps or crude extracts prepared from psyllids or plant tissue. Comparisons with other detections will be discussed.

2.11 P

Occurrence of *Diaphorina citri* Kuwayama in an unexpected ecosystem: the Lake Kissimmee State Park Forest, Florida

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In July 2012, we captured Asian citrus psyllids (ACP), *Diaphorina citri*, at the Lake Kissimmee State Park (Polk county, FL). ACP were captured on yellow sticky traps deployed in a wet flatwood ecosystem. Specimens were sent to the Florida Department of Agriculture and Consumer Services (FDACS) and were all identified as *D. citri*.

From the 12 July through 8 October 2012, we monitored the ACP population at this location. Capture of ACP on 19 July reached a maximum of 1.3 ACP per trap per week. ACP collected were submitted to qPCR and 20% of captured ACP in this forest were positive for *Candidatus* Liberibacter asiaticus (Las).

After exploration of the surrounding area, we found four non-cultivated tangerine plants on the border of Lake Rosalie, 1 km away from the original ACP collection site. These four plants were tested for Las and all were negative. Yellow sticky traps were also deployed on these citrus trees but no ACP were collected at this location.

Plants found in the original area of collection were identified, and to our knowledge, none are currently known as alternative hosts of ACP. We are performing bioassays and thus far, we found that ACP were able to feed and survive on gallberry (*Ilex glabra* L.). These results suggest that ACP may have a wider alternative host acceptance range and / or higher dispersal ability than previously thought and occur within a dense Florida forest in the absence of surrounding citrus groves within at least 3 km.

2.12 P

Efficiency of different set of primers in PCR detection of *'Candidatus* Liberibacter asiaticus'

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Huanglongbing (HLB), caused by the bacteria '*Candidatus* Liberibacter spp', is one of the most destructive diseases for citrus production around the world. Early diagnosis of diseased citrus trees is of utmost importance for the control of HLB. Molecular techniques, such as PCR, offer quick and specific detection and identification of '*Ca*. Liberibacter spp.'. Furthermore, different sets of primers have been used for detection of '*Ca*. Liberibacter asiaticus', the main agent of HLB. However, neither of the described sets of primers may detect all cases of HLB. Thus, conventional PCR using three common sets of primers, A2/J5, OI1/OI2c, and Lp1c/HP1, which amplify fragments of 703 pb, 1200 pb, and 2400 pb, respectively, were examined for HLB diagnosis. A total of 969 samples from different citrus cultivars, tree ages and regions of the State of Paraná, Brazil, were included in this study. The total number of samples tested positive for HLB by any set of primers were 598, representing 61.7% of the samples examined. Among these positive samples, 96.7% were identified with the primers A2/J5, 86.6% with OI2c/OI1, and 81.4% with HP1/Lp1c. When combined, 99.8% of the samples were HLB positive based on the sets of primers A2/J5 and OI2c/OI1. Based on the results obtained, the set of primers A2/J5 showed the highest efficiency in the detection of HLB for the bacterium that occurs in the State of Parana, Brazil.

Citation

Bastianel C., Garnier-Semancik M., Renaudin J., Bové J.M., Eveillard S., 2005. Diversity of "C*andidatus* Liberibacter asiaticus", based on the Omp Gene Sequence. *Applied and Environmental Microbiology* 71:6473-6478.

Bové J.M. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol 2006;88:7-37.

Jagoueix S., Bové J.M., Garnier M. PCR detection of the two '*Candidatus*' Liberobacter species associated with greening disease of citrus. Mol Cell Probes 1996;10:43-50.

2.13 P

Home Detection Kit for *Candidatus* Liberibacter asiaticus (LAS) Associated with Citrus Huanglongbing from Psyllids

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Management of citrus huanglongbing (HLB) requires rapid detection of infected psyllids and trees in an orchard. Detection of HLB associated bacteria (LAS) can be done using psyllids since detection in infected trees is usually delayed (Manjunath et al., 2008). We have developed an easy to use, rapid and affordable detection kit for grower use for testing psyllids for LAS at a reasonable price for initial investment and an operating cost of about $2 per sample.. Eight psyllid samples can be simultaneously tested within 45 minutes. The psyllid DNA extraction and detection of LAS are conducted using a SmartDART™ unit which is operated by software installed on any android device for visualizing real time results. The test results can be e-mailed for both storage and analysis. The DNA prepared can be stored refrigerated and sent to a laboratory for validation. No other equipment (even pipets) is required for the test. The detection system was validated using a large number LAS isolates from many citrus varieties, from different countries; the results were comparable to that of traditional real time PCR data. Development of methods for multiplex detection of the pathogen and the host DNA from both psyllids and plant host are in progress. We believe the detection system will be useful for growers in intra-orchard management, for extension workers, nurserymen, and in areas where the disease has become endemic as well as in those areas where the disease has been recently introduced.

Citations

Manjunath K.L., Halbert, S.E., Ramadugu, C., Webb, S., and Lee, R. F. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus Huanglongbing in Florida. Phytopathol. 98(4): 387-396. (doi: 10.1094/PHYTO-98-4-0387)

2.14 P

Effect of Temperature on Lighted Sticky Traps (TransTrap®) used to Detect Asian Citrus Psyllids in Shipping Containers

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Abstract

The Asian citrus psyllid (ACP) is an insect native to tropical and subtropical regions in Asia, which has spread into most citrus producing regions around the world. The main concern with ACP is the spread of Huanglongbing (HLB), a disease vectored by the psyllid. The volume of citrus shipments from Mexico into the U.S. prompted questions about the risk of moving ACP and HLB on this pathway. Our goal was to determine temperatures at which ACP would be active and thus fly to lighted sticky traps inside a transport trailer.

Our results found that temperature significantly affected the number of adults captured. No psyllids were recovered on the traps at 12˚C (53˚F), which is the average temperature of citrus shipments crossing the border. This is compare with an average capture of 43, 47, and 42% of the released adults at 24, 28, and 32°C (75, 82, and 90°F), respectively. Only 1%, 2% and 18% were captured at 18, 20 and 22°C (64, 68, and 72˚F), respectively. Given that the average temperature of limes arriving at the border is 12˚C, it is unlikely that ACP adults would fly to a lighted sticky trap placed inside a refrigerated trailer. In addition, the majority of the packing houses in Mexico place the fruit into coolers prior to loading. We believe a lighted sticky trap would not be effective in detecting ACP within refrigerated citrus shipments for the period of time from loading at the packing house to trap recovery at the border crossing.

2.15 P

Effect of time and storage methods on the detection of *Candidatus* Liberibacter asiaticus in *Diaphorina citri* by qPCR*

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The assessment of bacterialiferous Asian citrus psyllid (ACP) frequency is important for (i) studies of bacteria acquisition and inoculation by ACP, (ii) disease detection in disease free areas but with ACP presence, (iii) efficiency evaluation of inoculum reduction strategies, (iv) evaluation of frequency of *Candidatus* Liberibacter asiaticus (Las)-positive ACP and the abundance of inoculum sources or putative new HLB infections relationships. Depending on the conditions and time of storage of collected psyllids, Las DNA in ACP could degrade and Las-false negative results might occur. Thus, this study was conducted to evaluate the detection of Las in ACP adults submitted to different storage methods and time of storage by real-time PCR (qPCR). Two 2x3x7 factorial experiments were conducted. Factors were 'Ethanol' (with or without 70% ethanol), 'Temperature' (-20°C, 4°C and 26°C) and 'Time' (0, 3, 7, 14, 21, 28 and 35 days). For each treatment, 20 samples with 3 ACP adults from nymphs reared on Las infected trees were tested for Las presence by qPCR. No significant differences in percentages of psyllids samples positive for Las were observed among the storage methods up to 35 days, except a slight trend of decline in Las detection in samples storage without ethanol at 26°C after 14 days of storage.

*Part of first author's dissertation in the Fundecitrus Professional Master on Control of Citrus Diseases and Pests.

2.16 P

Candidatus Liberibacter asisticus detection in the leaves, roots from infected trees and leaves of new shoots from the stumps of the infected sweet orange trees in Texas

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A total of 108 root and corresponding symptomatic leaf samples from four different quadrants were collected from 27 6 year old sweet orange trees in which the presence of *Candidatus* Liberibacter asiaticus (CLas) was previously confirmed by quantitative polymerase chain reaction (qPCR). There was no significant difference ($p>0.05$) in the level of infection as determined by threshold cycle (Ct) values between different types of tissue tested. Analysis of variance (ANOVA) showed that there is no significant difference ($p>0.05$) in test results among different distances from the trunk or quandrants where the root samples were collected. The stumps of the infected trees were covered in a psyllid-proof cage and leaves from the new shoots emerged from these stumps did not show the presence of CLas where as the roots showed the presence of CLas. Moreover, there was no significant difference between roots from infected trees and roots from infected stumps. Additionally, there was a significant difference with an average Ct value difference of 2.97 cycles between the DNA samples extracted from roots using two different commercially available kits.

2.17 P

Detection of *Candidatus* Liberibacter asiaticus in *Diaphorina citri* caught on yellow sticky traps during the winter and summer of Sao Paulo State Brazil*

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Fundecitrus, Araraquara, Brazil

The assessment of bacterialiferous Asian citrus psyllid (ACP) frequency is important in epidemiological and management studies because it can be related with the abundance of inoculum sources and with putative new HLB infections. For that, ACP can be collected directly or on yellow sticky traps (YST) commonly used by Brazilian growers to monitor psyllid population. The YST are usually left in the field for 2 weeks after which time YST are visually evaluated for the ACP presence, and if present, the psyllids are removed from the YST and tested by real-time PCR (qPCR) for liberibacter presence. Previous studies in Florida showed that the incidence of Las-positive ACP declined with increasing time on the YST (Irey et al., 2011). Thus, the objective of this work was to determine if time ACP is keep on YST affects qPCR results for Las and if it was related to weather conditions during winter and summer of Araraquara-SP (Brazil). ACP adults from nymphs reared on Las infected trees were placed on YST (BUG-Agentes Biológicos) in the field and 20 samples with 3 individuals were tested after 0, 1, 3, 9, 12 and 15 days. The results were compared with samples directly collected without trap glue. Experiments were done in June, July and August (winter) and in January, February and March (summer). In contrast with previous report in Florida, no difference on the incidence of Las-positive ACP samples was observed up to 15 days on the YST in both seasons.

*Part of first author's dissertation in the Fundecitrus Professional Master on Control of Citrus Diseases and Pests.

Session 3:

Epidemiology and Survey

3.1

Optimised regulatory surveys for the regional-scale early detection of Huanglongbing

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Prior to the arrival of HLB in a region large-scale surveillance programs are usually instigated in order to detect the disease as early as possible. Early detection is necessary to minimise the impact of the disease and facilitate any containment or eradication interventions. Large-scale surveillance surveys are however expensive, covering large geographic regions and stretching fiscal and manpower resources. Available resources must thus be deployed in the most optimal way. The choice of which locations within a region to survey is a complex problem since there may be hundreds of thousands of possibilities to choose from. Predicting how the epidemic will spread through a heterogonous landscape of citrus plantings and how this relates to where sampling resources should be deployed to find the 'needle in the haystack' is challenging and most surveys are consequently sub-optimal. We bring together state of the art epidemiological modelling and stochastic optimisation techniques to determine the optimal pattern of sampling deployment across a landscape. We find that the optimal pattern of sampling resources in a region is often counter-intuitive; for example simply targeting the highest risk locations is rarely the optimal course of action. We show how the optimal pattern depends subtly on epidemiological factors such as the spatial pattern of citrus plantings and vector densities in a region. We also show how geo-referenced information on likely entry points into a region, e.g. trade and travel hubs, can be incorporated to improve the probability of achieving early detection.

3.2

Commercial risk-based survey for HLB and implications for efficacy of Citrus Health Management Areas (CHMAs)

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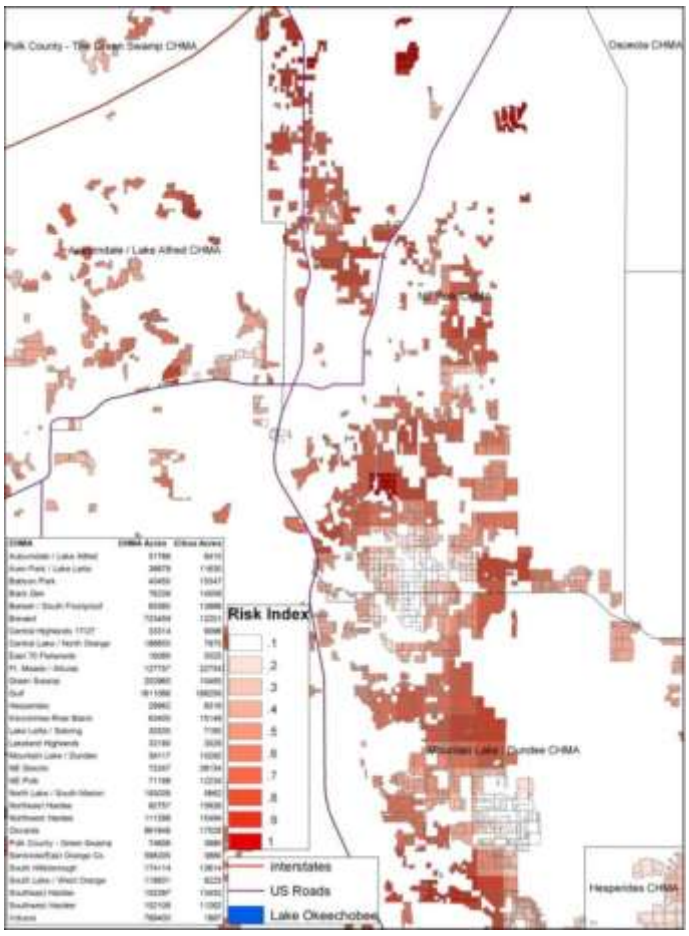
The USDA, APHIS, PPQ has been conducting a risk-based multi-pest survey (MPS) for the past two years. The initial 6 statewide survey cycles were conducted on a 6 week basis, whereas the subsequent 20 cycles have been conducted on a 3-week basis. HLB and ACP are the two main pests addressed by the MPS but other citrus pests and diseases, i.e., Asiatic Citrus Canker (ACC), Citrus Leprosis Virus (CLiV), Citrus Black Spot (CBS) and Citrus Variegated Chlorosis (CVC) were also given lesser emphasis during the survey.

Commercial Survey model design and implementation

a. For the commercial citrus survey, similar risk factors are calculated as used for the residential survey (See Gottwald et al., 2013 IRCHLB III abstract) and the risk reversed to reflect the impact of urban populations and activities on commercial plantings. The MPS is used to assess incidence, to predict future spread and disease increase, and for commercial citrus management and regulatory decision-making.

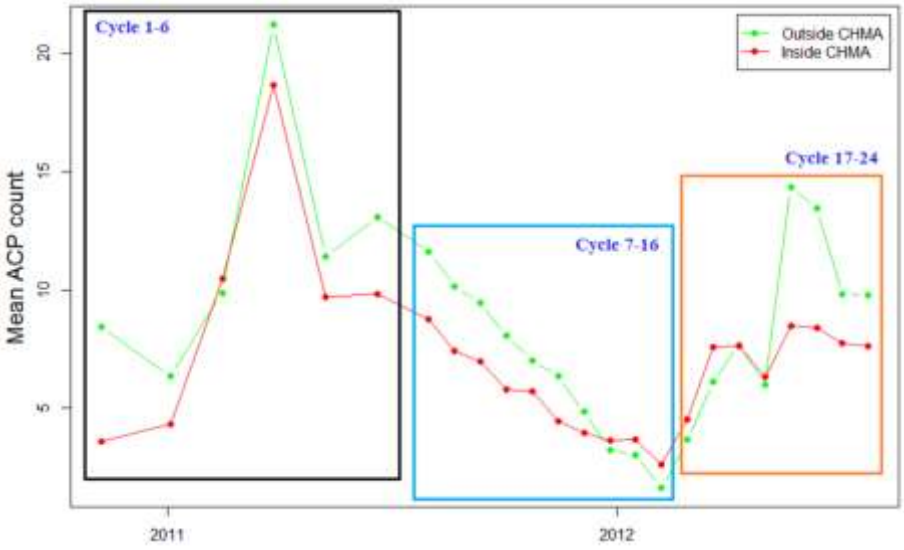
b. *Stratification:* To apply the MPS statewide, the citrus industry is first parsed into strata and sampled based on a risk-bias algorithm previously designed and deployed in Florida (Parnell et al.). A stochastic algorithm is then used to prioritize sample locations (individual blocks) within each strata. Thus there is a weighted stratified sampling applied across the entire citrus industry. The prioritization of individual blocks within each strata is via a risk calculation as described immediately below.

c. *Individual risk factors*: Risk factors considered are cultivar susceptibility, planting size (in hectares or acres), planting age, proximity to ACP populations (both residential and commercial citrus), proximity to HLB



infections (if and when discovered), risk from Asian populations, proximity to abandon citrus if any, and proximity to residential citrus. The model already exists for the state of Florida and is being adapted to and amended for California and Texas. A risk index factor (also known as a biasing factor) is calculated based upon the sum of all criteria listed above. This risk factor is calculated for each commercial block which takes into account the relative importance or weighting of each factor.

d. *Additional risk factors:* Other risk factors includes proximity to nurseries, home centers, military installations, Indian reservations, transportation corridors, and collection of other factors used in residential risk survey.
e. *Statewide HLB risk model validation and improvement*: The initial survey was used for model validation of the existing predictive model within the Florida, California, and Texas environments. As data are collected from each state, post-survey analyses are used for adjustment of weighting of individual risk factors. Subsequent surveys will then have the benefit of an improved model. Thus, the model is dynamic and can be continuously improved until it approaches a steady state, where subsequent analyses add little or no significant benefit in changing risk factor weightings.
f. Output from the model can be used for overall risk mapping such as the map (See above) which was generated for commercial citrus blocks in central Florida. The output can portray ACP population density, HLB population density, and combinations of other risk factors to generate a cadre of overall risk maps.
g. When a sufficient number of surveys have been conducted through time, spatiotemporal predictions can also be generated such that the maps can be predictive estimates of future ACP/HLB risk areas as well as evaluation of the overall efficacy of the CHMA program (See figure to left). These efficacy and predictive maps can be utilized by production managers and regulatory agencies as an attempt to offset emerging ACP and HLB hot spots. Such maps can also be utilized to aid in optimizing CHMA size, location, and number of CHMAs needed. Although CHMAs have been set for Florida, these data can aid in CHMA development for other citrus producing states.



3.3

Predicting the establishment and spread of plant disease from regulatory sampling

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Invasive plant diseases can have devastating consequences on the local plant populations, in both agricultural and natural landscapes. Knowledge of the spatial patterns of pathogen spread can be used to guide more time- and cost-effective disease management strategies. Based on disease dispersal principles and consideration of host pattern, an improved plant disease epidemiological model was developed and tested for plant disease mapping. The model is able to characterize the disease dispersal gradient and predict infection risk, with indication of uncertainty, through heterogeneous environments without reference to the source of infection. As a result, sampling methods can be informed by the predicted prevalence map of the disease. In order to better describe the shapes of the dispersal gradients, three different dispersal functions (Exponential, Modified power law, and Cauchy distribution) were considered in the model. Two data sets of disease observations of Huanglongbing (HLB) of citrus in different landscapes (Southern Garden and Devils Garden plantation) in Florida were used to evaluate the performance of the improved method for disease mapping. The results showed that the improved model provided estimates of greater precision for unsampled hosts. With all different dispersal models compared, the exponential dispersal gradient gave the most satisfactory performance. All the determined information can help decision makers understand the spatial aspects of disease processes, and formulate decisions about disease control accordingly.

Methodology

The methodology of the original developed model is described exhaustedly in Parnell et al., 2011 and Luo et al., 2012. As a result, this section is only focused on describing improvements to the original model.

Host density and susceptibility

The performance of disease modeling is greatly affected by the way in which transmission between infected and susceptible hosts is modeled. In order to increase computational efficiency and ease the effort in host sampling, the improved model uses density as an alternative way to represent host distribution. To do this, we aggregate the entire host population by regular grids, and then calculate the density (D), infection ratio (R) and average host spatial location with each grid. Each grid is treated as an individual host, and the disease map is estimated following the

exact procedure of the original model. In addition to density, host susceptibility, *S*, (including environmental, biological and climatic factors) may influence the efficiency of disease dispersal. The susceptibility determines the probability of the disease becoming established when a pathogen arrives. For strong resistant host varieties, the disease will die off or fail to reproduce. The combined effect of host density and susceptibility on disease spread can be expressed as

$$y_i = a\sum_{j\neq i}\frac{D_j}{D_i}R_j \exp(-bd_{ij})S_i \qquad (1)$$

and the infection ratio R_i can be calculated by back transformation

$$R_i = 1-\exp(-y_i).$$

Here d_{ij} is the distance from grid location *i* to *j*, and R_j is the infection probability of grid *j*. Positive parameters *a* and *b* are used to describe the shape of the exponential curve, with *a* representing the magnitude of the source and *b* measuring the steepness of the gradient. According to equation 1, the occurrence of an epidemic is strongly tied to host density and susceptibility. This adds a second source of stochasticity to the disease dynamics, which better described the disease spread characteristics. There are different ways to summarize host density and susceptibility relative to spatial scale. With sufficient information, it is possible to empirically estimate the suitable spatial size to contrast host density and susceptibility.

Dispersal model

Information about the form of dispersal gradients is an essential component of spatially explicit epidemiological models (Sackett & Mundt, 2005). Determining a suitable dispersal function is key to understand the spread of plant diseases in space, as well as in time. Instead of focusing on the exponential function, two other dispersal functions with different tail shape patterns were used to characterize disease spread. The first was a modified power law model (Gregory, 1968)

$$y_i = a\sum_{j\neq i}\frac{D_j}{D_i}R_j(1+d_{ij})^{-b}S_i \qquad (2)$$

which has non-exponentially bounded tails, but has a finite value at the source. The second was a Cauchy model (Shaw,1995; Xu & Ridout, 1998) which allows for the proportion of healthy hosts to be taken into account

$$y_i = a\sum_{j\neq i}\frac{D_j}{D_i}R_j\frac{1}{(1+(\frac{d_{ij}}{b})^2)}S_i \qquad (3)$$

where *b* is the median dispersal distance parameter.

All above disease dispersal functions assume that disease intensity tends to decrease with increasing distance from the source of inoculum (Fig. 1). The biggest difference between the

exponential and modified power law function can be found in the tail of each gradient. The modified power law usually has a sufficiently long fat tail, indicating possible long distant dispersal. Conversely, the exponential has a short tail with an exponential decay, where the distant host would almost become irrelevant to disease spread. The shape of the Cauchy model looks similar to exponential, but it has a slightly heavier tail.

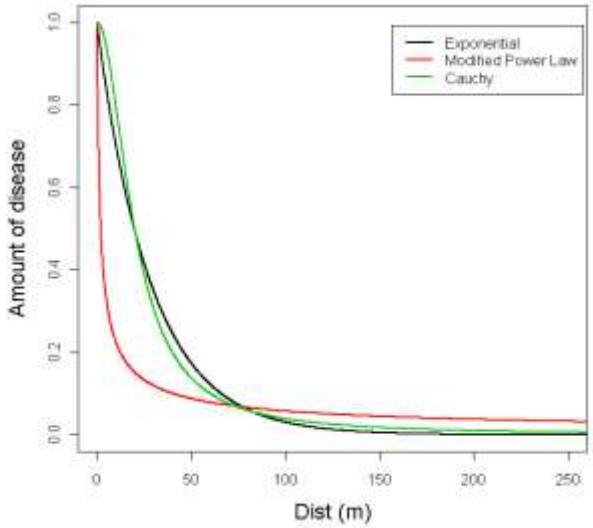

Figure 1. Graphical comparison of different dispersal functions used to model disease spread.

Some observed disease gradients and dispersal patterns are adequately fit by all models, but others are better explained by one over the other (Fitt *et al.*, 1987; Ferrandino, 1996). The spatial spread of disease with the exponential distribution has been studied extensively (van den Bosch *et al.*, 1988; Zadoks & van den Bosch, 1994), and simulation studies have shown that the exponential distributions produce a more regular radial-spatial pattern of disease spread, while long-tailed distributions such as the modified power law and Cauchy produce more long-distance dispersal and the resulting spatial pattern appears to be clumped or fractal (Shaw, 1995; Xu & Ridout, 1998). Without further investigation on the actual data, it is difficult to claim a single method is superior to the others.

Applications

A mechanistic modeling approach was improved for disease mapping with consideration of host and susceptibility. An efficient C++ program was produced for the modeling framework, which allows flexibility in appropriate model selection for future pathogen threats at a range of scales from local to regional. In addition, the model provides plant health authorities and policy makers with a set of protocols and computer programs that address: (a) the mechanisms of pathogen dispersal, (b) the distance and pattern of disease spread, and (c) predictions of infection probabilities for unsampled hosts with uncertainty analysis. Through better understanding of the spatial aspects of disease processes, we improve our capability to handle disease appropriately.

References

Ferrandino, F.J., 1996. Length scale of disease spread: Fact or artifact of experimental geometry. *Phytopathology*, **86**, 806-811.

Fitt, B.D.L., Gregory, P.H., Todd, A.D., McCartney, H.A. & MacDonald, O.C., 1987. Spore dispersal and plant disease gradients: A comparison between two empirical models. *Journal of Phytopathology*, **118**, 227-242.

Gregory, P.H., 1968. Interpreting plant disease dispersal gradients. *Annual Review of Phytopathology*, **6**, 189-212.

Luo, W., Pietravalle, S., Parnell, S., van den Bosch, F., Gottwald, T.R., Irey, M.S. & Parker S.R., 2012. An improved regulatory sampling method for mapping and representing plant disease from a limited number of samples. *Epidemics*, **4**, 68-77.

Parnell, S., Gottwald, T.R., Irey, M.S., Luo, W. & van den Bosch, F., 2011. A stochastic optimisation method to estimate the spatial distribution of an invasive plant pathogen. *Phytopathology*, **101**, 1184-1190.

Sackett, K.E. & Mundt, C.C., 2005. The effects of dispersal gradient and pathogen life cycle components on epidemic velocity in computer simulations. *Phytopathology*, **95**, 992-1000.

Shaw, M.W. 1995. Simulation of population expansion and spatial pattern when individual dispersal distributions do not decline exponentially with distance. *Proceedings of the Royal Society B*, **259**, 243-248.

van den Bosch, F., Frinking, H.D., Metz, J.A.J. & Zadoks, J.C., 1988. Focus expansion in plant disease. III: Two experimental examples. *Phytopathology*, **78**, 919-925.

Xu, X.M. & Ridout, M.S., 1998. Effects of initial epidemic conditions, sporulation rate, and spore dispersal gradient on the spatio-temporal dynamics of plant disease epidemics. *Phytopathology*, **88**, 1000-1012.

Zadoks, J.C. & van den Bosch, F., 1994. Expansion and spatial spread of disease. *Annual Review of Phytopathology*, **32**, 503-521.

3.4

HLB βioMαth: Sentinel network and research

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The citrus Huanglongbing (HLB), recognized as the most devastating citrus disease worldwide, was detected in Sao Paulo state, Brazil in 2004. The HLB management strategy employed in São Paulo is based on preventing new infections by reducing the inoculum (certified planting seedlings, psyllid control and removal of symptomatic plants). However, HLB continues to disseminate, reaching two neighbor states. In Brazil, citrus is cultivated country-wide (88% of the microregions produce citrus, responding for more than 30% of the planted area). If this dissemination pattern persists, there is a risk of emergence of HLB in areas not yet affected. To face this problem, exclusion strategies and early detection/eradication are crucial, specifically, tools, information and support for the action of phytosanitary defense agencies. The objectives of this Network are generate information that allows to defense phytosanitary agencies prioritize, anticipate or reassess actions relating to the exclusion or eradication of HLB, focusing on preventive actions to areas still unaffected. Since 2010, dataset (presence/absence of vector and bacteria, vector population measurements, etc.) are being obtained from different eco-regions of Brazil: south (cold), northeast (including semi-arid region) and north (amanzon). The analysis of the data until now shows that in the south and north regions the presence of the vector is uncommon or even rare. In contrast, in the northeast the presence is very common, and in the semi-arid region, the vector occurs, but in less abundant and sporadic fashion. Symptomatic plants and insect collected in all regions were tested and did not show the presence of the bacteria.

Citations:

Castro, M.E.A., Bezerra, A.R., Leite, W.A., Mundin Filho, W., Nogueira, N.D. 2010. Situação e ações do estado de Minas Gerais frente ao *huanglongbing*. Citrus Research & Technology 31: 163-168.

Coletta-Filho, H. D., M.L.P.N. Targon, M.A. Takita, J.D. De Negri, J. Pompeu Jr., A.M. Amaral, G.W. Muller & M.A. Machado. 2004. First report of the causal agent of *huanglongbing* ("Candidatus Liberibacter asiaticus") in Brazil. Plant Disease. 88:1382.

Nunes, W.M.C., Souza, E.B., Leite Junior, R.P., Salvador, C.A., Rinaldi, D.A., Croce Filho, J. & Paiva, P.G. 2010. Plano de ação para o controle do *huanglongbing* no estado do Paraná, Brasil. Citrus Research & Technology 31: 169-178.

3.5

A new method for spatial analysis of irregularly spaced HLB data and biological implications

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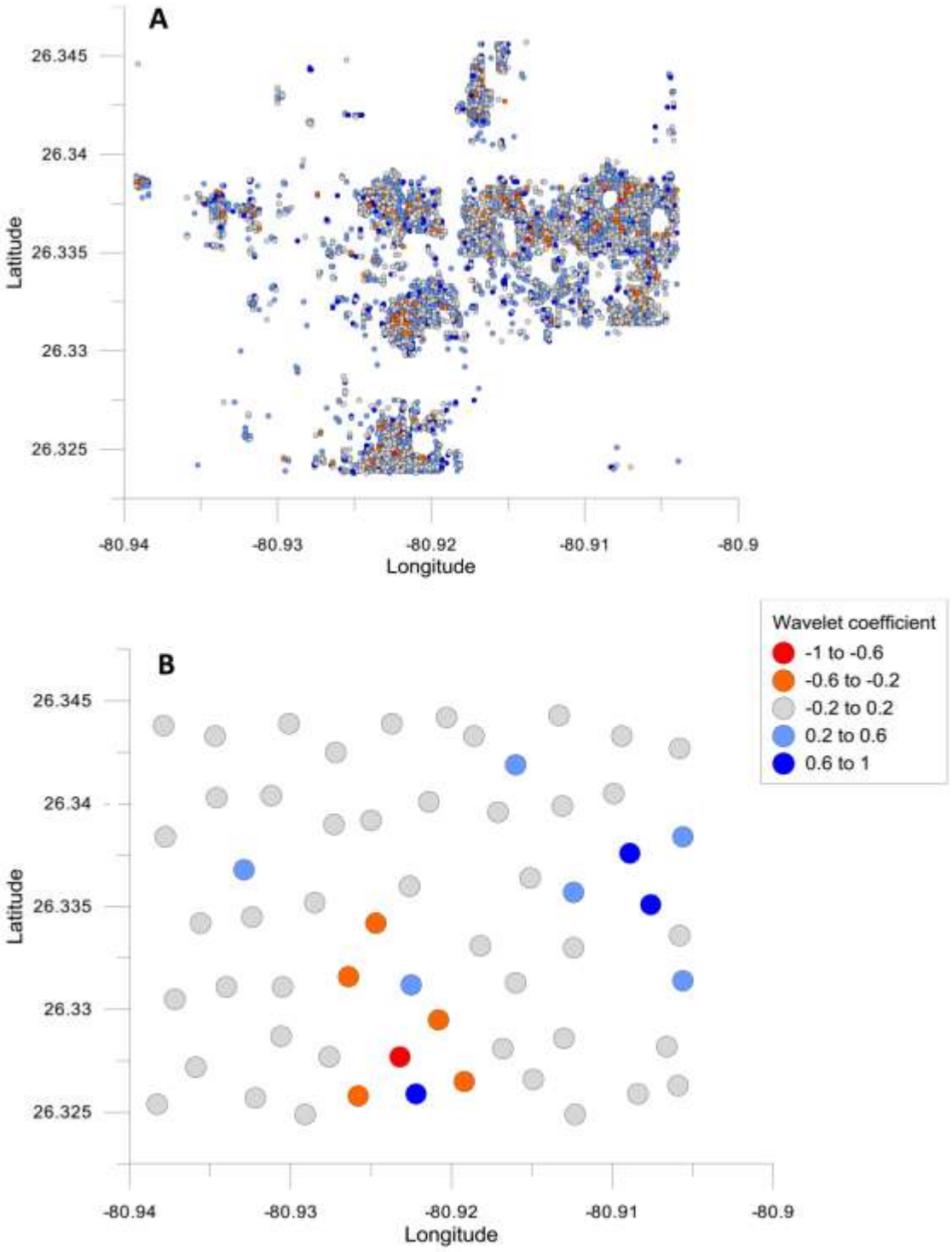
Field data on intensity of plant diseases is very often irregularly spaced (i.e., there are varying amounts of distance between rows, ponds, voids, roads, houses, or other land areas). Typically, this type of data is gridded and the average disease intensity of the plants within the grid is used instead of the original data on each separate plant. This is done because the underlying statistical assumptions in the analysis of spatial data usually require that data be equally spaced. However, a new method of analysis, sometimes called second-generation wavelet analysis, can be used on irregularly spaced spatial data. Wavelet analysis is a method used to analyze variations in scale and position of non-stationary spatial signals (non-stationary for our data means the statistical properties can vary based on location within the orchard), and the second-generation refers to an iterative process, called a lifting scheme (1), which allows for the irregular spacing. Irregular spacing is often found in citrus groves as spacing within and between rows is often not uniform, and on a larger spatial scale, distance between blocks and plantings are not necessarily simple multiples of distances between rows and trees. In addition, there are a number of other issues such as missing trees, the presence of irregular roads, ponds, staging areas, etc., that cause citrus groves to have irregular distances between trees when viewed at the plantation or regional scale. Therefore, to test this new method, we conducted a second-generation spatial wavelet analysis on a large irregularly spaced citrus planting (Southern Gardens) in Florida where over 260,000 trees were assessed for incidence of huanglongbing (HLB) over five sampling times.

The primary result from this analysis is a wavelet coefficient for each diseased tree in the original data. This coefficient essentially represents the difference between the observed density of disease around the tree and the density predicted from neighboring diseased trees and their respective densities. The neighboring trees can simply be the nearest neighbors, or the analysis can take multiple clusters of neighbors into account. The coefficients are very useful for multiple subsequent analyses, and one of which is to identify the clustering pattern of HLB infected areas over several spatial scales. For this data, the scales ranged from 17.7 square meters (i.e., the trees at this spatial scale were completely surrounded by diseased trees on all sides) to 1,712,195 square meters. Two example maps in the figure below indicate where clustering is located within the grove at some of the A) smallest scales and B) largest scales. Interpretation of the wavelet coefficients will be presented. Results for this analysis are superior to some traditional analyses for clustering that only explore a few spatial scales. The spatial scales at which clustering is identified has often been used in plant pathology as a basis for not only theoretical understanding of epidemics, but also in identifying the most appropriate sampling methods for disease control and the scale at which management practices should be applied. For large land areas like citrus

groves with highly irregular spacing and widespread diseases such as HLB, these types of applications using previous methods of analysis may lead to some incorrect interpretations.

1. Sweldens, W. 1998. The Lifting Scheme: A Construction of Second Generation Wavelets. SIAM J. Math. Anal. 29:511-546.


A
26.345
26.34
26.335
26.33
26.325
Latitude
-80.94
-80.93
-80.92
-80.91
-80.9
Longitude
B
Wavelet coefficient
-1 to -0.6
-0.6 to -0.2
-0.2 to 0.2
0.2 to 0.6
0.6 to 1

2.

3.6

Risk-based residential HLB/ACP survey for California, Texas and Arizona

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The recent discoveries of HLB in the Los Angeles Basin and the Rio Grande Valley of Texas underscore the imminent danger of HLB spread in these two States and the urgent need for highly sensitive survey methods for early detection of new residential infections of HLB combined with rapid intervention to contain and eliminate further spread. The Arizona citrus industry is also at considerable risk due to the proximity to the Mexican border and continued immigration of ACP from Mexico. The 2008 economic downturn has led to dwindling fiscal resources for many regulatory agencies including those tasked with conducting the survey for HLB. Therefore, sampling efforts need to be deployed based on potential risk introduction and threat to commercial citrus to optimize early detection. A risk-based residential survey has recently been constructed and deployed in Southern California and the Rio Grande Valley of Texas, and is being designed for Southern Arizona.

Filtering of the survey area

Residential areas surveyed were determined by a human population density map generated from 2000 and 2010 U.S. Census data. This map was then filtered to remove areas where residential citrus would not exist or only contain rare or minimal numbers of trees; including:

h. Water bodies, such as major lakes, ponds, rivers, and reservoirs.
i. Park and recreation areas including national parks and forests, community centers, golf courses, zoos, amusement parks, and convention centers.
j. Transportation areas such as airports, airfields, train and bus stations, and parking lots.
k. Living areas that would not support citrus such as hotels and resorts, hospitals and care centers, nursing and retirement facilities, Tyumen oil institutions, jails and prisons.
l. Commercial workplaces such as shopping and retail centers, industrial areas, office spaces, vineyards, and non-citrus agricultural areas.
m. Community areas such as colleges, schools, churches, and cemeteries.
n. Areas higher than 700 m (2300 feet) which is the reported elevation above which ACP cannot survive due to either temperature or atmospheric pressure (Albert 1883).
o. Areas where yearly minimum temperatures (based on 10-year temperature averages) fall below the tolerance threshold, (temperature and duration) for survival of ACP.
p. Military installations, Indian reservations, and other places that cannot be surveyed due to lack of access. However, these areas will be used in the risk calculation that follows (see below).

The result is a fully filtered residential population map which includes only residential areas that are presumed to be able to sustain residential citrus.

Calculation of Risk

The overall risk algorithm is constructed considering several major components of risk. Initially, each of these components will be simply given equal weighting, because it is difficult to quantify the relative influence of each risk factor compared to the others without substantial data. The risk model is dynamic and can be easily changed over time. As data is collected during subsequent survey cycles, we reassess the various contributions of each individual risk factor and then apply appropriate weightings accordingly. This will allow us to dynamically change and enhance the survey model through time, thus making it more accurate and robust relative to mapping and prediction.

a. *Estimation of residential citrus populations:* Based on data provided by CDFA, in the LA basin 60% of the households have residential dooryard citrus and of these households the average is approximately 2 trees per household. Initially for Texas and Arizona similar population structures were used to estimate the population density of residential citrus statewide. However, residential citrus population density is not a direct linear relationship with human population but rather varies nonlinearly with human population density. The nonlinear relationship was estimated for the state of Florida and the same mathematical function applied initially to California, Texas and Arizona until such time as more data are collected directly from each state and the function corrected specifically to reflect their individual situations. Using the human population we can then map all or part of each state as a function of residential citrus population density.

b. *Estimation of risk due to potential ACP spread*: Risk was evaluated due to potential ACP spread from commercial nurseries, home centers, packinghouses, other citrus production or commercial vendors (e.g., big box stores or flea markets) and green waste facilities. In addition risk was evaluated for military

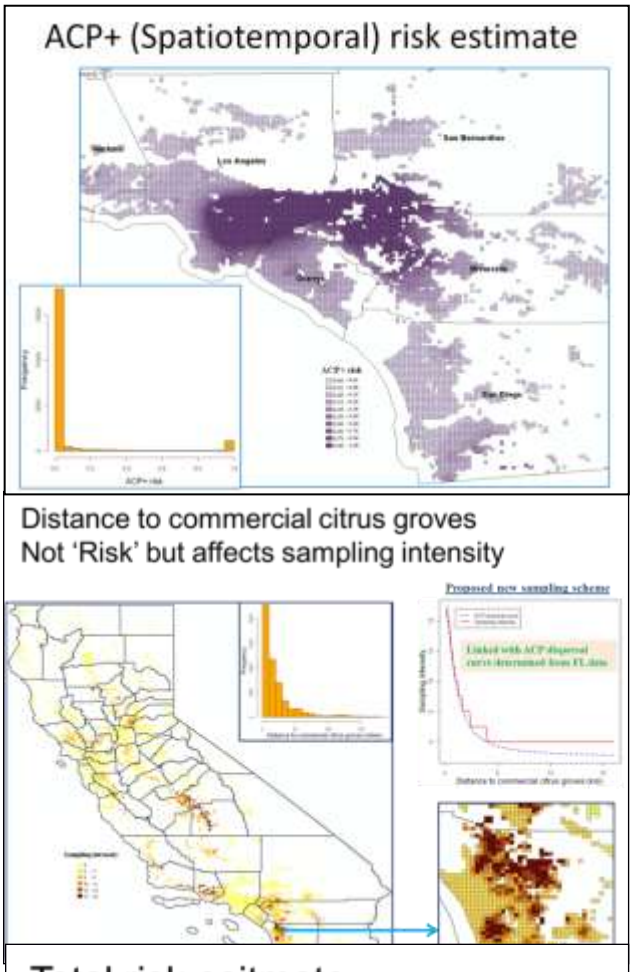

Figure 1. Top) ACP+ risk in Southern California. Middle) Inverse distance-based function from commercial citrus used to adjust sampling intensity. Bottom) Total risk estimate presented on a1-mi^2 grid.

installations and Indian reservations, both of which will be excluded from survey due to lack of access. Neither of which are subject to customs and/or import/export regulation, which suggests that they could act as unknown sources for introduction of HLB and ACP. From prior data collected in Florida we know that ACP risk decreases with distance from the source following an adjusted power law function up to approximate 16 km. This function is applied to estimate and weight risk as a function of distance from commercial citrus production and sales centers. However, not all of these areas are given the same risk weighting. Obviously nurseries that produce citrus have a high risk as do retail centers with high traffic of citrus sales, whereas, small retail nurseries and incidental retail vendors would have much lower risk.

c. *Estimation of risk due to known ACP population prevalence and dynamics*: From 2010 to 2012, data from ACP traps in Southern California and ACP incidence in Texas and Arizona were considered. The spatial positions of prior ACP populations and their duration were combined in an overall spatiotemporal disease dispersal model. Thus, it is not only the presence of ACP but its duration (temporal function) that ascribes risk to a particular location. An overall map layer of ACP risk was created for each state. This is used both for residential and commercial citrus surveys dynamically. Data collected in future surveys will be incorporated in risk calculation and as a result the ACP risk maps will change over time. Risk from ACP spread is considered an inverse power law function over distance (Fig. 1 Top).
d. *Transportation corridors*: The primary and secondary roads and expressway system used for commercial citrus production movement is considered the transportation corridor. Based on analyses of this system in Florida, a negative exponential function (extents to be determined) was used to estimate risk over distance perpendicular to transportation corridors of concern for each state.
e. *Climatological effects* were accounted for where appropriate. From previously published data (Hall et al., 2011) we can extrapolate minimum temperature thresholds below which ACP cannot survive. Residential and commercial survey maps will be adjusted by minimum temperature thresholds to represent the likelihood of ACP development and spread.
f. *Population demographics* are especially important. From prior data in a number of locations in various countries, we know that residents with Asian heritage have ties and connections to Asian countries that have HLB, and thereby pose a higher threat of introduction (unintentional and often unknowingly). Therefore higher sampling intensity and risk calculations resulted from areas where Asian populations are prevalent. The initial HLB find in Los Angeles Basin was within one such high risk Asian population area.
g. *Risk of HLB positive find(s) and Las positive ACP* are added as they occur. To date there is one in California, four (two commercial and two residential) in Texas, and none in Arizona, but more will likely occur over time. A distribution function was calculated based on data in Florida that provides a description of risk of HLB as a function of distance from HLB positive detections via an inverse power law function. More sampling effort will be emphasized on each area and surrounding areas with HLB infection.
h. *An adjustment for sampling intensity* was also developed based on proximity to commercial citrus plantings. An inverse distance weighted function from commercial citrus was used provide a higher sampling intensity near commercial citrus areas (Fig. 1 Middle).

Distribution to stakeholders:

a. *Overall mapping of cumulative total risk* were calculated for each of the regions of concern for each state (Fig. 1 Bottom).
b. *Survey protocol:* Risk maps were provided to each state/agency based on STR (1 mi.² areas described by section – township – range). The calculated risk impacts the probability of STR selection for residential survey, i.e., the higher the risk, the higher chance such STR will be selected. Therefore 'hot' disease STR areas are the areas predominately covered and extra assurance is provided via a stochastic selection of a small proportion of STRs in low risk areas. An output data set in Excel is also provided that lists each STR and its estimated total risk. This can then be used by survey teams as a protocol to perform a systematic risk based survey. If more fiscal and manpower reserves can be dedicated to survey, then regulatory agencies can simply select more STRs and include lower risk areas.
c. *Multiple interactive maps* that can link to Google Earth were also provided so the regulatory agencies could target survey teams more precisely via visual representations of risk. Figure 2 shows a satellite view of a 4 mi² area of residential Los Angeles and a corresponding residential risk map for the same area. Note manufacturing and nonresidential subareas have been filtered out.

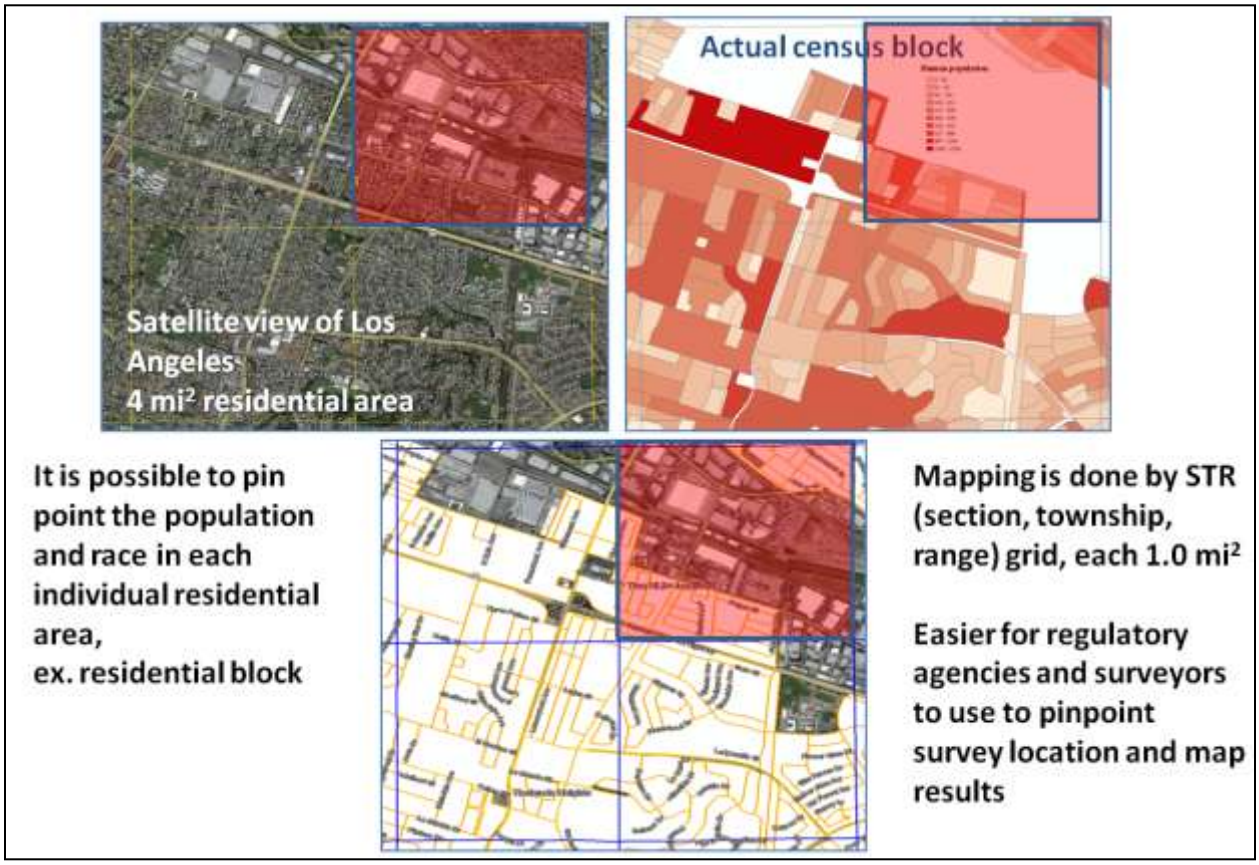

Figure 2. Multiple interactive mapping

The survey models described above provide a modeling framework for development of surveys for other citrus producing areas and industries such as areas in Central and South America and the Caribbean. A similar framework can be easily transferred to apply to survey other non-indigenous diseases when required. In the global sense, surveys that can predict and detect introductions before or while in low incidence will afford improved chances of disease suppression/management prior to areawide or regional spread that can eventually act as sources for future introductions into the US.

References

Hall DG, Wenninger EJ, Hentz MG (2012) Temperature studies with the Asian citrus psyllid, Diaphorina citri: cold hardiness and temperature thresholds for oviposition. *Journal of Insect Science*, 11:83.

3.7

Edge Effects and *Huanglongbing*

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Huanglongbing (HLB), spread by a psyllid vector, is globally considered a major threat to commercial and sustainable citrus production. Better understanding of the vector-mediated patterns of HLB spread is essential to inform and maximize disease management. From previous studies, edge effects are a significant characteristic of the HLB pathosystem and have been observed predominately in larger plantings. In this study, we investigated 1) the impact of different edge classes and orientations, 2) the quantitative influence of distance from edges, and 3) the temporal dynamics of each edge effect. Spatial analyses of edge effects were conducted on two years of HLB incidence data from the Southern Gardens plantation in south Florida. The Southern Gardens dataset consists of multiple surveys for more than 250,000 citrus trees in 180 blocks. The blocks are arranged in six rows of 30 blocks, and each block typically contains around 1500 trees. Each block usually contains trees of the same age and variety. Based on the shape and orientation of the plantation (Fig. 1), five different edge types were classified, including ponds, main roads (NS and WE), and internal plantation edges (voids) between blocks (NS and WE). With variation due to variety and tree age taken into account, results clearly showed significant edge effects for ponds, NS and WE main roads, and the estimated distance of influence from an edge (i.e., 120, 130 and 90m, respectively). These edge effects were consistently significant across different assessment dates, although the magnitude of the effect varied temporally. Compared with tree age and variety, higher intensity of infected trees was initially found at the borders of ponds. No obvious edge effects were found for internal plantation edges, which was probably due to their associated small void width. To further examine the temporal nature of within-grove edge effects, an experiment was designed to determine if *Diaphorina citri* populations along edges of groves vary according to time-of-day and time-of-year in relation to the azimuth of the sun. Three citrus groves, each divided into nine sampling areas (corresponding to four cardinal directions, four intercardinal directions [e.g., northeast], and an interior sampling area) consisting of 30 trees each, were surveyed for *D. citri* using a stem tap method (1). To maximize exposure to incident sunrays, sampling areas consisted of trees within the two outermost rows (except for the interior sampling area). Groves were sampled three times per day (during the 3h including and after sunrise, surrounding solar noon, and before and including sunset) and three times per year (corresponding to intervals of time surrounding the summer solstice, autumnal equinox, and winter solstice that maintain a 5° error range of the azimuth during the midpoint of each time-of-day sampling period). Preliminary results indicate there were more *D. citri* found in the south, north, east, and northeast sampling areas at sunset within the summer solstice sampling period than in other sampling

areas. Differences were seen among sampling areas for other time-of-year and time-of-day combinations but were not substantial within the currently unrepeated experiment. More data will be collected over the 2013 to 2014 growing seasons. The determined edge effects can be identified and investigated to better understand the general HLB risk and contribution to potential epidemics. Placing more emphasis on management practices at plantation edges should result in improved HLB disease control.

1. Hall, D. G., and Hentz, M. G. 2010. Sticky trap and stem-tap sampling protocols for the Asian citrus psyllid (*Hemiptera: Psyllidae*). Journal of Economic Entomology 103(2):541-549.

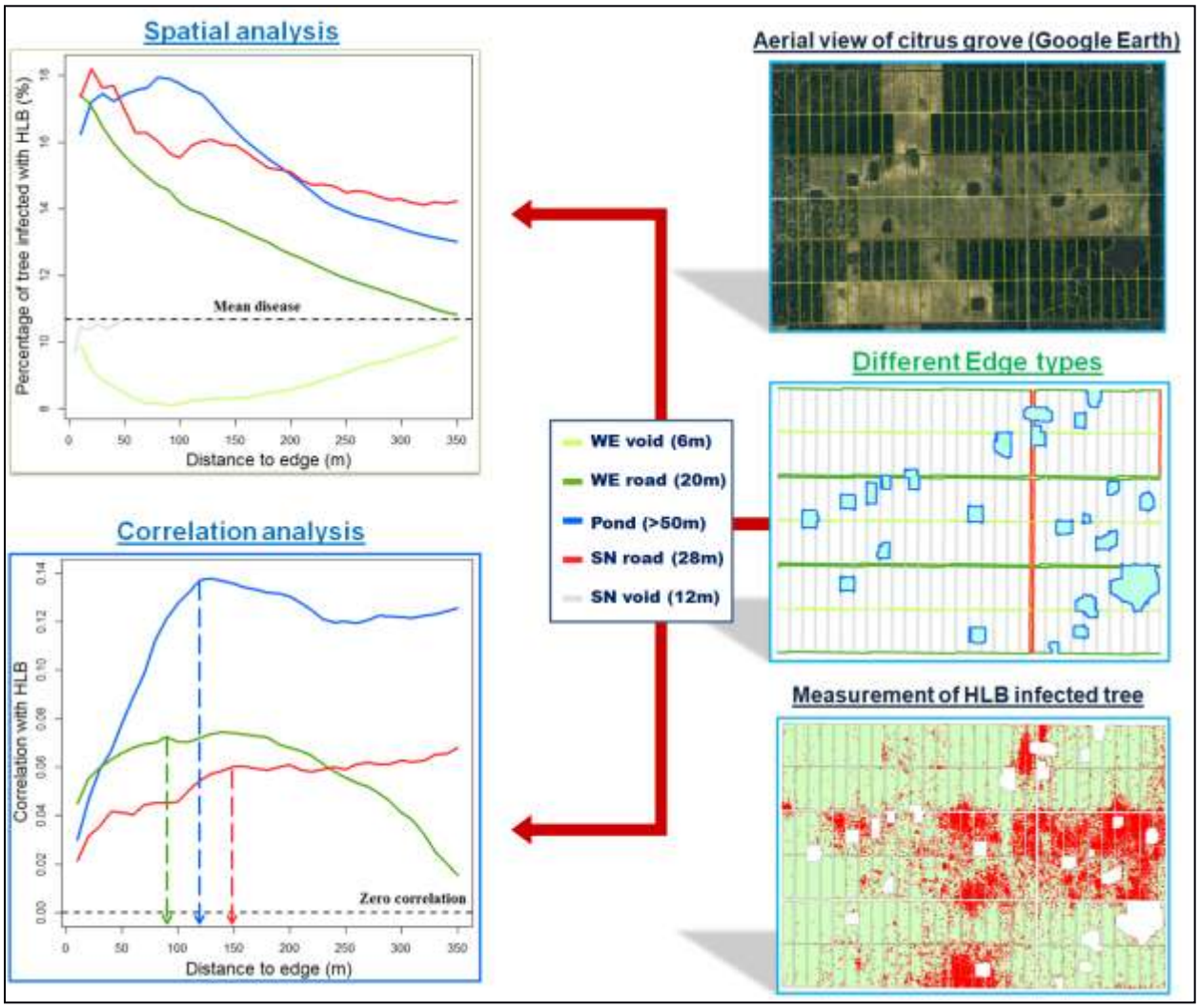

Figure 1. Edge classification and modeling for the Southern Gardens plantation in South Florida.

3.8

Variability of direction of tree-to-tree spread of HLB over time

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Candidatus Liberibacter americanus and *Candidatus* Liberibacter asiaticus are two bacterial species that cause huanglongbing (HLB) disease in citrus-growing regions of Brazil. A concentrated sampling plan of a grove in Matao, Brazil was initiated to evaluate the spatial and temporal spread of these bacteria. The exact location of each of 8697 trees was recorded, and each symptomatic tree was assessed by PCR for the presence of *Ca*. L. americanus and *Ca*. L. asiaticus during 17 different months from April 2006 to May 2008 (Fig. 1). In the first month, only five trees were confirmed to have *Ca*. L. asiaticus. The first trees with confirmed cases of *Ca*. L. americanus were not found until February 2007. By the end of the study, 43 trees were confirmed to have *Ca*. L. americanus, 1164 trees were confirmed to have *Ca*. L. asiaticus, and three trees were coinfected. For the new trees each month that were confirmed to have *Ca*. L. asiaticus, their distance (m) from previously infected trees was calculated. This process continued for each of the 17 months and for all lag times from 3 to 24 months (lag is defined here as the time from visual symptoms on one tree to visual symptoms on a newly infected tree). For the example shown in Figure 2 with a lag of 6 months, 95% of the new infections were within 50 m of a previously infected tree. Therefore, it was assumed that a new infection could be from psyllids and bacteria of any previously infected tree within a 50 m radius. Directionality of disease spread was then examined. The absolute difference between the direction (North = 0°) from an infected tree to other trees it was determined to possibly infect and the direction from that infected tree to a newly infected tree (Fig. 2A) were found. Results indicate that predictions of tree-to-tree spread should take the direction of previously found infections into account versus assuming that all trees around an infected tree (i.e., a 360° circle around an infected tree with some radius) have equal probability of being infected by psyllids from a symptomatic tree. The example in Figure 2A indicates that about 80% of new infections (*i'*) appeared to emanate from a prior infected tree (*i*) that was already found to infect other trees within ±45° (or 90° total) cone extending from the older infection (*i*). Therefore, in future development of spatio-temporal models for HLB, it is suggested to attribute higher probabilities of infection for trees that are in the same direction as previous suspected travel routes of psyllid populations. One cause for the results found may be that new infections from a prior infected tree all occurred during one or a few spread events where the psyllids moved in similar directions (possibly due to similar environmental influences), but the variability in lag period among the trees resulted in symptoms identified during different scouting times. Similar results as shown for the lag period of 6 months

were also found for most of the other lag times, but the shortest periods of 3 and 4 months had more uniform results than is shown in Figure 2A. Other possible reasons for the findings were investigated but were found to not be the cause. First, the direction of the infections has no pattern (i.e., uniform distribution) in this data (Fig. 2B), which indicates there is variability in psyllid movement over the 2 years of the study. Second, it was thought the results might be dependent on the angle of the rows (Fig. 2C). However, only 11% of new infections were indicated to be caused by a tree within the same row. That is 244 out of the 270 observations in the first bin of Figure 2C (i.e., there were 270 cases where the angle between the row and the new infected tree was between 0 and 5° and 244 of those were within the same row), resulting in an overall uniform distribution. Third, the results found were the same when trees near the edges were excluded (since the evaluations could only be in a single direction) (data not shown).

Acknowledgement

The authors greatly appreciate the collaboration of S. A. Oliveira, the manager of Boa Vista farm, for his many contributions.

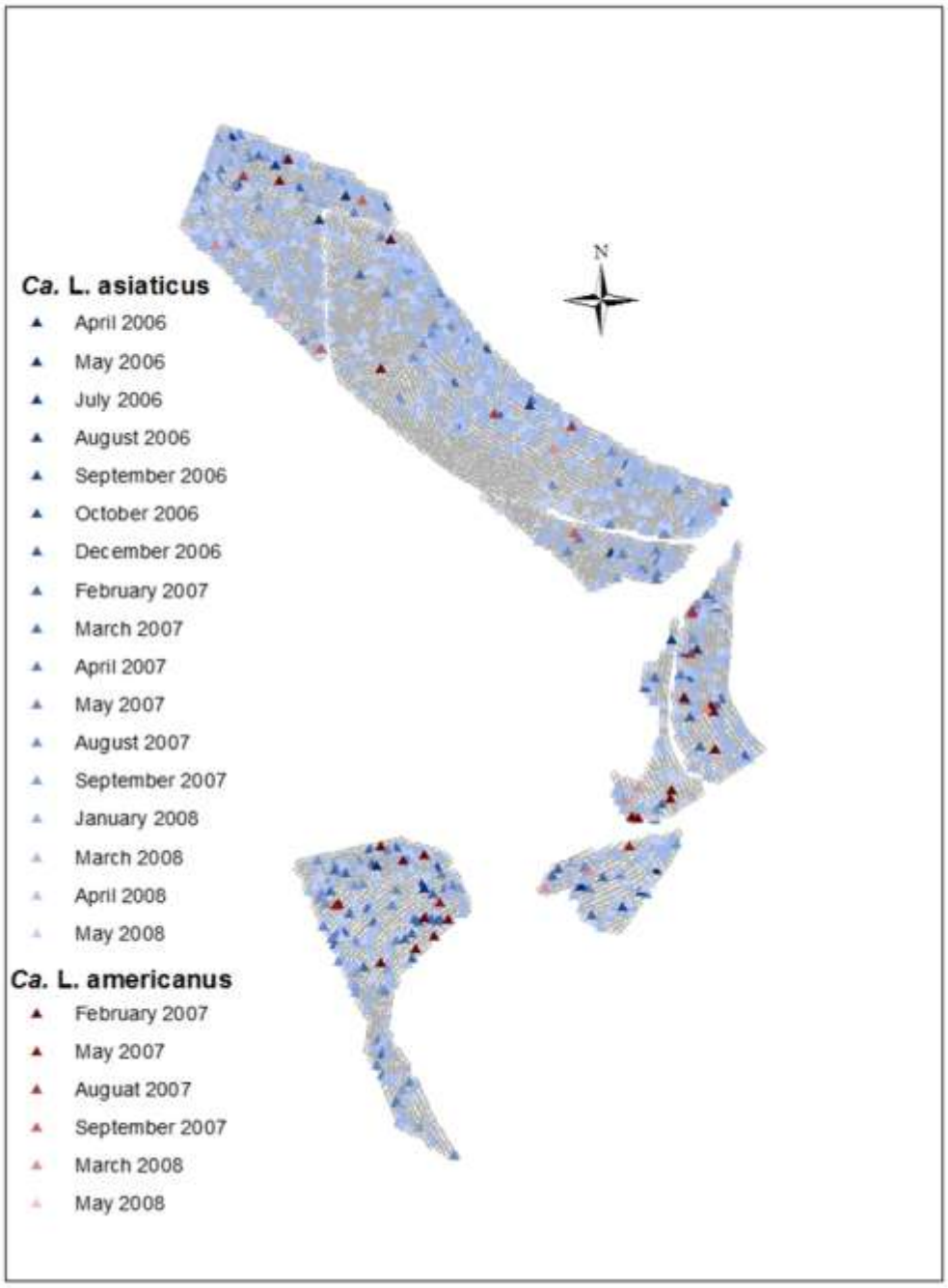

Figure 1. Citrus orchard in Matao, Brazil with 8697 trees. Those confirmed to have the bacterial species *Candidatus* Liberibacter americanus and *Candidatus* Liberibacter asiaticus are colored red and blue, respectively. Note variable and complex planting patterns, i.e., row directions along elevation contours.

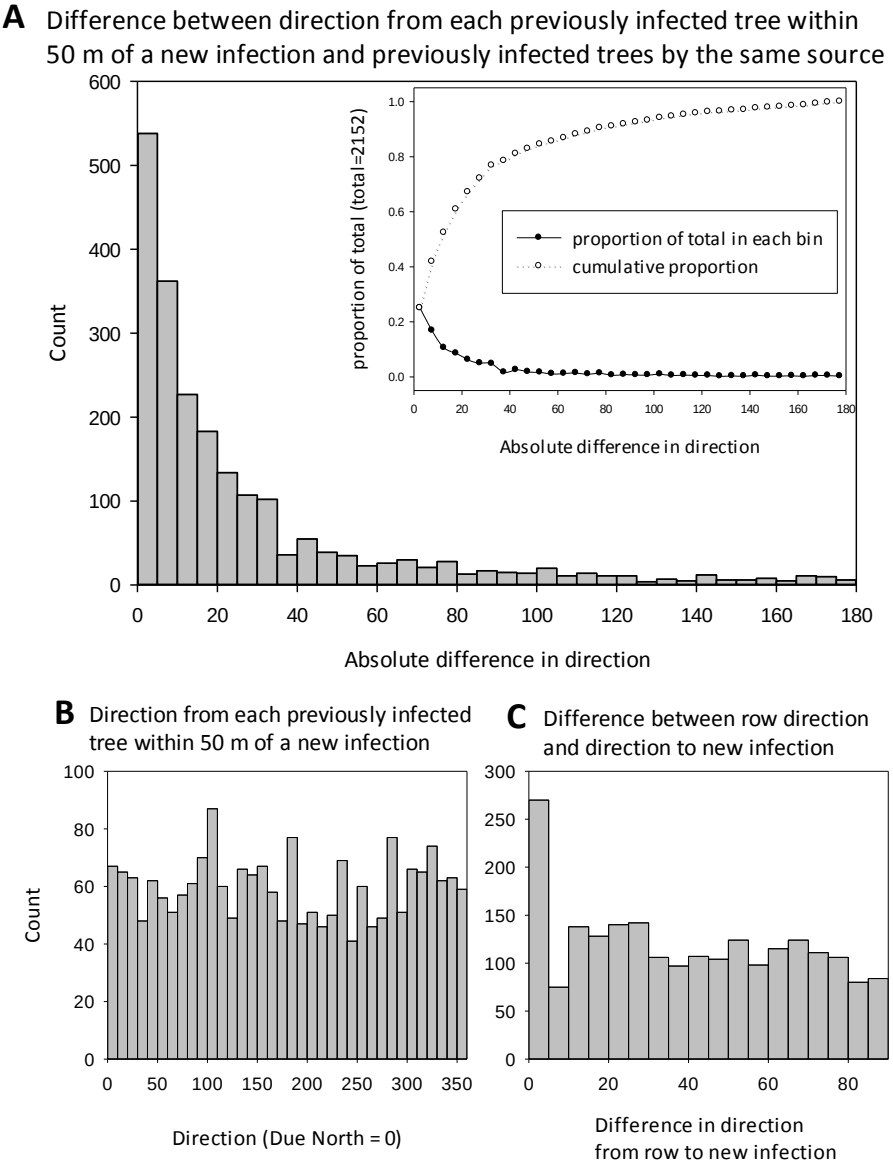

Figure 2. A) Directional (North = 0°) differences from an infected tree to newly infected trees that are within 50 m. B) Direction from an infected tree to newly infected trees that are within 50 m. C) Directional difference between the angle of the row the infected tree is in and the angle to the newly infected tree.

3.9 P

Early detection surveillance for Huanglongbing in a plantation; from theory to practice.

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The detection of a new HLB epidemic in a planting often occurs when the disease has already reached high incidence. This is problematic and once the epidemic has become established it is difficult to recover the economic productivity of the grove. However, if new epidemics can be detected early enough then more can be done to control the disease. Early detection requires that surveillance surveys be in place before the disease arrives; that is, a number of trees within a healthy grove should be inspected at regular intervals for symptoms of HLB. Exactly how many trees should be surveyed and how frequently this should be done is a non-trivial problem and one that has not previously been addressed in plant pathology. We present a theoretical method that relates the dynamics of an invading epidemic to the dynamics of a monitoring program. The method determines exactly how an early detection survey should be designed in order to achieve a high probability of detecting an epidemic whilst it is at an early stage. We compare the theoretical method to a complex simulation model which replicates the spatial and temporal dynamics of HLB in the field. By running the model thousands of times we can make probabilistic predictions on early detection survey design that can be directly compared with the theoretical method. We find striking similarities between the simple theoretical and more complicated simulation approach that enables us to make valuable new insights as well as deliver methods for transfer into practice.

Session 4:

Asian Citrus Psyllid Biology and Genomics

4.1

Behavioral, Ultrastructural, and Chemical Studies on the 'Honeydew' Excretions in Nymphs and Adults of the Asian Citrus Psyllid

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The Asian citrus psyllid (ACP) *Diaphorina citri* (Homoptera, Psyllidae) is the main vector of citrus huanglongbing (citrus greening) bacterium, the most serious citrus pathogen worldwide. Behavioral and ultrastructural studies on 'honeydew' excretions by ACP indicated interesting differences between nymphs, males and females. The anal opening in ACP, near the posterior end of the abdomen, is on the ventral side in nymphs and on the dorsal side in adult males and females. Video recordings show that males produce clear sticky droplets of honeydew gently laid behind them on the leaf surface, whereas the females powerfully expel whitish, different shaped, pellets that travel away from the female, probably to get these sticky excretions away from their eggs and newly hatched nymphs. ACP nymphs produce long ribbons or tubes of honeydew excretions that frequently stay attached to the exuviae after molting. Honeydew excretions of both nymphs and adult females are covered with a thin layer of whitish wax-like material ultrastructurally composed of a convoluted network of thin filaments apparently produced by the "wax" glands underneath the anal ring which is absent in males of this and other psyllids. The chemical composition of these excretions are being investigated using infrared microscopy and gas chromatography/mass spectroscopy.

4.2

Sequencing and annotation of the Wolbachia endosymbiont of Diaphorina citri by the CG-HLB Genome Resources group reveals candidate sources of interaction with the insect host

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The CG-HLB Genome Resources group serves as a bioinformatics resource for diverse projects related to the biology of citrus greening/HLB. A major recent project concerns the generation and annotation of a draft genome sequence for the Wolbachia endosymbiont (wDi) of the Asian citrus psyllid, of particular interest given the potential for control of psyllid behavior through manipulation of its bacterial endosymbionts. The Wolbachia draft genome was assembled and contigs aligned using the wPip strain from mosquito, its closest relative among completed Wolbachia genome sequences. OrthoMCL analysis of the annotated draft genome sequence confirmed the presence of 670 genes common to all sequenced Wolbachia genomes. Candidate host interaction factors include 54 predicted ankyrin proteins hypothesized to play a role in host reproductive manipulation, a Type IV secretion system linked to ankyrin protein export, and a bacterioferritin linked to host iron homeostasis. Several metabolic capabilities were identified in wDi that are absent from Liberibacter. FtsZ and Wsp phylogenies indicate that the Wolbachia strain in the Florida D. citri isolate falls into a sub-clade of supergroup B, distinct from Wolbachia present in Chinese D. citri isolates, supporting the hypothesis that the *D. citri* introduced into Florida did not originate from China. The Wolbachia sequence and annotation can be viewed on the CG-HLB Genome Resources Website (http://citrusgreening.org/), together with the sequences of publically available Liberibacter genomes sequenced to date. Future plans involve development of a searchable Liberibacter diagnostic sequence database using the over 1700 publically available *Ca*. Liberibacter gene sequences.

4.3

Sexual transmission of a plant pathogenic bacterium, *Candidatus* Liberibacter asiaticus, between conspecific insect vectors during mating

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Candidatus Liberibacter asiaticus (Las) is a fastidious, phloem-inhabiting, gram-negative bacterium transmitted by Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). The bacterium is the presumed causal agent of huanglongbing (HLB), one of the most destructive and economically important diseases of citrus. In this study, we investigated whether Las is transmitted between infected and uninfected *D. citri* adults during courtship. Our results demonstrate that Las is sexually transmitted from Las-infected male *D. citri* to uninfected females at a low rate (4%) during mating. Sexual transmission was not observed following mating of infected females and uninfected males or among adult pairs of the same sex. Las was detected in genitalia of both sexes and in eggs of infected females. A minimum latent period of 7 days was required to detect the bacterium in recipient females. Rod shaped and spherical structures resembling Las were observed in ovaries of Las-infected females with transmission electron microscopy, but were absent in ovaries from uninfected *D. citri* females. The size of the rod shaped structures varied from 0.39 to 0.67 mm in length and 0.19 to 0.39 mm in width. The spherical structures measured from 0.61 to 0.80 mm in diameter. This investigation provides convincing evidence that a plant pathogenic bacterium is sexually transmitted from male to female insects during courtship and established evidence that bacteria persist in reproductive organs. Moreover, these findings provide an alternative sexually horizontal mechanism for the spread of Las within populations of *D. citri*, even in the absence of infected host plants.

Citations

Mann, R.S., K. Pelz-Stelinski, S.L. Hermann, S. Tiwari, and L.L. Stelinski. 2011. Sexual transmission of a plant pathogenic bacterium, *Candidatus* Liberibacter asiaticus, between conspecific insect vectors during mating. *PLoS ONE.* 6(12): e29197.

4.4

Responses of Asian Citrus Psyllids to Substrate-borne Vibrational Communication Signals

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The Asian Citrus Psyllid (ACP), *Diaphorina citri* Kuwayama, vectors a harmful bacterium, Candidatus Liberibacter asiaticus, which causes huanglongbing, an economically devastating disease of citrus. Improved methods for detection and trapping of ACP could significantly reduce the damage associated with the spread of this disease. One previously unexploited method of detection involves the vibratory, substrate-borne signals by which adult male and female ACP communicate over 10-50-cm distances within their citrus tree hosts. Mate-seeking males begin calling while moving along the tree branches, searching for females. When a receptive female detects these signals, she replies within about 0.5 s in a duet that facilitates location of her position. A series of studies was conducted in a quiet laboratory setting to manipulate these vibrational communications for attraction and trapping of male ACP. First, male recorded calls were played back to females and the frequencies, durations, and loudness of calls that elicited the greatest female response were analyzed for further study. In addition, female recorded calls were played back to males, attracting them to the source of the calls. Based on these results, we began development of an automated system with a computer-driven vibratory element that replies to male calls immediately after detecting them. Searching males move quickly towards the source of such calls and can thereby be trapped. To facilitate trap development we have begun testing the relative attractiveness of carefully constructed synthetic calls. We present preliminary results of the attraction bioassays.

4.5 P

An evaluation of different plant species for rearing Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)

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Many research projects concerning the Asian citrus psyllid (ACP) are dependent on a steady supply of ACP being readily available. ACP is not a difficult insect to rear in a number of respects, and basic information on rearing procedures has been published (Skelley and Hoy 2004). Skelley and Hoy (2004) reported on rearing procedures using the host plant *Murraya exotica* (=*paniculata*) L. USDA-ARS in Fort Pierce, Florida has reared ACP on *M. exotica* (Hall et al. 2007) and also on *Citrus macrophylla* Wester (Hall et al. 2012). Recently, a field study of 87 genotypes showed that a number of these were vastly favored over others by ACP for colonization (Westbrook et al. 2011). An experiment was recently initiated to compare nine favored genotypes as potential rearing hosts. These genotypes were: *Afraegle paniculata* (Schumach.) Engl. (CRC #297), *Bergera koenigii L.* (CRC #3165), *Citrus aurantifolia* (Christm.) Swingle (CRC #3822), *C. macrophylla* (CRC # 3842), *Citrus maxima* (Burm.) Merr. (CRC #3945), *Citrus medica* L. (CRC #3523), *Citrus taiwanica* Tanaka & Y. Shimada (CRC # 2588), *Citrus reticulata* Blanco (CRC #2590), and *M. exotica* (CRC # 1637). This presentation will present quantitative data on the following plant parameters: percent and speed of seed germination; speed of seedling growth; number of branches produced per plant without trimming; speed of flushing after being trimmed; number of branches per plant stimulated by trimming; number of leaflets per flush shoot; and flush shoot length. Additionally, qualitative data will be presented on general aspects of working with these plant species.

Citations

Hall, D. G., and M. L. Richardson. 2012. Toxicity of insecticidal soaps to the Asian citrus psyllid (*Diaphorina citri*) and two of its natural enemies. J. Applied. Entomol. doi: 10.1111/j.1439-0418.2012.01749.x

Hall, D. G., S. L. Lapointe, and E. J. Wenninger. 2007. Effects of a particle film on biology and behavior of *Diaphorina citri* (Hemiptera: Psyllidae) and its infestations in citrus. J. Econ. Entomol. 100: 847-854.

Skelley, L. H., and M. A. Hoy. 2004. A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine. Biological Control 29: 14-23.

Westbrook, C. J., D. G. Hall, E. Stover, Y. Duan, and R. F. Lee. 2011. Colonization of *Citrus* and *Citrus*–related germplasm by *Diaphorina citri* (Hemiptera: Psyllidae). HortScience 46: 997-1005.

4.6 P

Dispersal behavior of *Diaphorina citri* Kuwayama (Homotera: Psyllidae) under laboratory condition

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Abstract: Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, is the vector of Huanglongbing (HLB), the most devastating disease of citrus worldwide. Knowledge of ACP behavior to search host plants is helpful for understanding HLB spreading within or between citrus trees. In present research in laboratory, the behaviors of ACP adults to evaluate host plants were observed. ACP adults could disperse to seedlings of *Rhododendron simsii* (non host plant for ACP), *Murraya panciculata* L. and *"*Lugan"*Citrus reticulata* Banco when the plants were put together. The mean number of adults per plant on *R. simsii* was significantly lower than those on citrus and murraya at 18 h and 42 h after ACP were released, respectively. The numbers of adults on citrus and murraya became significantly different 90 h after treatment. The numbers of ACP adults per plant were not significantly different among the 3 types of murraya seedlings classified by development degrees of flushing shoots. Within a tree, the ratio of ACP adults on the new flushing shoots did not significantly increased in comparison to other parts of the tree during 1-4 d after the psyllids were released. Even on 7th day after ACP release, about 30 % adults habituated on the parts of trees other than flushing shoots. The above indicated that ACP adults spent less time to differentiate between host and non-host plants in comparison to the differentiation between different host plants species or different parts of a host plant.

Keywords: *Diaphorina citri* Kuwayama; Huanglongbing, Dispersal behavior

4.7 P

The Asian Citrus Psyllid Genome (*Diaphorina citri*, Hemiptera)

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The Psyllid genome is a scientific breakthrough in that it opens the psyllid genetic blueprint to investigations of all questions ranging from taxonomic origins to the understanding of developmental biology, to the acquisition and transmission of pathogens. The Asian citrus psyllid, *Diaphorina citri* (Hemiptera) threatens the citrus industry as a vector of the plant-infecting bacteria, *Candidatus* Liberibacter asiaticus, associated with the devastating disease, Huanglongbing. Psyllids are major disease vectors of many fruit tree crops yet their genetics have remained poorly studied. The first genome draft of *D. citri*, DIACI_1.0 was completed in 2011 (ARS, Ft. Pierce, FL), however, gaps in the assembly prompted additional sequencing using the long run PacBio system at the Los Alamos National Lab, NM. The newly assembled genome DIACI_2.0 was assembled using the new software PB-Jelly, with an improved N50 of 38 kb, up from 25kb, and the number of resolved bases increased by over 10 million. The genome and transcriptome have been submitted into the public domain at the National Center for Biotechnology Information, NCBI, to be processed - **[**http://www.ncbi.nlm.nih.gov/genome?LinkName=bioproject_genome&from_uid=29447**]** and for access by the larger research community. The psyllid transcriptome identified over 25,600 predicted genes, and is supported by an additional 19,598 previous EST's. Life stage specific transcripts were identified for Adults, Nymphs and Eggs. BlastX analyses showed the most similarity to the Pea Aphid, *Acyrthosiphon pisum*, another hemipteran. The transcriptome data was provided for the **Inno**centive® Challenge program 2011, to increase efforts for RNAi development against psyllids. Other researchers are also using these data to develop strategies to suppress psyllid populations. Efforts are now focused on annotation of the psyllid genome which will provide more information on the genetic basis of psyllid biology.

4.8 P

Molecular interaction between citrus bacterial pathogen *Liberbacter asiaticus* and its insect vector Asian citrus psyllid *Diaphorina citri*

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Abstract

Huanglongbing (HLB), the most serious disease of citrus, involves Candidatus Liberibacter asiaticus(CLas), a Gram-negative phloem-restricted α-Proteobacterium transmitted by Asian citrus psyllid *Diaphorina citri*. Despite insect vector as an important factor in HLB transmission, to the best of our knowledge, little research has so far been conducted on the molecular interaction between CLas and Asian citrus psyllid. Many Gram-negative bacterial pathogens have been shown to adhere to insect cell surface by interactions between receptors and ligands, establishing protein complexes that help them enter into insect cells. In the present study, Far-western (protein overlay assay) was used to seek receptors, two-dimensional blue native/SDS-PAGE to explore complexome (receptor-ligand), and MALDI TOF MS/MS to identify the receptors and ligands. We showed how Clas adhered to psyllid cells and which protein complexes were established on the cell membrane. Understanding how CLas interacts with the insect cells, will lead to build up a new strategy to control the disease.

Session 5:
Asian Citrus Psyllid Ecology and Transmission

5.1

A Comparative Transcriptomic Approach to Elucidate Psyllid-*Ca*. Liberibacter Interactions

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Citrus greening is the most destructive disease of citrus crops worldwide. The introduced Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama transmits the (putative) causal bacterium, *Candidatus* Liberibacter asiaticus. A close relative, *Ca.* L. solanacearum, is the pathogen associated with Zebra chip disease of potato and vein-greening disease of tomato. It is both transmitted by and propagative in the endemic (western U.S) potato psyllid (PoP) *Bactericerca cockerelli* Sulc. The PoP occurs widely in the western U.S. and so has been used as a parallel study system for the quarantined ACP-greening complex. To identify proteins involved in global psyllid-*Ca*. Liberibacter interactions, the ACP and PoP transcriptomes were sequenced, yielding a total of 45,976 and 82,224 Illumina unique ACP and PoP transcripts, respectively. Cluster analysis revealed a high degree of sequence and transcript conservation suggestive of roles in core growth and developmental processes, providing the first molecular snapshot of the specific psyllid genes responsive to parasite invasion and circulation in the host. Evidence of inter-psyllid molecular conservation substantiates the suitability of PoP as a study system for ACP-*Ca. L. asiatcus*. Comparative *in silico* expression analysis within and between psyllid species revealed predicted functions involved in *Ca*. Liberibacter parasitism that were both unique and shared in common among adult and nymphal instars. In addition functional characterization based on Gene Ontology analysis has revealed a number of genes associated with host-parasite interactions that could mediate *Ca*. Liberibacter infection, propagation, and circulation in the psyllid, as well as transmission processes.

5.2

Induced release of a plant-defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen

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In this investigation, we experimentally demonstrated specific mechanisms through which a bacterial plant pathogen induces plant responses that modify behavior of its insect vector. *Candidatus* Liberibacter asiaticus, a fastidious, phloem-limited bacterium responsible for causing huanglongbing disease of citrus, induced release of a specific volatile chemical, methyl salicylate, which increased attractiveness of infected plants to its insect vector, Asian citrus psyllid (*Diaphorina citri*), and caused vectors to initially prefer infected plants. However, the insect vectors subsequently dispersed to non-infected plants as their preferred location of prolonged settling because of likely sub-optimal nutritional content of infected plants. The duration of initial feeding on infected plants was sufficiently long for the vectors to acquire the pathogen before they dispersed to non-infected plants, suggesting that the bacterial pathogen manipulates behavior of its insect vector to promote its own proliferation. The behavior of psyllids in response to infected versus non-infected plants was not influenced by whether or not they were carriers of the pathogen and was similar under both light and dark conditions. Feeding on citrus by *D. citri* adults also induced the release of methyl salicylate, suggesting that it may be a cue revealing location of conspecifics on host plants. Collectively, our results suggest that host selection behavior of *D. citri* may be modified by bacterial infection of plants, which alters release of specific headspace volatiles and plant nutritional contents. Furthermore, we show in a laboratory setting that this apparent pathogen-mediated manipulation of vector behavior may facilitate pathogen spread.

Mann, R.S., J.G. Ali, S.L. Hermann, S. Tiwari, K.S. Pelz-Stelinski, H.T. Alborn, and L.L. Stelinski. 2012. Induced release of a plant defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen. *PLoS Pathogens*. 8(3): e1002610.

5.3

Disrupt the bacterial growth in the insect vector to block the transmission of Candidatus Liberibacter Asiaticus to citrus, the causal agent of citrus greening disease

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Genome of Candidatus Liberibacter Asiaticus (CLas) reveals the presence of luxR that encondes LuxR protein, one of a two components cell-to-cell communication system. But the genome lacks the second component; luxl that produces Acyl-Homoserine lactones (AHLs) suggesting that CLas has a solo LuxR system. Interestingly, we detected compound that may act as AHLs in the insect vector (psyllids) healthy or infected with CLas but not in the citrus plants. This finding suggests that the insect is the source for AHL. The fact that CLas forms biofilm on the surface of insect gut reveals the presence of cell-cell communication system. Here the system is solo LuxR. Moreover, we have confirmed the activity of CLas-LuxR by its expression in E. coli and detection of LuxR-AHL Complex. In order to block the vector transmission of CLas, we produced plants that express LuxR. Insects will acquire CLas and luxR. LuxR will compete with the bacteria for binding to AHL and consequently, CLas will not be able to colonize the insect or perform biofilm and fails in the transmission. We aim to provide an environmental friendly solution for the most destructive disease in citrus (Huanglongbing) by producing specific LuxR in citrus to interfere with the vector transmission. As an alternative way, we aim to use synthetic molecules that mimic the specific AHL as an application to disrupt CLas transmission from plant to plant by its vector. More AHL in insect may confuse the bacteria and induce a strong sticky biofilm that hardly release cells to plant during the insect feeding. Accordingly, the transmission from plant to plant will be diminished or blocked

5.4

Stylet penetration activities of *Diaphorina citri* associated with transmission of *Candidatus* Liberibacter asiaticus

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The electrical penetration graph (EPG) technique was used to determine *Diaphorina citri* stylet penetration activities associated with *Candidatus* Liberibacter asiaticus (Las) acquisition and inoculation in citrus. In a first experiment, healthy *D. citri* adults were connected to the EPG system and placed on Las-infected plants. Probes were artificially terminated after the following stylet penetration periods and waveforms: I) 20 min in waveform C (pathway phase through epidermis and parenchyma); II) C + 30 s in D (first contact with phloem tissue); III) C+D + 70 s in E1 (penetration and possibly salivation/ingestion in the phloem sieve elements); and IV) C+D+E1+ 1 h in E2 (phloem sap ingestion). The insects were tested for Las infectivity by real-time PCR 3 wks later. In a second experiment, 3rd-instar nymphs were first submitted to an AAP of 2 wks on Las-infected plants and then connected to the EPG system on healthy citrus seedlings during the same stylet penetration periods of the first experiment. *D. citri* acquired the pathogen only after penetration in the phloem sieve elements and mostly during waveform E2 (27 infective insects of 54 tested). Only 2 of 52 insects exposed to infected plants until waveform E1 (treatment III) were positive. In the second experiment, transmission (by 9 of 50 insects tested) was observed only for psyllids allowed to perform E2 on healthy seedlings. Overall, the data show that both acquisition and inoculation occur during the phloem phase, primarily during sustained sap ingestion (E2). This information is important to design efficient control tactics aimed to prevent Las transmission by *D. citri.*

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5.5

Characterization of the RNA Interference Response in the Asian Citrus Psyllid

Lindsay Shaffer, R. G. Shatters, Jr., C. Powell, R. Cave, D. Borovsky

The Asian citrus psyllid (ACP),*Diaphorina citri*, is a major pest of citrus since it is the only known vector of '*Candidatus* Liberibactor' species, the bacterium associated with citrus greening disease. Since control of the psyllid is the only effective defense so far against citrus greening, and heavy reliance upon pesticides is not sustainable, an RNA interference (RNAi) strategy for ACP control was investigated. RNA interference is an innate immune response triggered by the cellular uptake of double stranded RNA (dsRNA) and studies were conducted to determine if ACP could be killed by oral uptake of dsRNA that target essential ACP transcripts. An artificial diet system was designed that facilitated the ingestion of dsRNA complementary to genes involved in digestion. Using this system, ACP mortality was observed as a result of oral uptake of dsRNAs targeting an apparent essential ACP gene and this mortality was shown to be dose responsive, reaching a maximum of 37% at high dsRNA concentrations. There was also what appeared to be sequence independent ACP toxicity of large doses of dsRNA, concentrations above 48 ng/uL in the diet. However, this sequence independent mortality was not as high as that observed for the targeted ACP gene, never rising above 17%. These results provide support for the concept that RNAi could be adapted for use as a control strategy for the ACP.

5.6

Translating Anatomical Structures and Functional Genomics of *Candidatus* Liberibacter asiaticus and solanacearum Into Circulative, Propagative Vector-Mediated Transmission Processes

T. Fisher, J. Cicero, M. Vyas, R. He, W. Nelson, M. Willer, C. Soderlund, D. Gang, and J.K. Brown

Ca. Liberibacter asiaticus is the putative fastidious bacterial causal agent of citrus greening disease, also known as Huanglongbing (HLB), translated from Chinese as yellow dragon disease. The HLB bacterial pathogen is indigenous to Asia but has been introduced and dispersed to citrus throughout the Americas. A related bacterium that is indigenous to the Americas causes damage to potato (zebra chip) and tomato (vein-greening) and other solanaceous hosts. The causal agents are propagative and circulative in the psyllid vector, *Diaphorina citri* (Kuwayama) and *Bactericera cockerelli* (Sulc.), the Asian citrus and potato (or tomato) psyllid, respectively. The specific psyllid proteins that are indirectly or directly involved in the circulative, propagative transmission pathway are not known. However, if proteins were known that function at key points in the pathway e.g. post-ingestion, infection, biofilm formation, nutrition, circulation, and/or acquisition were known, such knowledge could be exploited to knock out their expression and abate pathogen transmission. To this end a combined approach involving functional genomics and anatomical localization of the bacterium is being implemented. Results indicate that *Ca*. Liberibacter establishes biofilms on the outer surfaces of the alimentary canal and salivary glands of the Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama and the potato psyllid (PP) *Bactericera cockerelli* Sulc. *In silico* transcript profiling of infected and uninfected ACP and PP identified a number of mis-expressed, unique transcripts (unitrans). Functional predictions (gene ontology associations) implicate certain of these unitrans in *Ca*. Liberibacter infection of the psyllid host and/or in psyllid-mediated *Ca*. Liberibacter transmission processes.

5.7

Low acquisition rates of '*Candidatus* Liberibacter asiaticus' by *Diaphorina citri* Kuwayama from citrus plants exposed to high temperatures

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'*Candidatus* Liberibacter asiaticus' (Las) is the most prevalent liberibacter species associated with huanglongbing (HLB) in Brazil. Within the state of São Paulo (SP), the disease spread more rapid to regions with relatively mild summer temperatures. This suggests that climate can influence disease spread. In order to test this hypothesis, Las titers in immature flush growth of Valencia orange plants exposed to different temperatures regimens, and Las acquisition by adult *Diaphorina citri* allowed to feed on flush growth of these plants, were determined in two experiments. The first experiment comprised plants with three levels of infection, three incubation periods (IP), and environments favorable (14.6-28°C) and unfavorable (24–38°C) to Las. The second experiment comprised plants with severe, late stage, infections, 10 IPs (based on 3 d intervals over 27 d) and 3 environments (12–24°, 18–30° and 24–38°C). After each IP, plants were removed from each environment, and adult *D. citri* were confined on new flushes for 48-h. After confinement, flushes and insects were analyzed by qPCR. Overall, Las titers were lower in flush growth of plants maintained in the 24–38°C environment than in the other environments, and the percentages of Las+ psyllids that fed on flush growth of these plants were lower than in psyllids that fed on flush growth of plants maintained in the other environments. The results indicate that the incidence and less rapid spread of Las in warmer than in cooler regions of SP may be related to the influence of ambient temperatures on multiplication of Las in leaves.

5.8 P

SEM- and TEM-informed anatomical observations of Ca. Liberibacter solanacearum (Lso) parasite localization in its psyllid host

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With SEM, we studied external midgut surfaces of all potato psyllid instars, and, additionally with SEM and TEM, the anterior alimentary system of adults. The ontogenetically earliest point of Lso detection was in the 3rd instar, then in consecutively greater percentages of 4th and 5th instars, then in a lower percentage of teneral and mature adults. Two age-based patterns of proliferation were identified in the oral region of mature adults- streaming, in which Lso were confined to passageways, and diffuse, in which Lso occurred inside of, and around, cells of mouthpart components, muscles and epidermis.

The route(s) by which Lso access the immature and adult midgut and stylets are unknown and may be multidirectional. A continuum of proliferation occurred along the adult external midgut, along the external esophagus, inside the salivary glands, and inside the oral region, indicating that Lso can 'strongarm' the anatomy during parasitism, potentially reaching any region of it, so that at least some can access key oral loci to complete the circulative, propagative pathway.

The oral region houses the convergence of foregut, salivary duct and stylet canals. Apparently, acquisition involves circumventing its cuticular linings to access its lumina. Circumvention might occur at the 'loadable' section of the salivary glands, and/or, potentially, several oral loci where the cuticle is thin and weak. As most of its construction is poorly understood, transmission pathway studies depend on its elucidation. We used SEM, TEM and LM to identify and map it for associating Lso with named components.

5.9 P

Seasonal shifts in *Candidatus* Liberibacter asiaticus prevalence in the vector *Diaphorina citri* in Florida

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Psyllid populations at six locations in central Florida and one location in southern Florida were sampled monthly and the proportion of adult psyllids carrying Liberibacter asiaticus was measured using QPCR of pooled samples. The Florida Automated Weather Network was used to estimate environmental conditions at these locations. Prevalence was highest during the last three months of the year, but psyllids with Liberibacter asiaticus could be found at all times. Fluctuations in prevalence associated with gender and abdominal color are also examined.

5.10 P

Stylet Morphometrics and Ultrastructure in Relation to Feeding Behavior and Pathogen Transmission by Nymphs and Adults of the Asian Citrus Psyllid *Diaphorina citr*i, Vector of Citrus Huanglongbing Bacterium

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The feeding behavior and stylet morphometrics were studied in nymphs and adults of the Asian citrus psyllid (ACP), *Diaphorina citr*i (Hemiptera, Psyllidae), vector of *Candidatus* Liberibacter asiaticus (Las) associated with citrus huanglongbing (HLB) disease. The stylet length of first instar nymphs averaged 266 µm (83% of body length) whereas that of 5th instar nymphs was 615 µm (34% of body length). Younger ACP nymphs feed only on young citrus leaves on smaller veins or on the sides of the midrib, whereas adults can feed anywhere on the veins of young or old citrus leaves. Epifluorescence microscopy of cross sections in citrus leaves indicated that the thick-walled fibrous layer around the phloem is much more prominent in older than in younger leaves. Additionally, first instar nymphs can reach the phloem because the distance to the phloem is shorter from the sides of the midrib compared to that from the top, and is considerably shorter in younger than in older/mature leaves. Ultrastructural studies on ACP stylets show that the width of the maxillary food canal in first instar nymphs is wide enough for Las bacteria to go through during food ingestion (and Las acquisition). However, the width of the maxillary salivary canal in first instar nymphs may not be wide enough for Las bacteria to go through during salivation (and inoculation of Las bacterium) into host plants. This may explain previous studies indicating that older ACP nymphs and adults can transmit HLB bacterium whereas younger nymphs probably cannot.

5.11 P

Acquisition and Transmission Efficiency of the HLB Bacterium, '*Candidatus* Liberibacter asiaticus' by the Striped Mealybug, *Ferrisia virgata*

Marco Pitino, Michele T. Hoffman, Lijuan Zhou, David Hall and Yong-Ping Duan

ABSTRACT

'*Candidatus* Liberibacter asiaticus' (Las) is the prevalent species of three different Liberibacter associated with citrus huanglongbing (HLB). Two psyllid species, *Diaphorina citri* and *Trioza erytreae*, are currently known to transmit Liberibacter bacteria. In this study, we tested the acquisition and transmission efficiency of Las by striped mealybugs (*Ferrisia virgata*) (Pseudococcidae; Hemiptera), another phloem-sap feeding insect with a broad host range of 264 species in 68 plant families. In our previous report, 63.0 % of striped mealybugs collected from the Las-infected periwinkle plants in USHRL greenhouse tested positive for Las using the HLBasp primers and probe, and the Las populations were estimated at 3.11×10^3 to 2.32×10^5 cells per mealybug. This was confirmed using conventional PCR with six primer sets targeting different Las loci and by the 100% identity of all seven PCR amplicons to the known Las sequences. However, attempts to transmit the disease in periwinkle and citrus using Las-infected mealybugs were not successful. To reveal the reason why Las-infected mealybugs were not able to transmit the disease, we used a leaf-disc bioassay in conjunction with typing of Las populations. Positive Las results were found in 100% of the mealybugs after feeding for 1-2 weeks on infected leaf discs that were obtained from infected periwinkle and citrus leaves. In addition, Las bacteria were detected in mealybug gut, salivary glands and body cavity, with the titer in the gut and body being higher than that in the salivary glands. It is of interest to note that mealybugs grown on infected leaf-discs for 1 week and then transferred to non-infected leaf-discs did not test positive for Las. However, mealybugs grown on infected leaf-discs for 2 weeks and then transferred to non-infected leaf-discs for 2 more weeks remained positive for Las. These results indicate that striped mealybugs share similarities and differences in comparison with the Asian citrus psyllids in terms of acquisition and/or transmission of the Las bacterium.

5.12 P

RNA Interference Screening Reveals Redox Processes to be Most Responsive to low dsRNA doses in Asian Citrus Psyllid

Ramos, John

The Asian Citrus Psyllid (Diaphorina citri Kuwayama) is an invasive Homopteran that has crippled citrus production in Florida with the spread of the Huanglongbing (Citrus Greening) disease, which yields small discolored and bitter fruit. The disease is associated with the bacterium '*Candidatus*' Liberibacter and is rapidly spreading to other citrus producing states. Gene targets were competitively deposited by experts from diverse fields for RNAi screening through an Innocentive challenge and the top fifty targets are presented here. Of these, seven targets resulted in the greatest mortality from dsRNA feeding trials, five of which are involved in redox biochemistry. Other targets that yielded in low dose with high mortality include those involved in ABC Transport, tetrahydrobiopterin biosynthesis and reproduction. These results suggest that system targeted gene silencing by RNA Interference can induce mortality at low doses, providing a promising new strategy for the development of non-chemical pesticides.

5.13 P

Composition of citrus phloem sap and honeydew produced by the citrus phloem sap feeder, the Asian citrus psyllid, *Diaphorina citri* (Homopetera: Psyllidae)

Hijaz, araj and Killiny, Nabil 10-5-2012(1)

The honeydew composition of Asian citrus psyllids (ACP), the vector of citrus Huanglongbing (HLB), was studied using gas chromatography-mass spectrometry (GC-MS). Honeydew samples were collected from healthy ACP that were reared on one-year Valencia trees inside an insectary at a University of Florida's Citrus Research and Education Center (CREC) grove in Lake Alfred, FL, USA. Dried samples (1 mg) were mixed with 30 µL of methoxyamine hydrochloride solution in pyridine (2%) and allowed to react for 17 h at room temperature. After methoximation, silylation reactions were induced by adding 80 µL of N-methyl-N-trimethylsilyl) trifluoracetamide (MSTFA) for 2 h at room temperature and 0.5 µL of derivatized sample was injected into the GC-MS running in the full scan mode. To check for amino acids, a 10 mg of alkaline honeydew sample was reacted with methylchloroformate (MCF) in a mixture of pyridine and methanol. The MCF derivatives were extracted with chloroform and analyzed with GC-MS. The moisure content was determined by drying the honeydew samples to a constant weight at a 100 °C. Most The major honeydew composition was as follows: 74.5 ± 2.8 sucrose, 12.4 ± 0.5 D-fructose, 6.4 ± 3.0 mannose, 1.8 ± 0.6 trehalose, myo-inositol 2.8 ± 0.8, ribitol 0.5 ± 1, galactose 0.4 ± 0.2, quinic acid 0.4± 0.3, and malic acid 0.3 ± 0.1. The moisture content of honeydew was 22.6 ± 2.6 No amino acids were detected as TMS or as MCF derivatives.

Session 6:
Asian Citrus Psyllid Management

6.1

Huanglongbing management on bearing groves based on favorable periods for symptomatic-trees removal and vector control

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In Brazil, Huanglongbing (HLB) has been managed by removal of symptomatic-trees and Asian citrus psyllid (ACP) control. Although new HLB-affected trees and ACP can be detected all year long, visual detection of HLB-affected trees has been more pronounced from March to August while ACP population densities are higher from September to February. Therefore, the aim of this work was to compare the efficiency of applying the control strategies only during the higher occurrence periods of new HLB affected trees and ACP adults with the control application during all year long. The experiment was carried out in a 4-yr old sweet orange Valencia/Rangpur lime grove and had a 2 by 3factorial design with 3 replications (1.4 ha plots). The factor "HLB-tree elimination" had 2 treatments: monthly elimination all year long; and monthly elimination from March to August, both based on visual inspection. The factor "Vector control" had 3 treatments: monthly ACP control all year long; monthly ACP control from September to February; and ACP control when 10% of 48 yellow sticky traps (YST) placed in the center of plots had at least one adult psyllid. All Treatments of ACP control were done alternating foliar sprays of Provado®, Dimetoato®, Trebon® and Marshal®+Micromite®. After 5 years, no significant differences were detected among different treatments for the variables mean cumulative HLB incidence and disease progress rate estimated by linear regression of the last 4 years cumulative disease incidence. The mean cumulative HLB incidence increased from 0.4% to 14.2% (Yr1 4.9%, Yr2 1.9%, Yr3 2.3%, Yr4 1.7%, and Yr5 3.0%). The number of caught ACP per YST per assessment and the area under the curve of percentage of YST with ACP were significantly higher for monthly ACP control from September to February (total of 34 sprays), but did not differed between monthly ACP control all year long (total of 65 sprays) and control based on ACP monitoring with YST (total of 21 sprays). We believe that HLB management wasn't better because there was a significant amount of new HLB-symptomatic trees (25.2%) found from December to February, and 12.3% of ACP caught in August. In conclusion, with some adjusts the management of HLB could be optimized according to the favorable periods for HLB-symptomatic trees detection and ACP populations.

Project supported by Fundecitrus, Fapesp (2007/55013-9) and CNPq (303675/2009-8).

6.2

Analysis of Methods/Systems for Delivery of Volatile Repellent Compounds to Protect Young Citrus Plantings from HLB

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Control of Asian citrus psyllids is critical for the citrus industry to survive. Citrus growers urgently need to be provided with new tools using recent technological developments for best control practices. One approach is the use of plant-based or natural volatile repellent compounds. Although there have been significant advances in laboratory studies, there is still no effective repellency system developed for field usage. Most importantly, even if the most effective psyllid repellent is utilized, its field performance may be less than desired if the most effective method of delivery is not employed. A system approach is required – the volatile repellent must be "matched" to the delivery methodology – to achieve the optimal overall repellency system, which also requires a physicochemical and engineering understanding of the repellency system employed to provide the sought-after control strategy. This presentation will examine both wax-based and vapor-based dispensing approaches. The volatile substances released from wax-based formulations are analogous to "contact repellents", whereas those released from vapor-based systems function as "spatial repellents" – two different approaches for delivery of volatile repellent compounds. We present a break-through in the art of controlled delivery of volatile compounds using a system approach. The Auburn vapor-based invention has many advantages, one of which is the flexibility it offers in the design and engineering of vapor delivery systems for biocontrol strategies in the citrus industry.

6.3

RNAi-Based Strategy for Asian Citrus Psyllid (*Diaphorina citri*) Control: A Method to Reduce the Spread of Citrus Greening Disease.

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Citrus greening disease is a serious bacterial disease of citrus worldwide and is vectored by the Asian citrus pysllid (*Diaphorina Citri*). The only effective control strategy includes vigorous control of the psyllid, primarily through heavy reliance on pesticides. As a more sustainable and environmentally friendly method of psyllid control, we evaluated a RNA interference (RNAi) approach based on psyllid oral uptake of dsRNA molecules that target specific psyllid genes. This approach is based on the finding that cellular uptake of dsRNAs, that match the sequence of essential genes, results in down regulation of those genes and can lead to cell/organism death. These dsRNA molecules were introduced into the psyllids through feeding on citrus engineered to express the dsRNA using a Citrus tristeza virus as a paratransgenesis vector. Increased toxicity was observed when adult psyllids were fed on citrus producing dsRNA targeting either gut protease genes. No increased psyllid toxicity was observed in psyllids fed on citrus producing green fluorescent protein (GFP) dsRNA. Toxicity related to specific psyllid gene knockout will be discussed. These results suggest that RNAi-based control may be a viable alternative to current pesticide use for control of psyllids and all phloem feeding pests.

6.4

Physiological selectivity of pesticides used in citrus culture on parasitoid *Tamarixia radiata* (Waterson, 1922) (Hymenoptera: Eulophidae)

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The Brazilian citrus culture is the second largest pesticides consumer, demanding roughly 17.5 kg of active ingredient per hectare annually. This research evaluated the physiological selectivity of 50 pesticides (22 insecticides, 16 acaricides, 10 fungicides, 1 mineral oil and 1 vegetable oil) used in citrus on parasitoid *Tamarixia radiata*. For that purpose, discs of the Valencia sweet orange variety, 3.5 cm diameter were sprayed using a Burkhard-Pottertower adjusted to a pressure of 15 lb.pol^{-2}, enabling the application of 1.8 ± 0.1 mg of chemical solution.cm^{-2}, according to the methodology proposed by IOBC/WPRS. After application, the discs were kept at room temperature for three hours to dry the residues. Next, the discs were placed in Petri dishes containing 2 mL of a not gelled agar-water solution at 2.5%. Then, adult parasitoids with no more than 48 hours after emergence were exposed to residues. The experimental design was completely randomized with 51 treatments and five replicates, and each replication comprised 10 adults of the parasitoid. The parasitoids survival was recorded 24 hours after exposure of adults to the toxic residues. Insecticides azadirachtin, etofenproxi, gamma-cyhalothrin, pyriproxyfen, tebufenozide, and diflubenzuron; the acaricides pyridaben, etoxazole, diflubenzuron, and fenpyroximate hexitiazoxi, and fungicides azoxystrobin, folpet, copper hydroxide, copper oxychloride, mancozeb + copper oxychloride, pyraclostrobin, thiophanate-methyl, and trifloxystrobin were innocuous to parasitoid *T. radiata*. The other pesticides should be evaluated under semi-field and field conditions to verify the impact on the parasitoid *T. radiata* in citrus.

Citation:

ABBOTT, W.S. A method of computing the effectiveness of an insecticide. **Journal Economic Entomology**, Lanhan, v.18, n.2, p.265-267, 1925.

HASSAN, S.A. Métodos padronizados para testes de seletividade, com ênfase em Trichogramma, p.207-233. In: PARRA, J.R.P.; ZUCCHI, R.A. (eds.). ***Trichogramma* e o controle biológico aplicado**. Piracicaba, FEALQ, 1997, 324p.

NEVES, M.F.; TROMBIN, V.G.; MILAN, P.; LOPES, F.F.; CRESSONI, F.; KALAKI, R. **O retrato da citricultura brasileira**, 2011. Disponível em: <http://www.citrusbr.com.br/download/biblioteca/o_retrato_da_citricultura_brasileira_baixa.pdf >. Acesso em: 14 jul. 2011.

6.5

Synthesis results from eight years of field testing insecticides against Asian citrus psyllid *Diaphorina citri* vector of huanglongbing: Considerations and Implications

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Diaphorina citri also known as Asian citrus psyllid (ACP) vectors *Candidatus* Liberibacter asiaticus, causal organism of the Asian "huanglongbing" or citrus greening disease and therefore needs to be managed effectively. Forty-three insecticides containing 39 active ingredients (a.i) recommended or experimental were tested during the growing season in foliar sprays (171 treatments, 35 a.i) targeted at flushing trees and soil applications (26 treatments, 6 a.i) to control ACP in citrus between 2005-2012. Psyllid suppression varied with product and lasted 1-7 weeks using foliar sprays on mature trees and 6-33 weeks using soil drenches in young trees. Experimental insecticides tolfenpyrad (Apta 15 SC), flupyradifurone (Sivanto 200 SL), sulfoxaflor (Closer 240 SC), cyantraniliprole (Verimark), and *Chromobacterium substugae* (Grandevo/MBI-203 EP) performed equal to or better than recommended products. Unfortunately, eggs and young nymphs are protected inside unfolded leaves such that insecticidal sprays may kill more predators and parasitoids common during growing season that would otherwise attack immature ACP and other citrus pests. Addition of new insecticides will broaden the range of products available to control ACP. Nevertheless, one to two sprays of broad-spectrum insecticides during dormant winter period when most mature trees are not producing new growth and beneficial insects are scarce can provide up to 6 months of ACP suppression into growing season and also conserve beneficial insects. Therefore, psyllid suppression using one or two dormant winter sprays of broad-spectrum insecticides followed by regular monitoring and rotation of relatively selective chemistries during growing season will help to reduce incidence of huanglongbing, pest resistance to insecticides and secondary pest outbreaks.

Citations

1) Qureshi, J. A., B. C. Kostyk and P. A. Stansly. 2012. Registered and experimental insecticides for control of Asian citrus psyllid and citrus leafminer on mature orange trees. Proc. Fla. State Hort. Soc. 125: (In press).
2) Qureshi, J. A., B. C. Kostyk and P. A. Stansly. 2011. Effectiveness of selective insecticides to control Asian citrus psyllid and citrus leafminer during leaf flushing. Proc. Fla. State Hort. Soc. 124: 85-89.
3) Qureshi, J. A. and P. A. Stansly. 2010. Dormant season foliar sprays of broad spectrum insecticides: An effective component of integrated management for *Diaphorina citri* (Hemiptera: Psyllidae) in citrus orchards. Crop Protection. 29: 860-866.

6.6

Asian Citrus Psyllid Management Strategies for California, 2012 and Beyond

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Asian citrus psyllid (ACP) was first detected in southern California in September 2008 in the urban landscape. Since that time, the California Department of Food and Agriculture has conducted an eradication program utilizing systemic imidacloprid and foliar cyfluthrin wherever psyllids are detected. This program was halted in Los Angeles County in 2011 because of the size of the ACP infestation. Psyllids have continued to spread to the east and the south into San Bernardino, Riverside, and Orange Counties. In areas such as San Diego and Imperial counties where urban treatments have been continuous, detections of ACP in commercial citrus are rare and a single treatment of a combination of two broad spectrum insecticides reduces psyllids below detectible levels for many months. In contrast, in San Bernardino and Riverside counties where re-infestation from urban areas is a continuing problem and where the treatments are not well-coordinated in an area-wide fashion, psyllids have become established and commercial growers must treat multiple times per year. The University of California in collaboration with the California Citrus Pest and Disease Prevention Committee have developed strategies for managing ACP once it establishes in commercial citrus. In the initial phase of invasion, when ACP densities are low, aggressive applications of two broad spectrum insecticides are recommended. In areas where the psyllid has become established, area-wide treatment programs utilizing 3-5 insecticides/year are necessary directed at periods of flush and also at the late fall and early spring overwintering populations. Insecticide choices are based on considerations of efficacy, control of other pests, costs, preservation of natural enemies and resistance management.

6.7

Extension Model to Improve Asian Ctrus Psyllid Control in Citrus Health Management Areas (CHMAs)

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Citrus health management areas (CHMAs) have been implemented throughout Florida to provide regional coordination to manage Asian citrus psyllid (ACP) and spread of HLB. The Gulf CHMA is going into its 5th season of cooperative action toward these goals. During the fourth (2011-2012) season we began providing GULF CHMA updates and interactive maps from CHRP data available on the CHMA website www.flchma.com, showing ACP levels and 'hot spots' (i.e. tap samples > 21 ACP for 3 consecutive cycles) on our website, www.imok.ufl.edu. The ring color of the proportional circle map designates the cycle, and the ring size the number of A adults per 50 taps. The largest ring represents psyllid numbers of 21 or greater. The map is readable by anyone with Adobe Reader, and it allows you to click on and off different cycle layers and view data for Cycle #, Cycle Date, County Name, and ACP # thus allowing comparison between two or more sets of data simultaneously and spatially. This project includes development and testing of a smart phone spray app for use by growers and consultants. The insecticide spray data will be converted to a map layer that overlays the Gulf CHMA psyllid counts to determine which growers may need help and what chemicals appear to be failing -- a precursor to predicting ACP resistance. We expect to build better working relationships with the growers by offering individual support of their economic efforts, ACP management, and HLB control.

6.8

Effect of Mineral Oil on Host Selection and Control of *Diaphorina citri* Kuwayama (Hemiptera: Psillidae) on Citrus

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This research was carried out to study the influence of mineral oil on landing and permanence; oviposition; and mortality of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) on citrus plants. For all experiments, mineral oil (Argenfrut®) was sprayed on sweet orange plants at 1% concentration. Landing-permanence and oviposition were assessed using choice and non-choice tests. For the first parameter, 50 adult psyllids were released in the center of the screen house (5mx2.5mx2m) (n = 10) and the number of psyllids/plant at different time intervals were counted. For the oviposition trial, which was conducted under laboratory conditions, 20 psyllids were confined in a cage to oviposit (n = 10) and after three days the number of eggs/plant were counted. The effect of mineral oil on psyllid mortality was assessed by confining 10 adult psyllids per plant (n=4) with fully expanded mature leaves after spray. Assessment was conducted 7 days after confining the insects, by determining the number of live and dead psyllids. The results of this research indicate that mineral oil has repellent effect on adults of *D. citri*, which prefer oil-free plants to land, remain and oviposit. Moreover, mineral oil was effective on *D. citri* control (mortality≥80%).

6.9

Morphological characterization of *Hirsutella citriformis* Speare Mexican isolates and evaluation against *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)

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Diaphorina citri, the vector of the pathogen causing Huanglongbing, has been found infected by the entomopathogenic fungus *Hirsutella citriformis* Speare in the Mexican citrus industry. The objective of this study was to characterize morphologically eight *H. citriformis* isolates and evaluate their potential for the control of *D. citri* adults. The fungal isolates were obtained from citrus groves located in the Mexican states of Campeche, Chiapas, Colima, Quintana Roo, San Luis Potosí, Tabasco, Veracruz, and Yucatán. The fungi showed mycelium composed by delicate hyphae measuring 1.18-1.88–µm in diameter; phialides 30.7-40.9 µm in lenght and neck length of 24.7-35.8 µm. Conidia measured 5.83-5.92 µm in length and 1.43-1.99 in diameter. The mucilaginous layer was 7.83-8.12 X 5.86-5.99 µm. The morphological characterization indicated that the isolates were related to *H. citriformis*. The experiments for the evaluation of pathogenicity were conducted under controlled conditions (25±2°C, 76±4% RH and 16:8 h L:D). Insects were inoculated by contact with sporulated cultures of the isolates. For each *H. citriformis* isolate, 15 adults of *D. citri* received the spores of the fungus. The results showed that mortality of the psyllids by the fungus began six days after inoculation; occurrence of the first *H. citriformis* sinnemata in the *D. citri* specimens was observed 10 days after inoculation. In the first bioassay, the final record of survivorship was performed 27 days after the beginning of the experiments; the mean rate of mortality was 98 and 70% for the Tabasco, and San Luis Potosi *H. citriformis* isolates, respectively.

6.10

Evaluating the Biological Control of ACP in the Rio Grande Valley of Texas

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Tamarixia radiata, a species specific ectoparasitoid of the Asian citrus psyllid (ACP), *Diaphorina citri*, was imported from Pakistan and permitted by the PPQ Permitting Unit for field release in Texas. Over 130,000 parasitoids have been released in the Lower Rio Grande Valley with establishment confirmed at 39 locations. Both open and closed releases are conducted and used to assess establishment and efficacy. Closed releases made in fine-mesh sleeve cages indicate parasitism levels at 10.4%. When compared to the controls, host mortality is reported at 64.9% in cages with parasitoids present versus 4.4% in cages with parasitoids absent. Further investigations into the host mortality of ACP nymphs have been explored by conducting visual observations on the behavior of female parasitoids (n = 30) for one-hour periods in arenas with suitable hosts. Data indicates that females will mount 3.1 $\pm$ 0.5 nymphs per hour. The parasitoid will either oviposit the nymph on the ventral side (36.5% of the time) or probe the nymph on the dorsal side (63.5% of the time). After probing, the parasitoid will either walk away (87.9% of the time) or host feed (12.1% of the time). Host feeding was documented at 0.43 $\pm$ 0.1 nymphs per hour. All nymphs that were host-fed were found to be eventually dead. Host mortality (64.9%) and parasitism rates (10.4%) combined can reduce ACP populations by 75.3%. Studies are still ongoing to help reduce both ACP populations and the incidence of citrus greening disease.

6.11

A grower question: So we are controlling the Asian Citrus Psyllid, but are we doing it well enough?

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Since Huanglongbing (HLB) was first found in Florida, growers have recognized the importance of controlling the Asian citrus psyllid (ACP) that vectors the disease. ACP management programs have been developed and applied over large acreages, yet in many instances, the incidence of the disease continues to increase. When asked, the growers invariably report that they are controlling ACP and it is obvious that the control programs are reducing the level of ACP in the groves. However there is an outstanding question as to whether the levels of ACP are being reduced enough to limit the spread of HLB. In most cases, growers have not implemented ACP scouting programs so the level of infestation is based on rough grove surveys and feelings. Even if scouting programs are in place, there are no established data-based thresholds to trigger additional applications of pesticides resulting in a "chasing" approach where applications are made after an infestation is found. Southern Gardens has had an ACP and HLB scouting program in place for many years. By comparing the annual ACP levels to resulting HLB infection succeeding years, it may be possible to elucidate thresholds and then design ACP control programs that effectively limit the spread of HLB. Data will be presented for three years of ACP scouting and four years of HLB scouting in an attempt to show the level of control that must be achieved in order to limit the spread of HLB. The bottom line is that control thresholds are very low and the level of ACP control that must be achieved in order to limit spread HLB is much lower than most growers realize.

6.12

The Flicker: A Vehicle-Driven, Mechanical Device for Detecting and Monitoring Adult Asian Citrus Psyllid and Other Arthropods in Citrus

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A number of different methods can be used to detect and monitor Asian citrus psyllid (ACP) including tap samples, sticky traps, sweep samples, visual searches, and flush shoot samples (Hall et al. 2012). Each of these methods has value in determining the presence and abundance of ACP, but there are advantages and disadvantages associated with each method. A number of factors may ultimately influence which sampling method to use. However, none of these methods can be used to quickly survey a large block of trees unless few trees are actually examined and/or many scouts are available for the survey. We report on a mechanical device for detecting and monitoring ACP in citrus. The device is attached to a vehicle and pulled through an orchard. Vertical baffles extending from the device are kept in contact with the outer edge of tree canopies while driving along a row. Adult psyllids are flicked (hence the name 'flicker') by these baffles onto large sticky traps positioned below the baffles. Moving at speeds up to 5.0 mph, the flicker can be used to sample a large number of trees at a fast pace. Among 51 blocks of trees studied, the flicker caught an average of 20 ACP but up to 160 ACP per 1,000 ft of trees. Limited data suggest that the flicker may be similar to other sampling methods with respect to detecting psyllids when populations are moderately high. Of interest is the efficiency of detection methods when ACP populations are small. Intuitively, the chances of detecting ACP should be increased by increasing the number of trees sampled across an area, which the flicker facilitates.

Citations

Hall, D. G., *E. D. Ammar*, *M. L. Richardson*, and S. E. Halbert. 2012. Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Psyllidae), vector of citrus huanglongbing disease. Entomologia Experimentalis et Applicata. (in press)

6.13 P

Generating Asian citrus Psyllid *Diaphorina citri* Kuwayama (Homoptera: Psyllidae) with twisting wings to prevent the spread of citrus greening disease

El-Shesheny, I.; Harjeri, S.; Gowda, S.; and Killiny, N.

Huanglongbing (HLB) is seriously threatening and causing considerable economic losses to the citrus groves. Its Management depends critically on the control of the Asian citrus Psyllid (ACP), the vector of the cause of HLB, *Candidatus* Liberibacter asiaticus bacteria (*C*Las). Silencing genes by RNA interference (RNAi) is a promising technique to control pests. In this study, the abnormal disk wing (awd) has been selected from the available psyllid annotated genome. It has been known that awd gene encodes a nucleoside diphosphate kinase and is associated with wing development. This research focused on the effect of RNAi of awd gene on ACP nymph instars that acquired dsRNA. The Results provide evidence that using the dsRNA of awd gene has diminished the development and survival of ACP nymphs. Moreover, knockdown of awd gene expression was observed through malformation of adult wings. Also, the expression of awd was messured by quantitative PCR (qPCR). Furthermore, we are conducting experiments to investigate awd's possible contribution in temperature tolerance. We attempt to establish effective practical application to prevent the spread of HLB in friendly environmentally strategy.

6.14 P

Novel synthetic compounds enhance the attractiveness of host-plant volatiles: An opportunity to boost detection and monitoring of Asian citrus psyllid?

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In the absence of pheromone attractants, host-plant volatiles offer the most likely means of improving capture levels of ACP with sticky cards and other types of visual traps. However, developing scent lures that can compete with the attractiveness of actual host-plants, especially those in flush, is challenging. We are developing a new class of synthetic scent lures that may enhance the attractiveness of naturally-occurring host-plant volatiles. These compounds are synthetic ligands that bind to chemosensory proteins (CSPs) found in the olfactory sensilla of target insects. These ligands may mimic naturally-occurring odorants and function as super-stimuli because of their strong affinity to CSPs. In our study, CSPs from ACP antennae were identified based on their reactivity to petitgrain oil (an essential oil extracted from sour orange leaves), an ACP attractant. Two behavioral assays were used to assess the biological activity of several candidate ligands. One assay measured ACP probing frequency into a line of emulsified wax (SPLAT®, ISCA Technologies) containing a test ligand, the other assay measured the retention time of psyllids in an airstream carrying the ligand. One ligand, nicknamed ‘Titan’, was more stimulatory than limonene, a common citrus volatile, while a mixture of Titan and limonene was significantly more stimulatory than either alone. Subsequent assays showed that Titan was as stimulatory to ACP as the odor emitted by flushing sprigs of orange jasmine, a favored host-plant. These results indicate that CSP ligands may synergize the attractiveness of naturally-occurring citrus volatiles and boost their effectiveness as scent lures for ACP.

ACP responded more strongly to low concentrations of Titan than to a higher concentration.

6.15 P

Effects of *Tagetes coronopifolia* and *T. lemmonii* (Asteraceae) essential oils in nymphs of *Diaphorina citri* (Hemiptera: Psyllidae).

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Worldwide, the management of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is performed mainly by the use of agrochemicals. Often, indiscriminate use of insecticides has resulted in the elimination of natural enemies and in the development of insecticide resistance in the target pest and in no target insects. This situation has motivated the generation and implementation of alternative strategies, such as the use of insecticidal plants. This study was carried out to evaluate the toxic effect of *Tagetes coronopifolia* Willd. and *T. lemmonii* A. Gray (Asteraceae) essential oils in *D. citri* third instar nymphs. Effect was evaluated by orange disc immersion method. Mortality was recorded 24 hours after applying the oils; the log dose response line Probit and the LC_{50} values were determined. *T. coronopifolia* and *T. lemmonni* oils were toxic to *D. citri* nymphs and the effect was positively related to the concentration. Highest nymph mortality (≥ 98%) with both oils was registered in concentrations of 10 mg mL^{-1}. The LC_{50} estimated for *T. lemmonii* and *T. coronopifolia* oils was 0.034 and 0.094 mg mL^{-1}, respectively.

6.16 P

Thresholds for HLB vector control in infected commercial citrus and compatibility with biological control.

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Control of the HLB vector, *Diaphorina citri* Kuwayama, is considered a basic component for management this disease, even in a high HLB incidence scenario. Such control is mostly chemically oriented. However, over use of insecticides would increase costs and be incompatible with biological control. Establishment of economic thresholds for psyllid control under different price scenarios could optimize returns on investment.

Two 3-year experiments are being conducted in commercial orange blocks with high HLB incidence. Experimental design is RCB with 4 treatments and 4 replicates: no insecticide, calendar applications, insecticide applications according to a threshold of 0.2 psyllids/stem tap sample, and applications according to a 0.7 threshold. Vector populations are monitored biweekly by tap sampling. Differences in vector abundance among treatments are being correlated to HLB infection levels estimated by Q-PCR and to fruit yields. Consequences of each vector control strategy on beneficial arthropod fauna are also being evaluated, as well as potential negative impacts on biological control processes in the crop.

After two years, a yield increase was observed with the calendar treatment, but so far additional costs would require high juice prices scenarios. Negative impacts of calendar sprays on biological control of mites and leafminers have also been observed.

6.17 P

Affordable Essential Oils for Management of the Asian Citrus Psyllid

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Plant essential oils are commonly used to manage insects; they are widely available and some are inexpensive. In this research we have selected five botanical oils costing less than $100 US per kilogram, to evaluate for repellency to Asian Citrus Psyllid (ACP), the insect vector of the causal pathogens of huanglongbing. In olfactometer assays, fir oil was repellent; clove and camphor oils were attractive; and litsea and citronella oils elicited no response from ACP females. In no-choice settling experiments, neither the low nor high fir oil treatment deterred ACP from settling. Subsequently, ACP were presented with a choice test between control plants and fir oil plants with a single dose of fir oil contained in a polyethylene vial. In this case, ACP disproportionately settled on control plants, avoiding fir oil baited trees completely. Finally, we conducted a field trial using yellow sticky traps baited with a high or low dose of clove or camphor oil deployed from seven mL polyethylene vials. We expected that the botanical oil baited yellow traps would catch more ACP than unbaited controls. There was no significant increase in trap capture over the course of our experiment in male, female, or total ACP capture. We hypothesize that this result may have been caused by sub-optimal release rates or the overriding visual cue elicited by yellow sticky traps. Our ongoing experiments are designed to improve the behavioral activity of release devices for these essential oils, which may have practical utility for ACP management.

6.18 P

Strategy of HLB management with insecticides in citrus groves in São Paulo, Brazil.

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Huanglongbing(HLB) , disease caused by bacteria *Candidatus* Liberibater asiaticus and *Ca.* L. americanus, has as vector *Diaphorina citri*. Since 2004 has caused huge losses on citrus industry of São Paulo state, Brazil. This research evaluated, during three consecutive years, an insecticide spraying program (Bayer® program – T1) consisted of two imidacloprid drench applications during the rainy season and one trunk application (Winner®) in the autumn, preceded and followed by foliar sprays of insecticides (Decis® or Provado®) based on an psyllid action threshold, compared to standard program of the grower, that used several foliar sprayings (Standard program – T2). This trial was carried out in 34 commercial groves in different counties of São Paulo state with trees at different age, variety/rootstock combination and canopy size. In each grove there was one plot per treatment consisted by 1000 trees. The incidence of *D. citri* and the number of HLB-symptomatic plants was fortnightly evaluated. The data were subjected to analysis by the test F and the averages comparated by Tukey (0,05). In average, after three years, it was observed in T1 a lower incidence of *D. citri* and consistent reduction of the number of HLB symptomatic plants (25,4) with 9 less insecticide applications. Even having been a consistent reduction of the vector population, added to the known effect of systemic acquired resistance (SAR) due to use of Imidacloprid in plants of citrus infected with *Xanthomonas citri* subsp. *citri*, this research shows an analogous process could be occurring on the pathosystem Citrus x *Candidatus* L. asiaticus e *C.* L. americanos, with positive contribution to growers and should be more detailed on further researches.

6.19 P

Brachygastra mellifica (Hymenoptera: Vespidae): Predation preference and feeding behavior on *Diaphorina citri* (Hemiptera: Psyllidae) in Mexico.

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In previous field studies in Northern Mexico, we found the wasp *Brachygastra mellifica* (Say 1837) (Hymenoptera: Vespidae) preying voraciously the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), the vector of the bacteria *Ca.* Liberibacter spp., the putative agent of Huanglongbing, one of the most devastating citrus disease in the world. As in Mexico, the ACP management considers the use of pest biological control, the availability of potential agents for the control of the vector remains as a priority. The objective of this study was to determine the predation preference of the wasp for the different developmental stages of *D. citri.* During 2011-2012, we performed experiments in field where we exposed manually to the predator new citrus flushes infested by eggs and diverse ACP nymphal instars. The predation behavior was recorded in videos and posteriorly analyzed in the lab. Other observations on its feeding attack were made directly on infested flushes in the trees, during the foraging activity of the wasp. Results indicated that *B. mellifica* preferred for predation 4th and 5th instar nymphs, and posteriorly 2nd and 3rd instars. Due to their size and position in the flush, eggs and first instar nymphs were practically discriminated by the wasp. Occasionally, *B. mellifica* consumed *D. citri* adults. This predator represents a potential agent for natural control of *D. citri* in the North of Mexico, and South of the USA, mainly through conservation strategies of beneficial insects.

6.20 P

Frequent Low Volume Sprays of Horticultural Mineral Oil (HMO) for Psyllid and Leafminer Control

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Low volume (LV) aerial and ground sprays have become an important method of application in Florida citrus. During Feb 2011, we started a trial in a 10.9 acre plot of 'Valencia' orange in Lee County comparing LV spray of 435 HMO with the Grower Standard (GS) and an Untreated check (UC) in 3x3 Latin square design. A Proptec rotary atomizer P400D spray machine was used for all treatments. HMO was applied every 2 weeks at 2 gpa. Significantly fewer ACP were seen on during May 2011 on GS trees than HMO trees, but during June these differences disappeared. Between Aug – Oct 2011, mean adult ACP populations were lowest for GS, followed by HMO and UC. Thus, LV oil treatments suppressed ACP, though not as effectively as the GS. However during winter 2011 HMO treatments were as effective as GS. Also, highest pound solids were seen from trees receiving HMO followed by UC and GS. Citrus leafminer (CLM) damage assessments (May/July 2011) using a modified Horsfall-Barratt scale showed less damage with GS compared with Oil which in turn was less than UC. However, CLM trap catches in Nov were significantly lower with HMO compared to GS and UC even though highest flush density was present on HMO-treated trees. Canker ratings (2012) for HMO and GS have been significantly less than UC. Thus, LV application of 435 horticultural mineral oil (HMO) for control of ACP and CLM have shown promising results the last 2 years.

6.21 P

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

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The Florida psyllid testing project (Manjunath et al. 2008, Halbert et al. 2012) showed that about 10% of regulatory samples of *Diaphorina citri* Kuwayama collected by Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS/DPI) inspectors from plants for sale in Florida were positive for *Candidatus* Liberibacter asiaticus (Las). Most of the commercial nurseries that produce the plants do not have psyllids or Las, so the most likely source of contamination is the retail venues themselves. If this is the case, great benefit could be achieved by preventing psyllid infestation in retail stores. Florida has a requirement that citrus plants for sale be treated with an imidacloprid-based soil drench (ISD). Producers are required to tag the plant with the date of treatment. The treatment expires in six months, but our data indicate that three months probably is more realistic. In 2009, there was an increase in plants infested with psyllids 30 days post-ISD treatment. In 2011, this increase in incidence of infested plants came after 90 days, indicating that growers were achieving better control with the ISD.

Citations:

Halbert SE, Manjunath KL, Ramadugu C & Lee RF. 2012. Incidence of huanglongbing-associated '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* (Hemiptera: Psyllidae) collected from plants for sale in Florida. Florida Entomologist 95: 620–627.

Manjunath, K.L., S.E. Halbert, C. Ramadugu, S. Webb, and R.F. Lee. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. Phytopathology 98: 387-396.

6.22 P

Asian Citrus Psyllid and Huanglongbing Management in California: How Psyllid Spread Will Affect Grower Costs

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The spread of Asian Citrus Psyllid (ACP) in California is beginning to reach commercial production. As a vector of the pathogen associated with Huanglongbing (HLB), spread of this insect puts at risk the state's billion dollar citrus industry wherever ACP establishes. Management of ACP and infected tree removal are the only known methods of limiting the spread of HLB, but these methods come with their own set of costs and risks. To reduce economic losses, ACP management options for growers are being developed before ACP and HLB spread throughout commercial citrus growing areas in California. This study will present an ex-ante cost comparison of current pest control practices, the IPM best practices for ACP, and the least cost ACP management program for different citrus growing areas in California. Partial budgeting will be used to estimate the costs under each scenario. Partial budgeting compares the changes in income and expenses that would result from implementing a specific alternative; hence it provides an indicator as to how the treatment is likely to affect the profitability of an enterprise (Kay et al. 2004). Because ACP treatments are applied to reduce the risk of HLB infection and ACP causes far less significant direct damage to citrus, changes in revenues are equal to zero and only costs are calculated. To calculate costs, data on material and application costs were obtained from meetings with pest control advisors in California's citrus growing areas.

Citations

Kay, R., W. Edwards, P. Duffy. 2004. Farm Management. Boston: McGraw Hill

6.23 P

Recommended pesticides persistence for integrated citrus production on ectoparasitoid *Tamarixia radiata* (Waterston, 1922) (Hymenoptera: Eulophidae)

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Tamarixia radiata (Waterston, 1922) is the main biological control agent of the psyllid *Diaphorina citr*i Kuwayama, vector of bacteria associated with Huanglongbing in citrus. However, indiscriminate use of chemicals affects its control rate. Thus, this study assessed the biological persistence of 22 insecticides, two oils and 16 acaricides recommended for the control of citrus pests, on the parasitoid *T. radiata*. For this, the parasitoid adults were exposed to waste products sprayed on citrus seedlings of the variety Valencia. After 3, 7, 10, 17, 24 and 31 days after spraying, leaves were removed and in the laboratory, leaf discs of 4.0 cm diameter were obtained with the aid of a metallic punch , and were placed in Petri dishes (4.5 cm diameter) on a gelled mixture of water-agar to 2.5%. Thereafter, 10 parasitoid adults of 48 hours of age were placed on each plate and, then, placed in a climate clamber (25 ± 1 ºC, 70 ± 10%, 14L10D). Each treatment had five replicates. Insect mortality was evaluated 24 hours after exposure to residues. Insecticides Saurus, Turbo, Mimic 200 SC and Azamax; mineral oil Argenfrut; vegetable oil Nortox and acaricides Vertimec 18 EC, Envidor, Sanmite, Torque 500 SC, Cascade 100, Borneo, Dicofol, Micromite 240 SC and Savey WP were classified as short lived, and insecticides Tracer and Dicarzol and acaricide Marshall Star were classified as persistent. Therefore, it is essential to use selective products in integrated pest management to preserve the parasitoid.

Citation:

ABBOTT, W.S. A method of computing the effectiveness of an insecticide. **Journal Economic Entomology**, Lanhan, v.18, n.2, p.265-267, 1925.

HASSAN, S.A. Métodos padronizados para testes de seletividade, com ênfase em Trichogramma, p.207-233. In: PARRA, J.R.P.; ZUCCHI, R.A. (eds.). ***Trichogramma* e o controle biológico aplicado**. Piracicaba, FEALQ, 1997, 324p.

6.24 P

Introducing DuPont Exirel™ and Verimark™ new insect control products for pest management and optimizing yield in Florida citrus

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Abstract

DuPont™ Exirel™ and Verimark™ insect control contain DuPont™ Cyazypyr™ insecticide, the second active ingredient from the anthranilic diamide class of chemistry, and the first to control a cross-spectrum of insect pests including Lepidoptera, Dipteran leafminers, fruit flies, beetles, whiteflies, thrips, aphids, leafhoppers, psyllids and weevils, while conserving key predators and parasitoids. Exirel™ and Verimark™ deliver a novel mode of action that impacts insect behavior by impairing muscle function. Intoxicated insects stop feeding rapidly, resulting in excellent plant protection. Exirel™ is designed for foliar applications to optimize leaf penetration and spray coverage. Exirel™ applied at the major flush periods provides excellent protection against Asian citrus psyllid adult and nymphs and citrus leafminer for extended periods, generally 3-4 weeks.

Verimark™ is designed specifically for soil applications to optimize root uptake and translocation. Verimark™ can be applied as a soil drench or injected through microsprinkler irrigation to young citrus trees from resetting in the field up to when trees reach a size of about 5 ft tall. Verimark™ provides excellent protection of continuously flushing young citrus trees against Asian citrus psyllid and citrus leafminer. New flush protection can last sixty days after a single application of Verimark™. The impact of Exirel™ and Verimark™ in the sustainability of citrus production will be discussed.

KEY WORDS: Exirel™, Verimark™, Cyazypyr™, Asian citrus psyllid, citrus, Feeding inhibition, disease transmission, crop protection

6.25 P

Perspectives to the use of entomopathogenic fungi for biological control of *Diaphorina citri* in Mexico

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Entomopathogenic fungi are natural enemies of *D. citri* adults in Mexico. Natural prevalence (%) of fungi on live adults in central Veracruz (summer) and southern Tamaulipas (fall) were: *Hirsutella citriformis* (7-35); *Isaria fumosorosea* (5-15); *Lecanicillium* (2 in Tamaulipas), and *Beauveria* (<1). *Torrubiella* (=*Sporothrix*) is a hyperparasite of *Hirsutella*. In inland Tamaulipas only *Isaria* was found. *Entomophthora* (<2%), a new report for *Diaphorina*, was found at Veracruz in October 2012.

On April 2011, spores (conidia) were applied either as water suspension or emulsion in PureSpray® oil at 0.5% in water; at $1x10^8$ (*Beauveria*, *Metarhizium*) or $5.6x10^7$ conidia/ml (*I. fumosorosea*), on lime trees against *D. citri* nymphs at Martinez de la Torre, Veracruz (MTV) (dry season). *Isaria* concentration was ca. 5X lower. Average infection (as sporulation) 72 h after application (AP) by *Beauveria*, *Isaria* and *Metarhizium* was near 70, 50 and 50% respectively. The fungus-formulation interaction was significant.

In 2012, spraying of conidia ($1x10^8$/ml in water) at MTV (rainy season) and General Teran, Nuevo Leon (GTNL) (dry summer) showed that infection (dense development of fungal hyphae inside nymphs) occurred in the field 48 h AP at MTV (% infection: *Metarhizium*= 31; *Beauveria*= 27; *Isaria*= 0); and 72h AP at GTNL (only *Metarhizium* applied; 42-76% infection). No sampling before at either place. Dead nymphs were collected and immediately examined or tissue-fixed. At GTNL, 30-65% of recovered conidia from foliage germinated 72h AP, and 30% after seven days. There, weather was >40ºC with rain. Since conidia remained viable for long periods on foliage, nymphs were examined immediately upon collection (2012), to avoid infecting insects stored in bags before examination. Other noise sources are: infection underestimates from falling off trees of infected (dead and sick) nymphs; and detection of fungal DNA on insects, from simple adhesion of conidia to insects without infection.

6.26 P

Identification and entomopathogenicity of newly-isolated fungi infecting *Diaphorina citri* Kuwayama (Homotera: Psyllidae) in Murraya orchards of Fujian, China

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Abstract : Among fungal isolates obtained from *Murraya panciculata* L. groves in Fujian, China, seven were tested pathogenic against the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Homoptera: Psyllidae). In the present paper, the isolates were identified for their taxonomic ranks and compared on their entomopathogenicity against ACP adults. Based on the analysis of conidia morphological data and ITS sequences of 18 S rDNA, the fungal isolates FJAT-9620, FJAT-9621, FJAT-9622, FJAT-9624 and FJAT-9719 were identified as *Beauveria bassiana*, FJAT-9623 as *B. asiatica* and FJAT-9720 as *Lecanicillium attenuatum*. Bioassays revealed that fungal isolates FJAT-9622, FJAT-9623, FJAT-9719 and FJAT-9720 infected adult psyllids with mortality of 95.00-98.33% at 27±1^{o}C and 100% relative humidity (RH) in the laboratory. Meanwhile, isolates FJAT-9620, FJAT-9621 and FJAT-9624 induced significantly lower mortality (3.33-40.00%) on the psyllids.

Keywords: *Diaphorina citri*; Huanglongbing; *Beauveria bassiana*; *Beauveria asiatica*; *Lecanicillium attenuatum*; Biologcial control

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6.27 P

Targeting juvenile hormone metabolic genes in the Asian citrus psyllid (Diaphorina citri Kuwayama) as a strategy to reduce the spread of citrus greening disease

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Diaphorina citri Kuwayama, the Asian citrus psyllid (ACP), is a devastating citrus pest due to its transmission of a phloem-limited bacterial pathogen, *Candidatus Liberibacter asiaticus*, that causes citrus greening. Psyllid control is a major part of effective greening disease management, and our research targets perturbation of insect juvenile hormone metabolism as a new psyllid control strategy. Previous studies have shown that application of a juvenile hormone (JH) analogue, pyriproxyfen, is known to produce ovicidal/nymphicidal effects, morphological abnormalities, and reduced fecundity in ACP adults. These observations prompted us to identify JH biosynthetic and degradative pathways as targets for biologically-based control strategies, including RNA interference, as alternatives to heavy reliance on broad-spectrum pesticides. First, candidate genes/cDNAs encoding the JH metabolic enzymes, juvenile hormone acid methyl transferase (JHAMT) and juvenile hormone esterase, were identified through computational analysis of the *D. citri* genome. Second, JHAMT cDNA was cloned, expressed in *E. coli* and a functional protein was purified. This JHAMT had a high affinity for substrates leading to JHI and JHIII synthesis, making it plausible that both juvenoids are present in the ACP. Results are discussed with respect to mechanism(s) of juvenile hormone biosynthesis/catabolism in the ACP and targeting this process as an interdiction point for a bio-rational ACP control strategy.

6.28 P

Entomophagous insects associated to *Diaphorina citri* (Hemiptera: Psyllidae) in citrus orchards with different weed management systems in Papantla, Veracruz, Mexico

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Huanglongbing (HLB), one of the most destructive diseases of citrus worldwide, is threatening the survival of the citrus industry in Mexico. *Diaphorina citri* is the primary vector of HLB; thus, control of the vector it's vital for disease management. This study was carried out to evaluate the influence of different management systems on the population psyllid density and entomophagous insects associated in orange orchards (*Citrus sinensis* cv. Valencia) in Papantla, Veracruz, Mexico. Five orchards with different management strategies were selected: 1) Manual and mechanical weed control and insecticide application, 2) Manual and mechanical weed control, with insecticide application, and high planting density, 3) Manual and mechanical weed control, without insecticide, 4) Constant herbicide application, without insecticide, and 5) Manual weed control, mechanical soil removal, herbicide application, without insecticide. Each orchard was sampled, monthly. Psyllids adults were captured on yellow sticky traps. Eggs, nymphs and adults of *D. citri*, and natural enemies were collected on flush shoots. Results show that the diversity of weeds varied according to the handling and sampling date and was higher in orchards and dates where herbicide use was reduced or null. *Cycloneda sanguinea*, *Azya* sp., *Scymnus* sp., *Curinus* sp., and *Brachiacantha* sp. were the predators collected. There was synchrony among populations of *D. citri*, predators and abundance of flush shoots. The presence of the parasitoid *Tamarixia radiata* was minimal as a result of the low *D. citri* nymphs density. The results suggest that weeds diversity guarantee the survival of predators, because they supply alternative food resources.

6.29 P

Soil Applied Systemic Insecticides for Control of Asian citrus psyllid in Newly Planted Citrus Trees

Phil Stansly and Barry Kostyk

Orchard renewal is a special challenge where HLB is endemic. Young trees are especially susceptible to the disease and continuously attractive to the psyllid vector due to frequent flushing. Heavy reliance is placed on systemic insecticides to protect young trees. However, all presently labeled for citrus are neonicotinoids (IRAC Group 4a) making resistance likely and the long term viability of this strategy questionable. Rotation partners are needed to forestall selection for resistance. Therefore, we conducted a multiyear study to evaluate rotations of neonicotinoid insecticides rotated with cyantraniliprole a Group 28 insecticide in a block of 'Hamlin' orange on 'US802' rootstock planted in May 2010. Four treatment programs using two rates of Verimark 20 SC, rotated with Platinum 75 and Admire Pro in two different sequences were compared to an untreated check in an RCBD with 4 replicates. Adult psyllid populations were monitored monthly and immatures counted when natural flush was present. Incidence of HLB was assessed by PCR in Aug 2011, and Jan, May and Aug 2012. Psyllid populations on treated trees averaged 1.43 per tap compared to a range of 0 to 0.8 on treated trees. Incidence of HLB reached 29% in Aug 2011 on untreated trees compared to 0-8.3% on treated trees. In May 2012 these numbers had increased to 67 % and 11-26% respectively. Diameter of scions was 33% larger on treated trees in Sep 2012. Although effective, the 90 day treatment regimen was not enough to protect trees from HLB and additional strategies are necessary.

Session 7:
HLB Management, Fruit Quality, Crop Loss, and Economics

7.1

Progress on Dissecting and Controlling the Citrus Huanglongbing Complex

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Abstract

Citrus huanglongbing (HLB) is a century-old and emerging disease that impedes citrus production worldwide. '*Candidatus* Liberibacter asiaticus' (Las) is the globally prevalent species of HLB bacteria. Here we describe our molecular characterizations of Las, and our newly-developed control methods for citrus HLB. From a genomics standpoint, we revealed Las has a significantly reduced genome (1.26Mb) and unique features adapted to its intracellular life style. Although the genome is small, Las contains at least two prophages that make up ca. 1/16 of the entire genome. Frequent recombination and reasssortment of these prophages/phages may contribute to Las's evolving diversity and plasticity. There are at least 9 different types of Las populations that may co-exist in a single infection, but some exist preferentially in different hosts and different geographical locations. Furthermore, different Las populations may account for titer variations, such as the extreme low titer of Las bacteria (detected by our qPCR method) from seed-transmitted citrus and infected *Murraya paniculata*. From a functional genomics standpoint, we revealed Las encodes a functional ATP translocase and acts as an "energy parasite". To modulate host energy biosyntheses and/or defense responses, Las encodes two novel autotransporter proteins that target to mitochondria. To compete for the limited zinc nutrient, Las encodes a ZuABC high affinity zinc uptake system. To avoid host defense machinery, Las encodes a functional flagellin that slowly triggers the citrus basal defense response. Although HLB is extremely difficult to manage, our newly-developed thermotherapy and chemotherapy methods provide potential components of an integrated control strategy for this devastating disease. In addition to the molecular characterization of the Las bacterium and its responses to stress, we have also revealed the dynamics of the microbial community (over 7000 OTUs-"species") in HLB-affected citrus plants and how the microbial community responds to antibiotic treatments and seasonal variations.

7.2

Citrus tristeza virus-based RNA-interference (RNAi) vector and its potential in combating citrus Huanglongbing (HLB)

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Citrus tristeza virus (CTV), a plus-sense ssRNA virus, is member of the genus Closterovirus, family *Closteroviridae*. RNA viruses are inducers as-well-as targets of gene silencing defense mechanism of host plants and this has been exploited as a tool in functional genomics. CTV was developed into virus-induced gene silencing (VIGS) or RNA-interference (RNAi) vector, which interferes with expression of endogenous genes in citrus or GFP-transgene in *Nicotiana benthamiana* (16c) in a sequence specific manner. Photobleaching phenotype indicative of silencing of endogenous gene, phytoene desaturase in citrus, and red color under UV indicative of silencing of transgene GFP in *N. benthamiana* (16c) was observed using CTV-RNAi vector. CTV-RNAi vector has great potentials in combating huanglongbing (HLB) disease through (1) enhancing basal defense of citrus by silencing of auxin signaling F-Box receptor genes while simultaneously overexpressing microRNAs; (2) down-regulation of overexpressed genes, callose synthase and phloem protein-2, responsible for phloem-plugging in citrus by HLB; (3) expressing dsRNA specific to essential genes of insect vector psyllid (*Diaphorina citri*) to disable transmission of '*Candidatus*' Liberibacter asiaticus pathogen. Simultaneous silencing of multiple endogenous genes of a metabolic pathway is possible through tandem engineering of potential siRNA eliciting regions in CTV-RNAi vector.

7.3

Pre-symptomatic fibrous root decline in citrus trees caused by Huanglongbing and potential synergistic interaction with *Phytophthora* spp.

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Huanglongbing (HLB) caused by *Candidatus* Liberibacter asiaticus (Las) was first detected in Florida in late 2005 and is now widely distributed throughout the commercial citrus-growing regions. In recent seasons, concurrent with freeze and drought episodes, symptomatic HLB-infected trees were much more affected by the extremes of temperature and moisture than trees without HLB. Symptoms exhibited by the stressed trees were excessive leaf loss and premature fruit drop even when HLB-infected trees are managed with enhanced nutritional programs which are thought to improve tree health of HLB-infected trees. This stress intolerance may be due to a loss of fibrous roots. To assess root status of HLB-affected trees, blocks of 2,307 three-yr-old Hamlin orange trees and 2,693 four-yr-old Valencia orange trees were surveyed visually and by real time PCR (PCR) to determine Las infection status. The incidence of presymptomatic (PCR+, visually negative) and symptomatic (PCR+, visually positive) trees was 89% for the Hamlin block and 88% for the Valencia block. HLB+ trees had a 30 and 37% reduction in fibrous root mass density for presymptomatic and symptomatic trees, respectively, compared to HLB – trees. In a second survey, 10- to 25-yr-old Valencia trees were identified within 3-6 months of canopy expression as HLB symptomatic (PCR+, visually positive) or non-symptomatic (PCR-, visually negative) in orchards located in the central ridge, south-central and southwest flatwoods. Pairs of HLB+ and HLB- trees were evaluated for PCR status, fibrous root mass density and *Phytophthora nicotianae* progagules in the rhizosphere soil. HLB+ trees had 27-40% lower fibrous root mass density and in one location higher P. nicotianae per root but Phytophthora populations per cm3 soil were high on both HLB+ and HLB- trees. Fibrous root loss results primarily from HLB damage which may be interacting with *P. nicotianae*.

7.4

Vector Control and Foliar Nutrition for Management of Huanglongbing in Florida Citrus

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Huanglongbing (HLB) or citrus greening is a bacterial disease vectored by the Asian citrus psyllid (ACP) and causing mottled leaves, tree decline, and yield loss. Vector control and foliar nutrition are widely employed in Florida and elsewhere to respectively slow the spread of HLB and mitigate debilitating effects of the disease. A replicated field study was conducted in a 5.4-ha commercial block of young 'Valencia' orange trees through four harvests employing a factorial design to evaluate individual and compound effects of a popular foliar nutrient program and threshold-based vector management.

ACP populations were maintained at contrasting levels in insecticide-treated and untreated plots despite proximity. Nevertheless, incidence of HLB, estimated by PCR at nearly 30% at the beginning of the study, rose to almost 95% early in the third year without measurable reduction from vector control. However, insecticide treatments did result in higher threshold cycle (Ct) values, indicating reduced disease intensity. Vector control significantly improved yields all but year one, while the nutrition only treatment made a significant contribution to yield only in year four. Combined foliar nutrition and vector control was the best treatment all 4 years, and provided yields close to the pre-HLB regional average in the 4th year. Although the combined treatment was not profitable at current juice prices, this shortcoming could be remedied by reducing costs of the nutrient package and/or the insecticide regime.

Here finally is evidence for salutatory effects of both nutrient enhancement and vector control on HLB-infected trees. Further research is necessary to establish economic thresholds for both insecticide and nutrient application under different market and environmental conditions.

7.5

Evaluation of enhanced nutritional programs for mitigating HLB damage

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Florida growers have reported that enhanced nutritional programs (ENPs) maintain productivity of HLB-infected trees. However, efficacy and sustainability of the nutritional approach for HLB disease management remains uncertain. Complementary studies of multiple ENPs and their individual components compared to the standard nutritional program (SNP) on nursery and field trees were initiated in 2010. Two independent nursery trials were initiated with final data collection of the second trial currently underway. The field site was chosen for its mix of healthy, presymptomatic, and HLB symptomatic trees to determine if observed differences resulted from effects on healthy or infected trees. We have found no evidence of reduced phloem plugging in ENP treated nursery trees. *Candidatus* Liberibacter asiaticus (Las) populations are similar for ENPs and the SNP. Minor differences in Las movement have been observed. Las invaded new flush tissue faster in ENP treated trees than SNP trees. Phosphite treatments have caused Las to favor early invasion of root tissue compared to other treatments. Preliminary observations of the second nursery trial suggest that foliar symptoms are more apparent on the standard nutrient program compared to ENPs; however, root and canopy decline are unaffected. Fruit yield and HLB symptoms in field trees treated with ENPs have not differed significantly from the standard nutritional program after two years. Third year yield data will be presented.

7.6

Nutritional management, HLB epidemics and crop loss: Two years results

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Despite the relative effectiveness of recommended measures of inoculum reduction and Asian citrus psyllid (ACP) control to manage HLB, growers still look for nutrient management practices to minimize losses due to expected progress of the Huanglongbing (HLB). However, clear evidence of positive effects of improved mineral nutrition on tree health and productivity is lacking. Therefore, in December 2010 an experiment was set up in a non-irrigated grove of 8-yr-old Valencia sweet orange trees on Rangpur lime to evaluate the effects of nutrients (K, Zn and Mn), phosphate and salicilate leaf sprayed to the trees four times in the year during spring and summer. The experiment has 8 treatments in 4 randomized blocks with 1280 trees/plot. ACP has been rigorously controlled in 3 of 4 blocks. At the beginning of experiment the incidence of HLB symptomatic trees was <2%, and 20 HLB-affected trees with mean disease severity <3% were marked. After two years, preliminary results demonstrated that there was no effect among different treatments and that nutritional treatments did not reduce the progress of HLB-symptomatic trees incidence, did not reduce the disease severity progress in marked trees, and did not improve yield of HLB-symptomatic trees. In June 2012, the mean HLB incidence was 8% and 18% for plots with and without ACP control respectively. In September 2012, the mean disease severity on marked trees was 37% independent on ACP control. Compared with 'healthy' trees, HLB-symptomatic trees had a mean reduction of 15% and 44% in yield respectively in the first and second years after the beginning of experiment.

7.7

Antimicrobial Compounds to Combat Citrus Huanglongbing

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Citrus Huanglongbing (HLB) is associated with the fastidious bacterium (*Candidatus* Liberibacter) that is transmitted by a phloem-feeding insect (Citrus Psyllid). An ideal solution to combat citrus HLB is completely to eliminate the bacteria after a single course of the chemotherapy, either active directly on the bacteria or indirectly through induction of host defense compounds. Twenty-seven antimicrobial compounds were screened to test for *in vivo* activities against HLB bacterium while assessing their phytotoxicity to citrus using the optimized graft-based chemotherapy approach (Zhang et al., 2012). The Las bacterial titers were quantified by qPCR from the leaf samples that were taken at 4-month and 6-month after inoculation, respectively. The Las-infected plants were considered as Las positive with threshold cycle (Ct) values less than 32.0. The efficiency against the HLB bacterium of the tested compound was evaluated by Ct values in the inoculated plants (both scions and rootstocks), scion infected percentage and HLB bacterial transmission percentage. The phytotoxicity was determined by the survival and growth of scions treated by antimicrobial compounds. The clustering results indicated that 27 antimicrobial compounds were divided into 3 groups. The first group including 12 compounds, such as Zineb, was not effective in eliminating the HLB bacteria, with high scion infection (67.9%±14.4% in average), Las transmission percentage (83.4%±13.85 in average) as well as the high bacterial titers. The second group of only two compounds was also not effective against the HLB bacterium, but had high phytotoxicity to citrus (less than 40% of the scion survival and 15% of the scion growth). The third group including 13 compounds, such as nicotine, was effective in eliminating the Las bacteria and had no phytotoxicity to citrus. The effective compounds will be further tested in the container-potted plants and in the field.

7.8

Progress towards the development of a routine process to discriminate juice originating from HLB-free and HLB-infected trees using sensory and analytical analyses

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There are many reports in the literature, both historical and recent, that indicate that fruit from trees affected by Huanglongbing (HLB) can have off flavors and result in off-flavored juice. Several recent studies from Florida where fairly comprehensive sensory and chemical testing has been done, have shown that there are differences in flavor and specific chemical components in juice from healthy and HLB infected trees in some varieties and during some times of the year. However, there are also recent reports from Florida, mainly from production associated research trials and demonstration plots, where juice quality from HLB infected trees is reported to be good and similar to that of juice from healthy trees. In virtually all of these production-related research trials, the variables measured for juice quality were Brix, acid and ratio and no other organoleptic components were considered. Although these are standard measures of juice quality, they do not encompass the wide range parameters that are considered by processors and the beverage industry in the evaluation of raw input and final products. In many cases, sensory evaluation by trained panelists is a routine procedure in the evaluation of input streams and the final product. Thus sensory components should not be ignored when considering HLB management options, especially as the Florida industry moves towards 100% infection with HLB. This paper will present some of the progress that has been made to develop methodology to discriminate juice produced from fruit from healthy and infected tree using methods that directly and indirectly measure compounds and characteristics that impact flavor.

7.9

Further Studies on the Effects of Greening on Juice Quality: Do Nutritional Sprays Ameliorate HLB-Induced Off-flavor?

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Citrus groves receiving nutritional sprays were compared with groves in the same areas managed with conventional fertilization treatments. Fruit were harvested from healthy and Huanglongbing (HLB)-affected trees. Within HLB-affected trees, fruit were sorted into asymptomatic (HLB-a) and symptomatic (HLB-s) fruit. Sensory tests were performed using the difference-from-control (DFC) method, where juice from HLB-affected trees was compared with juice from healthy trees. Results show that panelists could detect differences between juice from HLB-affected and healthy trees in the 2009-2010 and 2010-2011 seasons, regardless of nutritional treatments, for Hamlin and Valencia. Like in previous years, those differences were perceived as more bitter or metallic for early harvests of HLB-affected Hamlin, but those differences were less and inconsistently perceived as more bitter, sweeter or more sour for late harvests or Valencia HLB-affected fruit. In the 2011-2012 season, there were much less differences between juice from healthy and HLB trees, possibly due to a season with high Brix/TA ratio, or due to later harvests. Results will be discussed in relation to chemical analysis, sugars, acids, and limonoids. Nutritional treatments that mitigate HLB symptoms on trees did not have a consistent effect on the HLB induced off-flavor of the fruit and juice, necessitating more seasons of study.

7.10

Limited success of heat treatments for curing HLB affected trees

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A series of assays was conducted in an attempt to eliminate *Ca.* L. asiaticus (Las) from HLB affected plants using hot air (HA) or steam (ST). HA assays were conducted with 2-y-old greenhouse (GH) potted Valencia/Rangpur lime plants and 4-y-old Hamlin/Sunki field trees. GH plants were exposed to 38 to 44°C for 24 to 192 h in a growth chamber (GC). Field trees were exposed to sunlight under plastic cover sheets (≥40°C) for 24 to 96 h after the branches were pruned 1.5 m above ground. ST assays involved root-uncovered GH plants and 5-y-old pruned Hamlin/Swingle and Pera/Cravo field trees. GH plants were exposed to 45 to 60°C for 5 to 30 min and the trees to 55°C for 5 to 20 min and 60°C for 5 or 10 min. Plant and Las responses to heat were temperature/time dependent. All plants exposed to HA for ≥96 h at 44°C in GC and ca. 1/3 of the trees treated for ≥24 h at ≥40°C in the field died. Around 1/3 of the plants exposed to ST for 15 min at 50°C or 5 min at 55°C, and all plants at 60°C also died. In the field ST also damaged or killed the trees at 55°C for ≥30 min or 60°C for ≥20min. Most of the plants or trees showed typical HLB symptoms and were PCR+ 3 to 5 months after treatment. Under the trunk bark of field trees the temperature took over 10 min to reach maximum 50°C, which was apparently not enough to kill Las. Las survival in the trunk and roots may explain the lack of success of the heat treatments against HLB.

7.11

The Chemistry behind DNA Isolation from Orange Juice and Detection of 16S rDNA of *Candidatus* Liberibacter asiaticus by qPCR

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The current standard to diagnose Huanglongbing (HLB) for citrus trees is to take samples from midribs of leaves, which are rich in phloem tissues, and apply quantitative real-time PCR (qPCR) test to detect 16S rDNA of *Candidatus* Liberibacter asiaticus (*C*Las), the putative causal pathogen. It is extremely difficult to detect *C*Las in orange juice because of the low *C*Las population, high pectin concentration, low pH and possible existence of an inhibitor to DNA amplification. The objective of this research was to improve extraction of DNA from orange juice, and detection of *C*Las by qPCR. Homogenization using a sonicator increased DNA extraction by 86%, and stabilized quantification of 16S rDNA in comparison to mortar and pestle extraction, which showed wide variability of Ct values of 16S rDNA. Orange juices are rich in pectin which has a similar physiochemical features to DNA: soluble in water and precipitates in ethanol/isopropanol solutions. Thus, it is difficult to separate the DNA from pectin. However, DNA was successfully extracted by adding pectinase to hydrolyze the pectin. Without going through an elution column, the amplification of plant and microbial DNA in orange juice samples was inhibited by an unknown compound. Thus application of an elution column successfully eliminated the inhibitor. To eliminate errors caused by different methods of sampling, DNA extraction and qPCR procedures, Ct of a cytochrome oxidase (COX) to represent citrus plant DNA was detected as a reference, and a relative unit, $\Delta Ct_{16S\ rDNA\text{-}COX}$ was introduced to express the relative *C*Las population.

7.12 P

Estimating the Economic Impact of an Eventual Introduction of Huanglongbing (HLB) in the State of Bahia, Brazil.

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Bahia is the second most important citrus region in Brazil, accounting for 5,5% of Brazilian production. 80% of production comes from family based farms, which depend on this crop for economic support. Huanglongbing (HLB) was never recorded in Bahia, but is already spreading in three other citrus-producing states of the country, one of which borders the state of Bahia. Thus, this study aimed to estimate the potential economic impact resulting from an eventual introduction of HLB in Bahia. The mathematical model of Gompertz and logistic model were used to determine the epidemiological pattern of the disease, considering three scenarios. In scenario A, the efforts of the Bahia State Agency of Agricultural Defense were positive preventing the establishment of HLB (baseline scenario). In scenario B, there was the introduction of bacteria in the Bahia citrus orchards, and the absence of control measures, contributed to the expansion of HLB in the following years. In scenario C, after detection of the disease, the producers would adopt control measures: eradication of symptomatic hosts and the insect vector population suppression. The costs of disease control were measured by the need for sprays, carrying out periodic inspections and eradication of plants with symptoms. The net present value (NPV) was used for comparing different scenarios. The results showed that, if the HLB is introduced in Bahia, the losses would be very significant in the following 20 years. Should control and eradication procedures are not followed, losses of up to US $ 890,7 million could occur.

Index terms: *Candidatus* Liberibacter, cost, eradication.

7.13 P

The effect of nutritional spray programs applied to mitigate symptoms of Huanglongbing on fruit drop caused by HLB and citrus canker and on 'Hamlin' orange trees

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Huanglongbing (HLB) was detected in Florida in 2005 and has reached 100% incidence in certain citrus plantings in southwest Florida. The putative causal agent of HLB in Florida is the bacterium *Candidatus* Liberibacter asiaticus (CLa). Citrus canker caused by the bacterium *Xanthomonas citri* subsp. *citri* is endemic in Florida. In 2011 and 2012, fruit drop on young 'Hamlin' trees with symptoms of HLB and/or citrus canker was particularly severe, with more than 90% fruit drop recorded. Nutritional sprays containing mainly micronutrients applied to citrus flush has emerged as a practice to mitigate the effects of HLB on plant health. An experiment was initiated in 2008 to examine the effects of nine treatments containing various materials alone or in combination used in popular nutritional programs being applied by growers. Products included micronutrients, systemic acquired resistance inducers, and a commercial biological control agent. Trees were evaluated visually and by PCR for detection of CLa annually. Disease severity and fruit drop associated with citrus canker were recorded for 2011 and 2012. Most treatments reduced the severity of HLB symptoms and stimulated vegetative growth which increased the citrus canker susceptible tissue and fruit drop except one treatment containing primarily micronutrients. In this treatment, fruit drop due to HLB and/or citrus canker was significantly reduced compared to other treatments. These findings might indicate that the use of certain nutritional applications for mitigation of HLB might reduce the severity of citrus canker and fruit drop on young 'Hamlin' orange trees.

7.14 P

Metalized Polyethylene Mulch to Reduce Incidence of Huanglongbing and Improve Growth of New Citrus Plantings

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Polyethylene mulch was evaluated for deterring colonization by Asian citrus psyllid (ACP) *Diaphorina citri*, reducing incidence of huanglongbing (HLB) or citrus greening disease and accelerating growth of young citrus. UV reflective low density polyethylene mulch metalized with aluminum, low density whitefaced polyethylene mulch and bare ground all using drip irrigation in a randomized complete block design were tested and compared to the current grower standard using micro-sprinkler irrigation with four replications located at the Southwest Florida Research and Education Center in Immokalee, FL. Populations of ACP and other arthropods were monitored on new flush while ACP movement was monitored using yellow sticky cards. Incidence of HLB was evaluated twice during the 20 month study period using qPCR. Trunk cross sectional area, soil moisture, and surrounding weed biomass were also monitored. Metalized mulch reduced pest populations and HLB incidence compared to all tested alternatives. In addition, metalized mulch increased tree growth and soil moisture while reducing weed pressure. Results of this study present a good case for the use of metalized plastic mulch for young citrus plantings.

7.15 P

Field validation of a greenhouse demonstration of phytohormone-mediated restoration of naturally infected HLB citrus in Florida, Texas and Jamaica

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A model system based on restoration of the physiological phytohormone balance in the phloem-limited bacterium Candidatus Liberibacter asiaticus (Las) infected citrus (orange, mercot, tangelo and grapefruit) was evaluated in the field at Ft Meade, FL, Weslaco, TX and Kingston, Jamaica. We hypothesize that development of Citrus Greening Disease or Huanglongbing (HLB), is the result of the release of latent infection precipitated by phloem blockage, root disease and the demise of the MEV pathway for phytohormone production and transport. Earlier greenhouse investigations with citrus seedlings and an alternative host, Periwinkle (Catharanthus roseus), indicate that exogenous application of some of the products of this pathway restore the internal phytohormone balance, reverse root decline, induce new axillary buds and restore vitality and production to the tree. This treatment program was scaled up and evaluated in the field with three commercial preparations applied in the fall or in the spring. A single foliar application in the fall provided superior restoration of growth and development. Treated trees were symptomless after 3 months, flowered, set and held fruit and produced over 550 mature fruit/tree. Additional investigations of phytohormone therapy are in progress to evaluate the impact on Las titer in both old (pretreatment) and new growth. Foliar application of selected phytohormones is an effective remediation treatment for Las infected citrus.

Keywords: Citrus greening, HLB, MEV, periwinkle, remediation

7.16 P

Preliminary Research on Soil Conditioner Mediated Citrus Huanglongbing Mitigation in the Field

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Abstract The efficacy of a soil conditioner (Citrus type, Runxin, Beijing agricultural fertilizer (10) 0037), made by Run Zhe Xin Ye Biotech Company, Zaoqing, China) on different ages of huanglongbing (HLB)-affected citrus trees was evaluated at 3 orchards in Sihui and at different months post treatment in Longmen, Guangdong province. Two species, Shatangju (*Citrus. reticulate Blanco* cv. Shatang ju) and Chuntianju (*C. reticulate* cv. Chuntian Ju) were evaluated in completely random design. Symptomatical observation indicated that the treated plants, especially young trees had more new shoots and young leaves than the untreated plants. And, the young leaves on treated plants looked healthy, with few HLB symptoms, compared to the untreated plants. Real-time PCR results indicated a significant "*Candidatus* Liberibacter asiaticus" ("Las") reduction in the treated 2-year old citrus plants ($p=0.005$). "Las" titers were reduced by 2.19 and 2.45 times in the leaves of treated plants, compared to those of untreated 3 and 8-year-old affected Shatangju. Statistic data from different aged Shatangju showed "Las" titers were significantly positively related with treatment ($P=0.004$) and age of trees ($P=0.022$), but not with old and young leaves ($P>0.05$). Comparative analysis of the efficacy of soil conditioner treatment in 4-year-old Chuntianju at 2, 4, and 7 month-post treatment (mpt) showed that the quantities of "las" were significantly lower in newly growing leaves at 7 mpt (94.51% decrease or 2.59 times lower than those at 4 or 2 mpt, $p=0.002$). Bacterial titers in treated plants were significantly lower (34.12% decrease) than control plants, and 82.72% lower in young leaves than in mature leaves. The P values of treatment (treated and untreated), leaf part (old leaf and new leaf), and sampling time (at different months after treatment) were $P=0.014$, $P<0.001$, and $P<0.001$, respectively. Soil conditional test revealed that the P, N, K, organic matters, and Mn contents in the soil conditioner treated orchard soil were all significantly higher than in the non-treated soil at 2 mpt ($p<0.05$). Unexpectedly, microfloras at the sites of treated and non-treated in the orchards seemed no apparent difference in total viable colony numbers and microorganism types. Semi-quantitative RT-PCR found most defense response genes increased in the treated plants. On the other hand, most starch synthesis related genes including genes coding phloem-specific lectin PP2-like protein were expressed stronger in the untreated plants. This study suggests the soil conditioner not only work as a fertilizer, but also can play a role in "las" titer management.

Key words Huanglongbing; Soil conditioner; "*Candidatus* Liberibacter asiaticus"

7.17 P

Relations between behavior of HLB and Iron application to Citrus tree

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Citrus Greening Disease (HLB) is one of the most serious citrus diseases all over the world. There are no effective methods to cure and major countermeasures are initial detection and cutting down of infected trees. Thus, HLB delivers serious impact to agricultural economy.

It is well known that HLB infected tree shows specific symptom like micronutrient deficiency. We revealed Iron (Fe) content of citrus leaves showing symptom of HLB were decreased rather than non-infected leaves (Pustika et al., 2008, Masaoka et al., 2011), and the activity of Fe(III) chelate reductase in root was reduced on HLB-infected citrus tree.

In this time we tried to evaluate the effect of Fe application to recover infected tree. Fe additive were applied to HLB-infected citrus tree and the density of HLB bacterium were evaluated using PCR. In some infected trees, the HLB bacterium became undetectable after treatment. This result suggests that Fe nutrient affects the ecosystems of HLB bacterium.

Reference

Pustika AB, Subandiyah S, Holford P, Beattie G.A.C, Iwanami T., Masaoka Y., (2008) Interactions between plant nutrition and symptom expression in mandarin trees infected with the disease huanglongbing. Aust Plant Dis, Notes 3:112-115

Masaoka Y, Pustika AB, Subandiyah S, Okdada A, Hanudin E, Purwanto BH, Okuda M, Okada Y, Saito A, Holford P, Beattie A, Miyata S, Iwanami T (2011) Lower Concentrations of Microelements in Leaves of Citrus Infected with Candidatus Liberibacter asiaticus. JARQ, 45: 269-275

7.18 P

Evaluation of antibiotics against the bacteria, *Candidatus* Liberibacter for control of citrus Huanglongbing

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Citrus Huanglongbing (HLB) is one of the most serious diseases of citrus worldwide. The present study was undertaken to screen antibiotics against *Candidatus* Liberibacter asiaticus (Las) while simultaneously assessing phytotoxicity to citrus. Twenty-eight antibiotics from ten classes of medical-antibiotics and three agricultural-antibiotics were tested for *in vivo* activities against HLB bacterium using the previously optimized graft-based chemotherapy method (Zhang et al., 2012). First samples for DNA extraction were taken at 4 months after inoculation; subsequent samplings were taken at 2 month intervals. The Las-infected plants were considered as Las positive by real-time qPCR with threshold cycle (Ct) values less than 32.0. The efficiency against the HLB bacterium of each compound was evaluated by Ct values in the inoculated plants (both scions and rootstocks), scion infected percentage and HLB bacterial transmission percentage. The phytotoxicity was determined by the survival and growth of scions treated by antibiotics. The results showed that beta-lactam antibiotics (Ampicillin, Pennicillin and Carbenicillin) were highly effective in eliminating the HLB bacteria, with undetectable Las titers in the inoculated plants by qPCR, and had no any phytotoxicity to citrus, with more than 75% scion survival. Antibiotics sulfonamide and tetracycline suppressed the HLB bacterium with Ct values of 35.7 on average, less than 30% scion infection and 16.9% Las-transmission percentage. The effectiveness of some antibiotics, such as aminoglycoside and quinolone, depended on their absorptions and permeability through citrus. Peptide antibiotics were not effective in eliminating or suppressing Las bacterium with less than 28.0 Ct values by qPCR and higher scion infection percentages and Las transmission rates. Three agric-antibiotics, Actidione, Validoxylamine A and Zhongsenmycin, were also effective in eliminating the HLB bacteria. Antibiotic combinations, such as beta-lactam and aminoglycoside, are suggested as future applications.

7.19 P

Systemic Acquire Resistance - SAR in the Control of Huanglongbing

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In Brazil, '*Candidatus* Liberibacter asiaticus' is the main causal agent of Huanglongbing (HLB), responsible for major losses in the Brazilian citrus production. HLB management includes the elimination of diseased citrus trees and control of the insect vector, *Diaphorina citri*. These measures have allowed maintaining HLB incidence under very low levels in different citrus producing areas of Brazil, but with high economic costs. Therefore, the objective of this study was to examine the use of systemic acquire resistance (SAR) based procedure for the control of this disease under greenhouse conditions. For each treatment, ten one year old plants of Valencia sweet orange (*Citrus sinensis* Osbeck) grafted onto Rangpur lime (*Citrus limonia* Osbeck) were pre-treated with acibenzolar-S-metil (0.2 g/plant), imidacloprid (4 g/plant) and thiamethoxam (1.5 g/plant), alone or in combination. The SAR inducers were applied 2 to 4 times at 60 days interval. The inoculation was carried out by tissue graft using diseased plant material, seven days after the first application of the SAR inducers. HLB symptoms were observed 180 days after inoculation in the check citrus plants. Furthermore, '*Ca*. L. asiaticus' was also detected by PCR in these check plants, as well as in the thiamethoxam treated Valencia sweet orange plants. No HLB symptoms nor bacterium detection by PCR were observed for the plants treated with imidacroprid, acibenzolar-S-metil, imidacroprid plus acibenzolar-S-metil, and thiamethoxam plus acibenzolar-S-metil. Imidacloprid treated plants showed phytotoxicity under the conditions of this experiment.

7.20 P

Effect of beneficial bacterial isolates from citrus roots in Florida on citrus Huanglongbing disease development (poster)

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Huanglongbing (HLB) is the most devastating disease of citrus in Florida. HLB is caused by the phloem-inhabiting bacterium '*Candidatus* Liberibacter asiaticus' (Las), which is transmitted by psyllid vector *Diaphorina citri*. The current management strategies of HLB are to control psyllids and eradicate infected plants. However, these management practices have not been able to stop spreading of HLB (Duan et al. 2009). Alternative approaches are needed to control HLB. In previous studies we isolated multiple bacterial strains from roots of healthy citrus plants in HLB infected groves in Florida, which have the potential to enhance plant growth and suppress diseases (Trivedi et al. 2011). Recently, early infection of roots by Las leading to root decline was suggested important in HLB disease development (Johnson et al. 2012). We hypothesize that introduction of beneficial bacteria to roots of citrus plants could decrease root damage by promoting root growth and reducing Las infection and thus improve HLB management. To test this hypothesis, six beneficial bacteria were assessed for their plant growth-promotion ability; and, three were found to be able to promote growth of both grapefruit and *Arabidopsis* (Col-0 ecotype) in greenhouse experiments, with increases in root length and weight, compared to mock treated plants. Thus, the three isolates were selected to evaluate the effect on Las infection in greenhouse assays. Details of the beneficial bacterial isolates, plant growth-promotion activity, and effect on Las infection will be discussed.

Duan, Y., Zhou, L., Hall, D. G., et al. 2009. Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. Mol. Plant Microbe Interact. 22:1011–1020.

Trivedi, P., Spann, T.M., and Wang, N. 2011. Isolation and characterization of beneficial bacteria associated with citrus roots in Florida. Microbial Ecology. 62:324-336.

Johnson, E.G., Bright, D.B., Graham, J.H. 2012. Early root infection and damage in citrus huanglongbing disease development. (Abstr) Phytopathology (Suppl 4) 102:S4.59. http://dx.doi.org/10.1094/PHYTO-102-7-S4.59.

Note: We would like to have this one as poster.

7.21 P

In-Field Thermal Treatment of Huanglongbing (HLB) infected Trees

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To decrease *Candidatus* Liberibacter asiaticus titer and increase the productive life of infected trees, thermal treatment of orange trees was proposed. A moving greenhouse was developed to cover single trees during the summer of 2012. Four trees (~ 2.5×2.5×2.5 m) were treated, one tree per day, during the months of September (trees T1 through T3) and October (tree T4). From each tree, three symptomatic branches were sampled to determine microbial kill before (0 h) and 2, 3, 4, and 5 h during the treatment. Temperature distribution throughout the canopy and on the sampled branches was also recorded. Maximal temperatures in the ranges 50 to 53 °C were reached at the top (2.4 m) of the canopy whereas at the bottom of the canopy (i.e., 0.6 m) maximal temperatures ranged from 36 to 43 °C. Due to varied micro-meteorological conditions during the treatment, temperatures of the T1 through T4 sampled branches reached above 40°C for 217, 166, 35, 228 min, respectively. For T1, T2 and T4 trees, average temperatures of the sampled branches reached above 45 °C for 87, 35, and 49 min or more. Attempt to quantitatively determine microbial kill by determining percent live bacteria at selected time intervals during thermal treatment was unreliable due to the very uneven distribution of initial proportion of live-to-dead bacteria and analysis variability. However, overall, after thermal treatments, live microbial populations decreased. These findings indicate that adequate thermal treatment of trees required forced convection air flow and supplemental heating.

7.22 P

Thermotherapy and chemotherapy to control citrus HLB in the field

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Huanglongbing (HLB), a systemic and destructive disease of citrus, is associated with three species of α-proteobacteria, '*Candidatus* Liberibacter asiaticus' (Las), '*Ca*. L. africanus' and '*Ca*. L. americanus'. Previous studies have found distinct variations in temperature sensitivity and tolerance among these species[1]. Las, the most prevalent and heat-tolerant species, can thrive at temperatures as high as 35°C[1]. Our earlier work has shown that Las bacteria in potted HLB-affected citrus were significantly reduced or eliminated when exposed to continuous temperatures of 40 to 42°C for a minimum of 48 h[2]. To determine the feasibility and effectiveness of thermotherapy in the field, various portable greenhouses were placed over commercial and dooryard citrus exposing trees to higher temperatures through solarization. Within weeks after treatment, most trees responded with vigorous new growth. Flush post-treatment had significantly less Las DNA present in leaves one year after treatment and trees continued to grow well. Unlike with potted trees, exposure to high heat through solarization was not sufficient to eradicate the Las population in field conditions, most flush after treatment was qPCR positive for Las. This may be attributed to fluctuating day and night temperatures, and the citrus roots imbedded in the soil being inadequately exposed to heat (temperature and duration). To further combat the systemic infection, chemicals such as penicillin and streptomycin used in conjunction with thermotherapy were applied to HLB-affected trees in the field. Plants post-treatment were monitored and leaves and roots were periodically sampled and tested for Las. Preliminary results indicate some treatment regimes are promising.

[1]MCG Gasparoto, et al. *Plant Pathol* **61**, 658 (2012).

[2]MT Hoffman, et al. *Phytopathology* **In Press** (2012).

7.23 P

Evaluation of Thermotherapy against Huanglongbing (Citrus Greening)under Laboratory Condition

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Abstract: Huanglongbing (HLB, citrus greening) is the most destructive disease of citrus. The disease is associated with "*Candidatus* Liberibacterasiaticus". Few management options are available, besides preventive measures such as the removal of affected plants, planting disease-free stock and maintaining vector-free production in quarantine areas. In this study, we assessed the efficacy of thermotherapy against the disease under control laboratory conditions. A total of sixty, 2-year old graft-infected *Citrus reticulate* Blanco seedlings were used for the study. The plants were randomly divided into 3 treatment groups (45, 48°C, and untreated), with 5 plants/rep, 4 reps/trt. The treated plants were placed in temperature chambers for a 4-h treatment session, repeated weekly3 times. Disease remission was observed beginning 8 weeks post-treatment. Real-time PCR assays revealed that pathogen "*Ca*. L. asiaticus" concentration of all HLB-affected seedlings were significant reduced except 8 plants under45 and 48°Ctreatments at 4 weeks after treatment. In contrast, pathogen concentration in the untreated control plants exhibited a significant increase, with the highest increase of about 30-fold compared to the initial pathogen concentration (pre-treatment). Except for 7 plants (7 out of 40 total plants), pathogen concentration in the new flushes of the treated plants decreased 90% at ca. 8 weeks

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after treatment, compared to the initial pathogen concentration. Nested and real-time PCR were used for confirmation of HLB infection in the seedlings, and for pathogen titer assessment.

Although the result is considered preliminary, it provides a foundation for further work in developing the technique for HLB management. Complementary work will include exploration of additional exposure time and temperature combinations as well as treatments using commercial field settings.

Key words: Huanglongbing; citrus greening; real-time PCR; thermotherapy; temperature

7.24 P

Thermotherapy for HLB Management - Historical perspective, anecdotal evidences, and recent research progress

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Although Asian type HLB is regarded as heat-tolerant, our literature review and analysis of climate data suggest that high summer temperature appears to restrict HLB distribution and occurrence. HLB worldwide distribution and severity appear to be impacted by high summer temperature - HLB is the most severe in the subtropical and tropical regions with moderate summer temperature maxima. Florida in the US, São Paulo in Brazil, Guangdong in China, and severe HLB occurrence regions share this climate characters.

Using heat for HLB management was first explored by Chinese scientists in the early 1960s. Studies revealed the effectiveness in using hot water and/or hot air for producing pathogen-free propagative materials. Small scale field trials were conducted using plastic sheeting in around the 1980s. Results were inconsistent, due to lack of quantitative means for measuring the efficacy in the field condition. Recent lab and field studies by our group in Guangdong and Fujian, China, and Florida reveal interesting findings. Las titers can be undetectable in the best scenario in the lab studies. Field studies achieved significant Las titer reduction and symptom remission.

Citations

7.25 P

Study of Thermotherapy against Citrus Huanglongbing in Fujian Province, China

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Abstract: Huanglongbing (HLB) is a major threat to the world citrus production.In this study, we investigated using a heat treatment technique for managing HLB-affected citrus trees in the field. A total of 725-to-8-year old mandarin citrus, *Citrusreticulata* Blanco, trees were used for the study.Nine trees were regarded as a replicate or a block, four replicates per treatment. Randomized complete block design was used for field experiment design. The treated trees were covered by using plastic sheeting for 6-h at day time, repeated three times weekly. Positive results were observed, judging by disease symptom expression and titer changes before and after treatment. New flushes and healthy young leave were abundance in the treated trees 4thweek as well as 11th weeks after last plastic sheeting. Approximately 60% treated trees had more than 80% reduction of Las titers, with eleven trees (11 out of 36) showing a decline of more than 95%, and eight trees with a slight increase of Las titers 4th week after treatment. Whereas Las titers in the untreated plants exhibited a significant increase, with the highest increase of about 96-folds, compared to pre-treatment 4th week after treatment. Las titers in the treated citrus trees declined more significantly 11th week after treatment, compared to those of untreated. About 44% treated trees had a more than 90% of titer reduction. Change of Las titer in the untreated trees varied substantially 11thweek after treatment. Twenty trees (20 out of 36) had a wide range of Las titer reductions, Las titers in the remaining 16 trees were increased significantly, with the highest increase of 31-fold, compared to the Las titer level of pre-treatment.

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Although the result is preliminary, it confirms that heat treatment can significantly reduce Las titers in the HLB-affect trees. This studyprovides a foundation for further future work in developing HLB management technique based on the technology.

Key words: Huanglongbing; citrus greening; real-time PCR; thermotherapy; temperature

7.26 P

Effect of Enhanced Zinc Nutrition on Mitigation of Huanglongbing (HLB)-affected Citrus

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The growth decline of huanglongbing (HLB)-affected citrus trees is considered to associate with nutritional disorder, as typical symptoms of HLB such as stunting tree, chlorosis or blotchy mottle of the leaves resemble to those of zinc (Zn), iron (Fe) and manganese (Mn) deficiency and lower Zn concentration has been consistently reported in the HLB affected than healthy plants. Hydroponic culture studies were conducted to evaluate effects of enhanced Zn nutrition on the mitigation of HLB-affected grapefruit seedlings. Modified Hoagland nutrient solutions were used with three Zn^{2+} levels: 0, 1.0, and 1.5 times of standard strength. Both HLB affected and Healthy grapefruit seedlings were subjected to the treatments for 49 days. During the growth period, photosynthesis of plant leaves was measured and at day 49 of the culture, leaf tissues and cells were examined for structural changes using light and scanning electron microscopy. Enhanced Zn nutrition generally improved the growth of HLB plants with less severity of symptoms. Photosynthesis in term of leaf electron transfer rate and photochemical quantum yield was enhanced. The wax layer and cuticle increased, and the epidermis cell became better organized, with higher number of normal stomatal openings, as compared to the control. In addition, enhanced Zn nutrition resulted in more developed xylem and phloem transport systems, thus reduced starch grains and polyphenol substances in the leaf cells. These results indicate that

enhanced Zn nutrition can improve the structure of photosynthetic, transfusion and protective tissue systems and thus promote photosynthesis, transport of photosynthates, and other related metabolisms of HLB-infected citrus.

Key words: Grapefruit, HLB, Zn^{2+}, tissue structure, transport system

7.27

Benefit-cost analysis of Huanglongbing management in Sao Paulo, Brazil

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The Brazilian citrus history is marked by several phytosanitary outbreaks due to new pests entry, frequently with high potential to generate economic losses. Some of these pests have threatened even the viability of citrus in Sao Paulo State, like Huanglongbing (HLB) that was responsible for eradicating 18 million plants between January 2005 and July 2012. It requires development of new methods to control the disease and ensure the economic viability of the activity. This study aimed to analyze costs and benefits of a broad control of HLB in the state (66 % of orchards are inspected and have HLB-symptomatic trees eradicated) and compare this scenario to another one representing a lower level of control (reference scenario, considering that only 26% of orchards manage HLB and proceed the eradication). We chose the Benefit-Cost Analysis to evaluate the differences between scenarios. Losses caused by HLB comprised reduction in productivity, elimination of infected plants and increase in production costs (due to inspections, insecticide applications, trees elimination and replanting). Costs and benefits were calculated according to state's phytosanitary status in 2010. The epidemiological model proposed by Bassanezi & Bassanezi (2008) was used to project the disease progress, crop loss damage, the citrus orchards size, orange production and producers' costs of production to control the disease along 20 years. The ratio B/C was estimated at 4.07 accumulated for the whole period. This result shows that each R$ 1.00 invested by producers to manage the HLB, prevents them to lose R$ 4.07 in gross revenues.

Citations

Bassanezi, R.B.; Bassanezi, R.C. An approach to model the impact of Huanglongbing on citrus yield. *Proceedings of the International Research Conference on Huanglongbing*, Orlando, p.263-264, 2008.

Session 8:

Host-Pathogen Interactions

8.1

Analyses of proteomic expression profiles and nutrient status of citrus plants in response to HLB

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Huanglongbing (HLB) is a highly devastating citrus disease and represents a major threat to the citrus industries in US. The etiology of HLB worldwide is associated with three insect-transmissible phloem-limited members of the bacterial group 'Candidatus Liberibacter spp', prevalently 'Candidatus. Liberibacter asiaticus' (Las). All citrus cultivars are susceptible to HLB. To better understand the physiological and molecular processes involved in host responses to Las, proteomic analyses via 2-DE and mass spectrometry as well as ICP spectroscopy analysis were employed to elucidate protein expression profiles in leaves of Las-infected grapefruit and lemon plants in presymptomatic and symptomatic stages of the disease. A Las-mediated down-regulation of 56 proteins including those associated with photosynthesis, protein synthesis, and metabolism was correlated with significant reductions in the concentrations of Ca, Mg, Fe, Zn, Mn, and Cu, especially in symptomatic plants. Interestingly, a Las-mediated up-regulation of 13 proteins including those associated with pathogen response, redox-homeostasis and starch anabolism was correlated with an increase in K concentration in pre-symptomatic and symptomatic plants. Since starch synthase requires K for activation, this result highlights a coordinated accumulation of granule-bound starch synthase and K in Las-infected plants. Analyses of host responses to HLB provides new information concerning physiological and biochemical processes of citrus to HLB. Those proteins that are up regulated specifically in response to Las infection could be useful biomarkers to develop a host-based diagnostic tool for early detection of HLB-affected citrus plants.

8.2

Transcriptome analysis of Huanglongbing-infected sweet orange leaves using RNA sequencing and quantitative PCR.

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RNA sequencing (RNA-seq) methods provide a complete description of RNA transcripts including alternative splicing and small RNA characterization. RNA-seq performed in our laboratory on healthy and Huanglongbing (HLB)-infected young leaves revealed that 4,044 transcripts were up-regulated and 2,562 were down-regulated in the diseased trees. Moreover, a number of genes showed alternative splicing events including exon skipping, intron retention, and 5' and 3' alternative splicing. Furthermore, quantitative PCR (qPCR) performed on 20 randomly chosen genes with high differential expression (10 up and 10 down regulated) showed that all were consistent with RNA-seq data. Additionally, variation in levels of gene expression was observed between young and mature leaves. These early host plant response genes due to HLB-infection might be useful in the development of early HLB-detection methods before manifestation of disease symptoms in the infected plants.

8.3

Comparison of microRNA Profiles and Some miRNA Target Gene Expression levels in Roots of Tangerine (*Citrus reticulata blanco* cv.'Sanhu') Trees infected with and without Hunaglongbing Bacteria

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Solexa sequencing was used to reveal the changes in small RNAome profile in roots of mock-inoculated (CK) and Huanglongbing bacteria-inoculated (HLB) 'Sanhu' tangerine (*Citrus reticulata blanco*) trees. Results showed that the number of reads of both unique and total sRNAs decreased apparently in roots following infection with HLB. Distribution in length of sRNAs changed also remarkably, showing an increase in 22 nt and 21 nt sRNAs and a decrease in 24 nt sRNAs in HLB infected samples. A total of 42 known miRNAs belonging to 27 highly conserved miRNA families were identified. Comparisons showed that 33 known miRNAs exhibited a significant expression difference between CK and HLB. In addition, 34 novel miRNAs, among which 24 were differentially expressed, were also identified, and their expression levels were analyzed by qRT-PCR. Three hundred and eighty five potential target genes were predicted for most of the 57 differentially expressed miRNAs. GO and KEGG annotation analysis revealed that most miRNA-target genes were those implicated in developmental process, response to stress and stimulus, transcription and protein metabolism. The characterization of the miRNAomes between the healthy and HLB infected Sanhu tangerine roots provided new insight into the involvement of miRNAs in HLB infection of citrus.

8.4

Citrus leaf volatiles response to *Candidatus Liberibacter* asiaticus and to its insect vector Asian citrus psyllids.

Faraj Hijaz and Nabil Killiny

Plant volatiles play an important role in defending plants against insects and pathogens attack. Released volatiles from insect-damaged plants may result in direct or indirect defense against insect and volatile accumulation in pathogen-infected plants may inhibit the movement of the pathogens within plant tissues. However, available information about the response of citrus leaf volatiles to Asian citrus psyllids (ACP) feeding and *Candidatus Liberibacter* asiaticus (Clas) infection is limited. Here we investigate the effect of ACP feeding, Clas infection, and simultaneous attack by ACP and Clas on the volatile content of Valencia leaf. Leaf volatiles were extracted using hexane and analyzed with gas chromatography-mass spectrometry (GC-MS). Eighteen out of the twenty-seven detected volatiles were induced (2 to 10-folds) in ACP-infested plants. On the other hand, only four volatiles were induced in Clas-infected plants (d-limonene, β-phelandrene, citronellal, and undecanl). The abundance of previous compounds was induced 4-folds in Clas-infected plants, except for limonene which was induced to more than 20-folds. In addition, citrus plants attacked simultaneously by ACP and Clas reduced their volatiles production compared to those attacked only by ACP. Our results suggested that insect and pathogen attack not only changes the profile of plant released volatile, but also influences the volatile contents of attacked plants. The result of this study might contribute to better understanding of citrus response to ACP and Clas attack and provide more information about the relation between stored and released volatiles.

8.5

Development of Symptom Expression and Presence of *Candidatus* Liberibacter asiaticus in Recently Infected, Mature Orange Trees

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Huanglongbing (HLB) is a serious disease of citrus that is threatening the citrus industry worldwide, including in Florida. HLB management is based on regional coordinated sprays for psyllid control, and either enhanced nutritional programs (ENPs) to prolong the productive lives of infected trees, or removal of infected trees. As infection rates in Florida have increased dramatically, many growers have opted for using ENPs. However little is known about how long a Florida orange tree will survive or produce quality fruit, when infected as a mature tree. This study focuses on understanding the rate of spread of HLB within newly symptomatic, mature trees. Three trees were selected on which initial symptoms were recently found and localized on one branch only; all initial symptoms tested positive for HLB disease using PCR analysis. The tree canopies were divided into eight sectors; two samples were taken from each sector every two months for 20 months, and were analyzed for the presence of the bacterium *Candidatus* Liberibacter asiaticus (Las), using Real Time PCR. As new symptoms developed they were flagged, logged, and analyzed using PCR. Asymptomatic leaves consistently tested negative for Las. Results show a relationship between symptom expression and pathogen detection. A definite pattern of spread of Las from the initial point of infection to nearby connected branches was documented. An estimate of canopy volume and rate of spread of symptoms and decline suggests that trees on the Southern Gardens standard nutritional program may remain viable between 4 and 30+ years after initial infection.

8.6

Phloem disruption from HLB infection in canopy and root framework

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Phloem sieve element plugging from callose and phloem protein 2 ligand production have been demonstrated at the leaf level with some phloem necrosis occurring just after or nearly simultaneously. Phloem necrosis also has been reported near the bud union, but it has not been carefully characterized at the trunk, canopy and root scaffold nor canopy and feeder root support structure level. Phloem samples were taken from 1-2 cm, secondary and primary scaffold limbs as well as root flares, pioneer roots and feeder roots. Samples of these were fixed and embedded for light and electron microscopy. In both young potted and bearing field trees, phloem of HLB infected trees showed more phloem cell production (layers of cells) than did healthy trees. Production of new phloem cells appeared to be occurring from already differentiating cells as well as the cambium. Wall distortion and thickening, starch accumulation, cellular content disruption and sieve element plugging occurred primarily in the intermediate cellular zone, at least 6 to 10 cells away from the cambium in trunk and canopy framework limbs. In contrast to stems, intermediate zone healthy feeder root phloem cells had accumulated starch and HLB affected roots had not. Collapsed cells, thickened walls and disrupted cytoplasm were typical in the HLB affected feeder and pioneer root phloem. This intermediate phloem zone may represent the bacterial affected tissue in the scaffold and support structures while the newest tissues have not had time for bacterial invasion or the effects thereof.

8.7

Candidatus Liberibacter asiaticus titers in citrus cultivars in the field and in ACP inoculated greenhouse trees

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A survey of seven six citrus cultivars (C. sinensis, C. paradisi, 'Temple' tangor, 'Minneola' and 'Orlando' tangelos and, 'Fallglo' and 'Sunburst' mandarin hybrids) growing in commercial citrus orchards in Florida revealed a strong correlation between HLB incidence and severity and Candidatus Liberibacter asiaticus (CLas) titer (Stover and McCollum, 2011). Temple tangor and grapefruit consistently exhibited the least severe HLB symptoms and lowest CLas titers, followed by , in increasing order of HLB symptoms 'Fallglo', C. sinensis,

In the orchard, Candidatus Liberibacter asiaticus (CLas) is transmitted to citrus hosts via the Asian citrus psyllid (ACP); however, in most greenhouse studies, CLas inoculation of citrus has been conducted by grafting. In an attempt to more closely mimic the natural process of CLas transmission by ACP to citrus we conducted a greenhouse study that included CLas-infected citron (*Citrus medica*) to serve as a source of inoculum, free-ranging ACP to vector the pathogen and 16 citrus genotypes (*C. medica*, *C. reticulata*, *C. grandis*, *C. sinensis*, *C. x paradisi*, *Poncirus trifoliata*, and nine citrus hybrids) as hosts. Our objectives were to determine the incidence of CLas infection and titer among the 16 genotypes over time. The experiment was conducted three times. Leaf samples were collected at regular intervals over a period of ca. 300 days and each sample was assayed for the presence of CLas. In each experiment, CLas titer remained at less than 10^1 copies 16S rDNA g^{-1} fwt until 150 to 175 days after placing CLas negative trees into the greenhouse, and thereafter increased steadily for the remainder of the experiments. After 300 to 350 days in the greenhouse grand means for CLas titer ranged from 10^3 to 10^5 g^{-1} fwt, although HLB symptoms were not apparent. Significant differences in CLas titer among the cultivars were first detected at approximately 125 days and throughout the remainder of the experiment. Our results have significant implications for studies involving transmission of CLas by ACP and subsequent disease development.

8.8

Modulation of plant defense responses by Salicylate hydroxylase of *Candidatus* Liberibacter asiaticus

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Citrus huanglongbing (HLB), associated with pathogen *Candidatus* Liberibacter asiaticus (Las), is a devastating disease to the US citrus industry (1, 2). To gain knowledge on the mechanism(s) by which Las evades host defense responses we first expressed salicylate hydroxylase (*sahA*) of Las in *Escherichia coli.* Our data indicate that *Las* encodes a functional salicylate hydroxylase, which converts salicylic acid (SA) into catechol, a product that does not induce resistance. The *sahA* gene was highly induced *in planta* compared to psyllid vector suggesting its important role in disease progression. To determine expression level of defense related genes after Las infection, *Xanthomonas axonopodis* pv. *citri* strain A^W (Xac A^W) was used to induce *PR* gene expression. The *PR-1* gene expression in Xac A^w challenged plants which were previously infected with Las was lower than Xac A^w challenged healthy plants. Using SA biosensor strain (*Acinetobacter* sp. ADPWH_lux), 4 fold reduction in SA accumulation was observed in the Las infected as compared to healthy plants. To understand the possible synergistic effect of the presence of Las on the citrus canker [caused by *X. citri* subsp. *citri* (Xcc)] we inoculated Xcc in Las infected and healthy leaves of grapefruit. The population levels of Xcc were significantly higher during all the observation time points (up to 14 days) in Las infected as compared to healthy citrus. that modulation of SA production and subsequent regulation of defense related genes such as *PR-1* gene could be one of the mechanisms deployed by Las to evade plant defense responses. The Las infected plants compromised with defense responses could further succumb to the infection by other pathogens. We also conducted experiments to restore the SA level in Las infected plants using SA hydroxylase inhibitors and test their effect in controlling HLB.

Citation

1. Bové, JM. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. 2006. Journal of Plant Pathology, 88 (1), 7-37.
2. Gottwald, TR, da Graça, JV, and Bassanezi, RB. 2007. Citrus Huanglongbing: The pathogen and its impact. Online. Plant Health Progress doi:10.1094/PHP-2007-0906-01-RV.

8.9

Early root infection and damage in Huanglongbing disease development

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Huanglongbing in grove trees is initially identified by foliar symptoms, most commonly blotchy mottle. Detection of *Candidatus* Liberibacter asiaticus (Las) in leaf tissue by qPCR early in disease development is usually limited to symptomatic leaves and proximal young leaves. Over multiple years, disease symptoms spread to the rest of the canopy. Although Las has been detected in root tissue, the decline of roots has been assumed to happen later in disease development when photosynthate production and transport have been significantly diminished in the tree canopy. Observations of initial spread of Las from the bud-inoculation site in the trunk of 1-yr-old potted trees have revealed that Las is frequently detectable in roots months before detection of Las in leaves and foliar symptom development. Even after symptom development Las is more evenly distributed in root tissue than in the canopy. Preliminary evidence suggests that Las is also more evenly distributed in roots of grove trees. Asymptomatic 9 year old grove trees with root Las infection had 26-41% lower root density than asymptomatic trees without detectable root Las. The loss of root density was independent of Las detection in leaves. Root loss precedes carbohydrate starvation as evidenced by root starch concentrations, suggesting the bacteria may play a more active role in root loss than phloem plugging. These results suggest that early invasion of roots by Las leads to root decline before the appearance of foliar symptoms and is likely the cause of larger than expected yield reduction on trees with limited foliar symptoms.

8.10

Colonization and distribution patterns of *Candidatus* Liberibacter asiaticus in distinct citrus rootstocks

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A study was conducted to assess (i) patterns of Las colonization in graft inoculated 1-y-old seedlings of Cravo Rangpur lime, Sunki mandarin and Swingle citrumelo and (ii) patterns of Las distribution in naturally infected 4 and 10-y-old trees of the sweet oranges 'Pera' on Sunki and 'Folha murcha' on 11 rootstocks. The seedlings were inoculated at the trunk 40 cm above the substrate level. Samples of bark were collected at the inoculation site, 10, 20, and 30 cm below it, and from the root 45, 75, 105, and 135 days after inoculation (dai). Samples from trees included symptomatic leaves, and bark from the trunk (10 cm above or below the grafting line) and root. The samples were analyzed through qPCR to estimate log Las genome/gram tissue. In seedlings Las colonization pattern was similar for all rootstocks. Las was detected in the root 45 dai, or 3 months before the symptoms developed on leaves. In Swingle maxima titers were lower but reached a plateau faster (45 to 75 dai) than in Sunki or Cravo (75 to 135 dai) for all sampled sites. In the field the distribution pattern of Las in the trees was also similar for all rootstocks, with higher titers detected in the leaves (5.29±0.22) than in trunks (4.38±0.49; 3.78±0.27) or roots (3.38±0.28). No correlation existed between the amount of symptom on the canopy and Las titer in the root. This lack of correlation plus the fast movement of Las from the inoculation site down to the root may explain the failure of pruning to control HLB.

8.11

Spatial Imaging of Zinc and Other Elements in Huanglongbing-affected Grapefruit by Microscopically Focused Synchrotron X-Ray Investigation

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Huanglongbing (HLB) is a highly destructive, fast spreading disease of citrus, causing substantial economic losses to the citrus industry worldwide. Nutrient levels and their cellular distribution patterns in HLB-affected grapefruit were analyzed after graft-inoculation of infected lemon scions containing '*Candidatus Liberibacter* asiaticus', the heat-tolerant Asian type of HLB bacterium. After 12 months, infected plants showed typical HLB symptoms including leaf curl and blotchy mottles on leaves. Zinc (Zn) concentrations in young, mature, and old leaves of grapefruit significantly decreased by HLB infection. **Micro-XRF imaging of Zn and other elements showed that preferential distribution of Zn was observed in the phloem tissues of leaves and stems collected from healthy grapefruit plants but absent from HLB-affected samples. Quantitative analysis of Zn intensity in the cross-sections of leaves using standard samples revealed that Zn concentration in phloem tissues of healthy grapefruit leaves was more than 10 times higher than that in the HLB-affected leaves.** No significant variation was observed for the distribution patterns of other elements such as K and Ca in stems and leaves of grapefruit plants before or after graft-inoculation of HLB infected lemon scions. These results suggest that reduced phloem transport of Zn is one of the most important constraints that contribute to HLB-induced Zn deficiency in citrus such as grapefruit. Our report provides the first *in situ* visualization of elemental variation within the tissues of HLB-infected citrus at cellular level.

Keywords: Distribution; grapefruit; huanglongbing; micro-XRF; zinc

8.12 P

Genome-wide Expression Profiling in Ponkan Infected by *Candidatus* liberibacter asiaticus

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Huanglongbing(hlb) is an economical and destructive disease on citrus in South China, such as Guangdong, Guangxi, caused by bacterium, *Candidatus* liberibacter asiaticus. The interaction in mRNA level between pathogen and citrus (Ponkan, *Citrus reticulata* Blanco) was primarily researched by Digital Gene Expression Tag Profiling. Ponkan leaves of 13 weeks and 26 weeks after HLB inoculation were used for analysis. The numbers of up-regulated genes were increased from 37% in 13 wpi (weeks post inoculation) to 64% in 26 wpi. The differentially expressed genes (DEGs) fold change increased more than 8 times from 16.7% to 87.3%. Gene ontology (GO) process molecular function enrichment analysis showed that the DEGs with oxidation reduction function increased from 4.41% to 8.48% and that DEGs responsive to stresses increased from 1.10% to 2.08%, but these related to defense responses decreased from 0.74% to 0.64%. However, those related to defense responses of down-regulated genes increased from 0.55% to 0.79%. Apparently, the expression level of resistance genes strengthened, while the defense ability of host declined along with enhanced stresses caused by HLB infection. Photosynthesis related genes were down-regulated in both 13 wpi and 26 wpi, which indicated that HLB infection greatly reduced the citrus photosynthesis, perhaps via feedback regulation of the accumulated starches resulted from blockage of sieve tubes by the bacteria in the phloem tissue. RIN4 is a negative regulator of plant immunity, also found up-regulated by approximately 9-fold.

8.13 P

Synthetic Peptides target ATP translocase of '*Candidatus* Liberibacter asiaticus' to block ATP uptake

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As an obligate intracellular pathogen, *'Candidatus* Liberibacter asiaticus' (Las) may act as an "energy parasite" by importing ATP from its host's cells. We previously demonstrated that the Las translocase NttA (gb|ACX71867.1) is functional in *Escherichia coli* and enables the direct import of ATP/ADP into the cell. Similar to other translocases, NttA was predicted to contain 12 transmembrane domains with 6 loops residing on the outer surface of the membrane. Using this structure, a 25-mer peptide was selected for synthesis based on the transmembrane (TM) hidden Markov model (HMM) and used as the target for the randomized 7-mer Ph.D.™ phage display library (New England Biolabs, USA) in an attempt to block the ability of NttA import ATP. Of the selected clones, 11 shared the HWGMWSY sequence, five shared the sequence SILPYPY, and four had unique 7-mer sequences. An ELISA was performed against the 25-mer using members of the two most highly represented sequences and all of the unique sequences. Of these phage, six appeared to have binding capacities. Radiolabeled ATP uptake assays were performed on *E. coli* expressing NttA using 10 small synthetic peptides based on the phage sequences, and the results indicated that peptides from the HWGMWSY grouping showed the most potential for blocking ATP uptake. Two peptides, HS-6 covering the first six aa (+1 charge) and GY-5 (neutral) covering the last five aa, were selected. At a concentration of 0.23 mM, both HS-6 and Gy-5 peptides decreased the amount of [α-^{32}P] ATP in the cells by 29.9 and 27.9%, respectively, while at a peptide concentration of 2.3 mM the amount of intercellular [α-^{32}P] ATP declined by 57.6 and 70.6%, respectively. Since NttA shares limited amino acid identity with other known proteins, we expect to have Las-specific inhibition when these peptides are expressed in citrus plants.

8.14 P

Characterization of the microbial community structure in '*Candidatus* Liberibacter asiaticus'-infected citrus plants treated with antibiotics

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The updated PhyloChip™ G3 were used to explore the differences in the relative abundance and phylogenetic diversity of the bacterial communities associated with HLB-affected citrus plants in the field over a growing season and those treated with antibiotic combinations of AG (Ampicillin at 1000 mg/L and Gentamicin at 100 mg/L) and PS (Penicillin at 1000 mg/L and Streptomycin at 100 mg/L). Both antibiotic treatments resulted in significantly lower Las bacterial titers ($Pr<0.05$) and their hybridization scores. Of the 50,000+ available operational taxonomic units (OTUs) on PhyloChip™ G3, 7,028 known OTUs in 58 phyla were detected from the field plants, and 7,407 OTUs in 53 phyla from the inoculated potted plants. Proteobacteria was the constantly dominant phylum of bacteria (38.7%~44.1%) vying for prevalence based on the season, followed by Firmicutes (23.5%~29.0%), Actinobacteria (12.4%~16.1%), Bacteroidetes (6.2%~6.6%) and Cyanobacteria (2.3%~3.2%). Circular tree comparing the Las-free and the Las-infected samples indicated that only 17 families present in the Las-free plants, such as *Cyanobacteriaceae*; but more than 137 families detected in the Las-infected plants, such as *Staphylococcaceae* and *Pseudonocardiaceae. Cyanobacteria* are believed to be responsible for introducing oxygen into the atmosphere and fixing nitrogen and phosphorus assimilation for plant growth. Both *Staphylococcaceae* and *Pseudonocardiaceae were* recognized as an emerging opportunistic pathogen of plant and animals. When compared to the bacterial populations in the leaves of citrus trees receiving the water control treatment, the *Bacteroidete* population decreased ($Pr<0.05$) by 59.6% and 51.8% in the plants receiving AG and PS treatments, respectively. The over-all diversity of bacteria also decreased with the antibiotic treatments. Bacterial cells in close proximity may be able to modify their microenvironment; thus, making the composition of the microbial community an important factor in the ability of Las to cause HLB progression. A low Las level was seen as a both a seasonal fluctuation, part of the bacterial population dynamics, and as a response to the antibiotic treatments.

8.15 P

Monitoring of *Candidatus* Liberibacter asiaticus in Citrus Seedlings at Greenhouse Conditions and Commercial Orchards of Sweet Orange and Tahiti Lime in the Northwest of Parana State, Brazil

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This study aimed to monitor the behavior of Ca. Liberibacter asiaticus in grafted seedlings in the greenhouse and in commercial orchards of sweet orange and Tahiti lime. Plants of sweet orange and Tahiti lime naturally infected with HLB were protected with screens aphid-proof to prevent the spread of disease. In greenhouse were used 17 Pêra variety seedlings that were inoculated in 2008 with infected budwood. The detection of HLB in the plants was carried out using conventional PCR. Ten leaves of each plant were collected for DNA extraction and for their full monitoring of the bacterial population by quantitative PCR for a period of 19 months. Ten leaves of each plant were collected for DNA extraction and for a period of 19 months were realized accompaniment of the bacterial population by quantitative PCR. Were observed erratic behavior of the bacteria. Even after bacterium detection in the plant, after a few months was not possible to verify the presence of the etiologic agent in the same place. In the seedlings the bacterium was not detected in 82.3% of the plants in certain months, coinciding with the warmer months of the year in Brazil, despite its presence have being detected at earlier dates. For the plants in commercials orchards, were expressed Ct between 18 and 33. The pathogen was even found when the expression of symptoms was small. The study of the population behavior of this agent contributes to the understanding of the epidemiology of this disease.

Support: CNPq, Capes, Araucaria Foundation

Topic Categories: Survey, Detection and Diagnosis

8.16 P

Nutritional Analysis of Flowers from 'Valencia' Orange Tree Infected with Huanglongbing.

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In the mid-2000s, Brazil was reported the disease huanglongbing (HLB), considered by its complexity one of the most destructive diseases in plants. As an agent associated with HLB, we have bacteria: "*Candidatus* Liberibacter americanus", "*Ca*. Liberibacter asiaticus "," *Ca*. Liberibacter africanus". Symptoms of greening may be masked by other symptoms generated from some diseases, besides, such symptoms are similar to those caused by various mineral deficiencies. The objective of this study was to verify if the HLB affects the accumulation of nutrients in citrus flowers. The experiment was in Valencia oranges trees (*C. sinensis*), in Rangpur lime (*C. limonia*) with 12 years of age. Treatments consisted of: 1) symptomatic branches of flowers (PCR+), 2) asymptomatic branches of flowers (PCR+) and 3) flowers of healthy plants (PCR -). The levels of macronutrients and micronutrients, in december 2011. The results showed, lower levels of N, P, K, Ca, Mg, B, Fe, Mn in the flowers of diseased plants (asymptomatic and symptomatic) compared with the levels of certain nutrients in healthy plants. The reduction in nutrient concentration mobile (N, P and K) as not mobile in the phloem (Ca and B) shows that the absorption and distribution of nutrients were reduced in young tissues and justify the diversity of symptoms found. The nutrients are involved in the activation / inactivation of enzymes related to metabolism and biosynthesis of plant hormone, auxin, gibberellins and cytokinins, which regulate the abortion of plant organs.

8.17 P

Transmission of Candidatus Liberibacter asiaticus to 16 citrus cultivars by Asian citrus psyllids in a greenhouse study

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In the orchard, Candidatus Liberibacter asiaticus (CLas) is transmitted to citrus hosts via the Asian citrus psyllid (ACP); however, in most greenhouse studies, CLas inoculation of citrus has been conducted by grafting. In an attempt to more closely mimic the natural process of CLas transmission by ACP to citrus we conducted a greenhouse study that included CLas-infected citron (*Citrus medica*) to serve as a source of inoculum, free-ranging ACP to vector the pathogen and 16 citrus genotypes (*C. medica*, *C. reticulata*, *C. grandis*, *C. sinensis*, *C. x paradisi*, *Poncirus trifoliata*, and nine citrus hybrids) as hosts. Our objectives were to determine the incidence of CLas infection and titer among the 16 genotypes over time. The experiment was conducted three times. Leaf samples were collected at regular intervals over a period of ca. 300 days and each sample was assayed for the presence of CLas. In each experiment, CLas titer remained at less than 10^1 copies 16S rDNA g^{-1} fwt until 150 to 175 days after placing CLas negative trees into the greenhouse, and thereafter increased steadily for the remainder of the experiments. After 300 to 350 days in the greenhouse grand means for CLas titer ranged from 10^3 to 10^5 g^{-1} fwt, although HLB symptoms were not apparent. Significant differences in CLas titer among the cultivars were first detected at approximately 125 days and throughout the remainder of the experiment. Our results have significant implications for studies involving transmission of CLas by ACP and subsequent disease development.

8.18 P

Colonization of Seeds of Citrus Rootstock Varieties by '*Ca.* Liberibacter asiaticus'

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Huanglongbing (HLB) is a disease of citrus associated with a systemic infection by the α-proteobacterium '*Ca*. Liberibacter asiaticus'. Infection of an individual tree can occur via psyllids (*Diaphorina citri* Kuwayama) carrying the bacterium or if the tree is propagated from infected budwood. Seed transmission is another possible mode of dissemination of the pathogen. Rootstock varieties are propagated from seed so we assessed the seed transmission among eighteen rootstock varieties using seeds from mature fruit collected in late winter and immature fruit collected in late summer. In dissected seeds real-time PCR detected pathogen DNA in seed coats at an incidence of 0-100%, whereas no pathogen DNA was detected in cotyledons or embryos from any variety. Seeds collected in late winter were germinated in a greenhouse and no pathogen DNA was detected in extracts of shoots of 425 seedlings harvested at 7-10 days post-germination whereas a small amount of pathogen DNA was detected in extracts from 6 of 425 roots. All six positive samples were the same rootstock variety which had 100% colonization of seed coats. The positive samples likely are a result of remnant seed coat which was not completely removed from cotyledons of the harvested seedlings. The data from this study suggest the pathogen can colonize the seed coat but it does not colonize embryos, which makes seed transmission unlikely.

8.19 P

Seasonal Concentration of Macro and Micronutrients in Different Vegetative Organs of Valencia Oranges Tree Affected By HLB

Medina, C.L.1,3; Saccini, V.A.V. 2, Dos Santos, D.M.M. 2, Machado, R.S. 1, Bataglia, O.C. 1, Furlani, P.R. 1 .1 CONPLANT, Training Consultancy, Agricultural Research and Development, Ltd., Campinas, SP, Brazil; 2 FCAV / Universidade Estadual Paulista, Jaboticabal, Brazil; 3 GCONCI/Citrus Consultants Group.

Plants infected with HLB have obstructions that affect the phloem transport of carbohydrates for developing organs such as fruit and other organs as the cambial region and roots. Some nutrients may be affected and it is usual the observation of foliar deficiency symptoms of minerals such as Mg and Zn. As the root system can also be harmed is possible that the deficiency symptoms are also caused by decreased absorption and not only by lack of redistribution through phloem. The objective of this study was to investigate throughout the year different nutrient concentrations of young leaves, mature leaves, young branches and the cambial region of Valencia Orange trees (*C.sinensis*) 12 years old, grafted on rangpur lime (*Citrus limonia*) . 1) symptomatic branches of flowers (PCR+), 2) asymptomatic branches of flowers (PCR+) and 3) flowers of healthy plants (PCR -). Healthy plants showed higher levels of nitrogen, calcium, magnesium, zinc and copper. There were variations in function the analyzed organs over time. The results are discussed according to the mobility of nutrients in plants.

8.20 P

Nutrient concentration in sap extracts of HLB-infected trees

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Huanglongbing (HLB) has affected approximately 4% of the citrus trees in São Paulo State, Brazil. Scouting, diagnosis and eradication of affected plants, as well as control of the Asian psyllid vector of *Ca*. Liberibacter spp. were established as required measures for suppression of disease inoculum and maintenance of fruit production in the orchards. Despite the relative effectiveness of those, growers still look for nutrient management practices to minimize losses due to expected progress of the disease. However, clear evidence of positive effects of improved mineral nutrition on tree health and productivity is lacking. A study was set up in the field with 8-yr-old sweet orange trees to evaluate the effects of nutrients (K, Zn and Mn), phosphate and salicilate leaf sprayed to the trees four times in the year during spring and summer. The orchard presented <2% of HLB infected trees beginning the study and experimental plots presented 1800 trees each, which allow access epidemiology of the disease within studied treatments. Temporal progress of HLB-symptomatic trees, fruit yield and nutritional status of trees has been evaluated. Preliminary results demonstrated that nutritional treatments did not improved vigor of HLB-symptomatic trees. Furthermore, nutrient concentrations in leaves and sap extracts were correlated, and major differences were observed for N, Ca, Mg, Mn and Zn in sap extracts with predominately lower levels in symptomatic compared to asymptomatic trees. These results have pointed out new research approaches of our research group.

Session 9:

Pathogen Genomics, Bioinformatics, Phylogenetics, and Culturing

9.1

Comparative genomics analysis of *Liberibacter* species to elucidate pathogenesis and culturability.

Michael T. Leonard[1], Jennie R. Fagen[1], Connor M. McCullough[1], Austin G. Davis-Richardson[1], Michael J. Davis[2,3], and Eric W. Triplett[1]

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Liberibacter crescens BT-1, a gram negative, rod-shaped bacterial isolate, was previously recovered from mountain papaya and sequenced. As L. crescens is culturable and is not a pathogen of citrus, comparative genomics of this strain with other uncultured, pathogenic *Liberibacter* should suggest genes involved in both phenotypes. Genomic comparison to *Liberibacter* sp. revealed differences in metabolic pathways and virulence genes. Specific differences in metabolism include the inability of *L. asiaticus* and *L. solanacearum* to synthesize histidine, tryptophan, and thiamine, as well as a reduced ability to produce other amino acids. *L. asiaticus* and *L. solanacearum* possess a thiamine ABC transporter not found in *L. crescens*, which may compensate for the inability to synthesize thiamine. Diversity in the flp pilus operon and metal ABC transporters were observed that may contribute to the pathogenesis of the uncultured species. These differences may contribute to variation in virulence and culturability among species. Finally, two putative prophage regions were found in *L. crescens* that share moderate functional similarity to the phage regions of *L. asiaticus*, but sequence similarity is not conserved. Additional genomic comparisons will expand our understanding of virulence and vector-interactions of the described *Liberibacter* sp.

9.2

The complete genome sequence of *Candidatus* Liberibacter americanus, a bacterium associated with Citrus Huanglongbing in Brazil.

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Fundecitrus, Araraquara, Brazil[1]; University of Florida, Gainesville, USA[2]; Universidade de São Paulo, São Paulo, Brazil[3]; INRA de Bordeaux, Villenave d'Ornon Cedex, France [4]; Universidade Federal do Mato Grosso do Sul, Campo Grande, Brazil[5]; Instituto Biológico, São Paulo, Brazil[6];

We used PFGE followed by CsCl bisbenzymide centrifugation to obtain sufficient DNA for pyrosequencing of the *Ca*. Liberibacter americanus (Lam) strain "São Paulo" genome. The complete circular genomic DNA sequence of Lam is 1,195,201 bp, with an average GC content of 31.12%, somewhat lower than other Liberibacters. There are 1,056 predicted Lam genes, with 1,002 encoding proteins, 9 encoding rRNA genes and 45 encoding tRNAs. The overall gene organization and structure of the Lam genome is more similar to Lso than to Las. There are 951 genes common to Lam, Lso and Las, 27 genes found in Lam and Lso but not Las, and only 8 genes common to Lam and Las but not found in Lso. Many pseudogenes or truncated genes were found among the unique genes of all 3 species. As with Las, two prophage were confirmed in Lam, with SP2 being 39,941 bp and SP1 being 16,398 bp in size; as in Las, the one that appears to replicate as an excision plasmid prophage carries putative lysogenic conversion genes, specifically peroxidases and a Type Vc secreted adhesin. These predicted peroxidases and adhesin were found in both Las and Lam, but appeared fragmentary or degenerated in Lso, indicating their potential for citrus host range determination. Liberibacter genomes seem to be under selective pressure to reduce GC% content and to lose unimportant genes. Although Lam have an outer membrane, most of the genes required for biosynthesis of lipopolysaccharide, which can trigger ROS production, are missing from the Lam genome.

Funding: The Citrus Research and Development Foundation (Grant #65).

9.3

Clues into the metagenome of Huanglongbing infected *Citrus* by analysis of ancillary sequences from Ion Torrent whole genome *Candidatus* Liberibacter asiaticus sequencing.

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Huanglongbing (HLB) is a globally devastating disease of citrus. Presently, three etiological agents are associated with HLB and include; *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter americanus; and *Candidatus* Liberibacter africanus. Attempts to determine alternate (non-Liberibacter) associated etiological agents of HLB have been performed, namely by metagenomic analyses with HLB phenotypic citrus of phloem tissue isolated from bark [1] and whole leaf midribs [2]. These reports indicated a strong correlation for Liberibacter species associated with HLB etiology, but they do not indicate the presence of other significant associated etiological agents. Utilizing both PCR and non-PCR based metagenomic strategies; these previous reports present an undersized view (relating to inherent technique limits, restricted sample scope, and/or bacterial bias) into the etiology of HLB. Here we report ancillary contiguous metagenomic sequences contained amongst whole CLas genome amplification contiguous sequences that were sequenced on the next generation Ion Torrent PGM sequencing system. Within these ancillary sequences, a diverse metagenomic community is present, indicating a rich diversity of both prokaryotic and eukaryotic organisms that are unique and in common within these globally isolated HLB citrus samples. Although this report does not represent a complete metagenomic study of HLB diseased citrus, it does suggest that the use of Ion Torrent PGM sequencing system can be employed for metagenomic analysis. These findings justify a more complete analysis of the metagenome of HLB etiology, which may help further elucidate the HLB disease complex.

References:

1. Tyler HL, Roesch LF, Gowda S, Dawson WO, Triplett EW (2009) Confirmation of the sequence of 'Candidatus Liberibacter asiaticus' and assessment of microbial diversity in Huanglongbing-infected citrus phloem using a metagenomic approach. Mol Plant Microbe Interact 22: 1624-1634.

2. Sagaram US, DeAngelis KM, Trivedi P, Andersen GL, Lu SE, et al. (2009) Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. Appl Environ Microbiol 75: 1566-1574.

9.4

The Dynamics of Prophages/Phages FP1 and FP2 of '*Candidatus* Liberibacter asiaticus' in Response to Stress Conditions

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'*Candidatus* Liberibacter asiaticus' (Las), the prevalent bacterial pathogen associated with citrus huanglongbing (HLB), harbors at least two prophages, named FP1 and FP2. Due to the fastidious nature of Las, little is known about the prophage's response to stress conditions. In this study, we used real time PCR to investigate the potential conversion of the FP1 and FP2 prophages under stress conditions by comparing the 16S rDNA copy number in HLB-affected periwinkle and citrus. When HLB-affected periwinkle was exposed to heat stress for 4.0 hours, more FP1 and FP2 phage particles were released at 42℃ and 45℃ than at 37℃. A temperature increase from 23℃ to 37℃ caused the relative copy numbers of FP1 and FP2 to increase six folds, while a shift from 23℃ to 42℃ or 45℃ caused the relative copy numbers of FP1 and FP2 to increase between 7.5 and 15-folds compared to the initial samples. Meanwhile, similar results were found when HLB-affected citrus scions were treated with tetracycline at concentrations of 500 ppm to 2000 ppm by soaking for three days. When treated with tetracycline for 7 to 9 hours, the relative copy numbers of FP1 and FP2 reached their highest levels with an increase of 6 to 11.1-folds compared to the initial samples. The results indicate that stress causes the prophages in Las to convert from the lysogenic to lytic cycle. Furthermore, transmission electron microscopy (TEM) provided direct evidence that an upward temperature shift is accompanied by the lysogenic to lytic conversion. This conversion from the lysogenic to lytic cycle may have applications in terms of modulating HLB populations in naturally occurring infections. The study gives new insight into the interaction of prophages and HLB, which may play a potentially important role in the control of HLB.

9.5

Exploiting the Las and Lam phage for potential control of HLB

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Huanglongbing (HLB) is a lethal disease of citrus caused by *Ca*. L. asiaticus (Las), *Ca*. L. americanus (Lam), and *Ca*. L. africanus. Our published results demonstrate that Las carries a prophage with a lytic cycle that can become activated in plants to kill the Las cell that carries it. Our more recent results analyzing the complete genome of Lam (refer Wulff *et al* abstract at this conference) demonstrates that it, too, carries a very similar prophage and apparent lytic cycle. Our goal is to try to develop a sensitive, multiwell, microtiter dish assay for high throughput screening of chemicals with ability to trigger the lytic cycle and potentially lead to a chemical treatment method to eliminate Las from infected trees, whether symptomatic with HLB or not. The intergenic region between the early and late genes of Las phage SC1 and SC2 (between locus tags gp120 and gp125) were cloned in both directions upstream of the *lacZ* reporter gene in *E. coli*. We then cloned and expressed predicted repressors and anti-repressors from Las to determine responsiveness of the reporter constructs. As expected, the predicted early gene reporters of both SC1 and SC2 were constitutively on and the late genes were constitutively off. However, the predicted repressors and antirepressors failed to function as predicted and a detailed examination of the intergenic region revealed that late gene expression is likely initiated in at least one other location. Additional putative late gene promoter regions are being analyzed.

Funding: The Citrus Research and Development Foundation (Grant #535)

9.6

Characterization of putative virulent factors of *Candidatus* Liberibacter asiaticus

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Citrus greening or huanglongbing (HLB) is a devastating disease of citrus, and poses a major threat to the citrus industry in the United States (1, 2). *Candidatus* Liberibacter asiaticus has been known to be associated with HLB in the United States (3, 4). Unsuccessful attempts to culture *Ca*. L. asiaticus have notably hampered efforts to understand its biology and pathogenesis mechanism despite some limited progresses in culturing. In order to characterize the putative virulence factors, we expressed putative virulent factors in *Nicotiana benthamiana*. Totally 24 putative virulent factors are being tested with most of them containing signal peptides. By transient expression of the candidates using TMV vector in *N. benthamiana*, we can screen the genes influencing plant development and morphology. Meanwhile, transformation of candidate genes into *N. benthamiana* driven by 35S promoter and phloem specific promoter respectively will further verify the function of putative virulence factors. Identification and characterization of the various virulence factors in *Ca*. L. asiaticus will advance the understanding of the biology and pathogenicity of the pathogen.

Citation

1. Bové, JM. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. 2006. Journal of Plant Pathology, 88 (1), 7-37.
2. Gottwald, TR, da Graça, JV, and Bassanezi, RB. 2007. Citrus Huanglongbing: The pathogen and its impact. Online. Plant Health Progress doi:10.1094/PHP-2007-0906-01-RV.
3. Sagaram, U., De Angelis, K. M., Trivedi, P., Andersen, G. L., Lu, S. E., and Wang, N. 2009. Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. Appl. Environ. Microbiol. 75:1566-1574.
4. Tyler, H. L., Roesch, L. F., Gowda, S., Dawson, W. O., and Triplett E. W. 2009. Confirmation of the sequence of '*Candidatus* L. asiaticus' and assessment of microbial diversity in huanglongbing-infected citrus phloem using a metagenomic approach. Mol. Plant-Microbe Interact. 22:1624-34.

9.7

Differentiation of "*Candidatus* Liberibacter asiaticus" isolates from Brazil, China, and the United States

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Abstract

"*Candidatus* Liberibacter asiaticus" is associated with citrus Huanglongbing (HLB, yellow shoot disease), a highly destructive disease currently threatening world citrus production. HLB has a long history in China and was found in Brazil in 2004 and U.S.A. in 2005. There is an urgent need to differentiate isolates of "*Ca.* L. asiaticus" from different geographical regions for effective control of HLB. In this study, isolates of "*Ca.* L. asiaticus" collected from Brazil, China and the United States were evaluated based on two previously characterized genomic loci, one locus (*trn*1) with variable tandem repeat numbers (TRNs), and the other locus (*snp*1) is characteristic in single nucleotide polymorphisms (SNPs). A total of 299 strains (84 Brazil, 132 China and 83 U.S.) were analyzed. At the *trn*1 locus, "*Ca.* L. asiaticus" strains were divided into TRN-A and TRN-B groups. TRN-A isolates dominated the China and U.S. populations but were not detected in the Brazil isolates. In contrast, TRN-B dominated the Brazil isolates but occurred at low frequencies in China (3%) and U.S. (6%). SNP Analyses at the *snp*1 locus established Term-A and Term-G groups. Term-A group included all Brazil and China isolates, along with 6% U. S. isolates which were also TRN-B isolates. The remaining (94%) U. S. isolates were in Term-G group. By combining data from the analyses of the two genomic loci, it is shown that the TRN-A:Term A genotype was unique to China, TRN-A:Term-G genotype was unique to U.S., and the TRN-B:Term-A genotype dominated the Brazil isolates. No TRN-B:Term-B isolates were found.

9.8

'*Candidatus* Liberibacter asiaticus' Encodes Two Novel Autotransporters that Target to Mitochondria

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Abstract

As a phloem-limited, intracellular bacterial pathogen, '*Candidatus* Liberibacter asiaticus' (Las) has a significantly reduced genome and causes huanglongbing (HLB), a devastating disease of citrus worldwide. In this study, we characterized two novel autotransporter proteins of Las, and redesignated them as $LasA_I$ and $LasA_{II}$ in lieu of the previous names Hyv_I and $Hyv_{II.}$ Proteins secreted by the type V secretion system (T5SS), known as autotransporters, are large extracellular virulence proteins localized to the bacterial poles. Bioinformatic analyses revealed that $LasA_I$ and $LasA_{II}$ share the structural features of an autotransporter family containing large repeats of a passenger domain and a unique C-terminal translocator domain. When fused to the GFP gene and expressed in *E. coli*, the $LasA_I$ C-terminus and the full length $LasA_{II}$ were localized to the bacterial poles, similar to other members of autotransporter family. Despite the absence of the signal peptide, $LasA_I$ was found to localize at the cell surface by immuno-dot blot using a monoclonal antibody against the partial $LasA_I$ protein. Its surface localization was also confirmed by the removal of the $LasA_I$ antigen using a proteinase K treatment of the intact bacterial cells. When co-inoculated with a P19 gene silencing suppressor and transiently expressed in tobacco leaves, the GFP-$LasA_I$ translocator targeted to the mitochondria. This is the first report that Las encodes novel autotransporters that target to mitochondria. These findings may lead to a better understanding of the pathogenesis of this intracellular "energy parasitic" bacterium, and to more efficient characterizing new molecular targets for HLB control.

9.9 P

Prophage-mediated population dynamics of '*Candidatus* Liberibacter asiaticus' in plant and insect hosts

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As an intracellular bacterium, '*Candidatus* Liberibacter asiaticus' (Las) lacks known transposons and IS elements but contains at least two prophages/phages. In this study, we revealed the genetic diversity and population dynamics of this bacterium based on two prophage hyper-variable regions (HVRs) using separate libraries constructed from citrus, periwinkle and psyllid. A total of 9 variants were identified, including 4 abundance types A, B, C, D and 5 rare types E, A1, A2, C1 and C2. The two HVRs, Type A and B, share highly conserved sequences and are localize to the two prophages, FP1 and FP2, respectively. The most abundant type in the psyllid library was Type A (36.71%), followed by Type B (25.17%) and Type C (19.72), but there was no Type D. However, the most abundant type in citrus was Type B (64.24%), followed by Type A (20.14%), C (11.11%) and D (1.39%). More interestingly, the Type A sequence was a very rare group (0.36%) in the periwinkle library. The most abundant type in the periwinkle library was Type B (43.73%) followed by Type C (39.07%) and then Type D (9.32%). Sequence analysis of these variants revealed the variations were due to the recombination and reassortment between two prophages. Conventional PCR results using primers specific to the different types indicated that Type A, B, C and D were present in more than 94.6% of higher titer Las-infected plant hosts; however, only 16.7% of tested psyllids contained D variants, which were very low titer, and Las-infected psyllids possessed higher titer Type A, B and C populations . Typing results for Las-infected citrus field samples indicated that only the Type D population was associated with huanglongbing (HLB) symptoms: high titer of D with typical blotchy mottle and extremely low to no type D with vein yellowing or other atypical HLB symptoms. Our finding that Las population dynamics derive from the prophage/phage activities may lead to a better understanding of how these bacteria evolve and adapt in different ecological niches.

9.10 P

Improved methods for genome sequencing of Liberibacters by BAC library-based metagenomics approach

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Liberibacters have not yet been successfully cultured; their minimal genomes carry multiple copies of several genes. Sequences identical to phage genomes have been found in many Liberibacters. Available evidences suggest that the Liberibacter genomes are adapting rapidly in different hosts and environments. Characterization of genomes of rapidly changing unculturable organisms can be challenging. We have used a model system based on *Candidatus* Liberibacter psyllaurous associated with tomato "psyllid yellows" (Hansen et al., 2008) to develop methodologies using alternate techniques for sequencing metagenomes. We have constructed a BAC library from infected tomato psyllids (*Bactericera cockerelli*). The library consists of 57,600 clones arrayed in 150 plates each with 384 wells. DNA from individual clones were pooled for screening purposes. Initial identification of clones with Liberibacter sequences were conducted based on 16s ribosomal sequences, and contiguous clones were characterized by end sequencing and identified as containing Liberibacter genome fragments. Screening of additional clones from the library was based on probes developed on such sequences. A total of 245 clones with Liberibacter genome fragments have been identified. A total of 63 bar-coded BAC clones were sequenced by using Roche 454 technology. BAC clones from this library contain large inserts (average size 70 kb). Similarities and differences with other well characterized genomes of Liberibacters (Duan et al., 2009, Lin et al., 2011) will be presented.

Citations

1. Hansen A.K., Trumble J.T., Stouthamer, R., Paine, T.D.2008. Appl Environ Microbiol. 74(18):5862-5865. A new Huanglongbing Species, "*Candidatus* Liberibacter psyllaurous," found to infect tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Sulc).
2. Duan Y., Zhou L., Hall, D.G., Li W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C.M., Gabriel, D.W., Williams, K.P, Dickerman, A., Sun, Y., Gottwald, T. 2009. Mol Plant Microbe Interact. 22(8):1011-20.
3. Lin, H., Binghai L., Jonathan M. G., Doddapaneni, H. Civerolo, E.L. Chen, C., Duan, Y., Zhou, L. and Vahling, C.M. 2011. PLoS One. 2011; 6 (4): e19135. The Complete Genome Sequence of '*Candidatus* Liberibacter solanacearum', the Bacterium Associated with Potato Zebra Chip Disease

9.11 P

Identification of small molecule inhibitors against SecA of *Candidatus* Liberibacter asiaticus by structure based design

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Abstract

Huanglongbing is the most devastating disease of citrus caused by *Candidatus* Liberibacter asiaticus (Las) (1, 2). In the present study, we report the discovery of novel small molecule inhibitors against SecA ATPase of Las by using structure based design methods. We built the homology model of SecA protein structure of Las based on the SecA of *Escherichia coli*. The model was used for *in-silico* screening of commercially available compounds from ZINC database. Using the glide flexible molecular docking method, twenty structures were chosen for *in vitro* studies. Five compounds were found to inhibit the ATPase activity of SecA of Las at nano molar concentrations and showed antimicrobial activities against *Agrobacterium tumefaciens* with MBC ranging from 128 to 256 □g/mL. These compounds appear to be suitable as lead compounds for further development of antimicrobial compounds against Las. To test the application potential of those compounds on plants, the phytotoxicity studies were performed on the five compounds against citrus. In addition, we are optimizing these five antimicrobial compounds to identify compounds higher antimicrobial activity.

Note: We would like to have this one as poster.

Citation

1. Bové, JM. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. 2006. Journal of Plant Pathology, 88 (1), 7-37.
2. Gottwald, TR, da Graça, JV, and Bassanezi, RB. 2007. Citrus Huanglongbing: The pathogen and its impact. Online. Plant Health Progress doi:10.1094/PHP-2007-0906-01-RV.

9.12 P

Detection of 16Sr IX phytoplasma (HLB phytoplasma) in Sunn Hemp (*Crotalaria juncea*) in São Paulo State, Brazil*.

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In São Paulo State, besides the occurrence of *Candidatus* Liberibacter americanus and *Ca*. L. asiaticus, a 16Sr IX group phytoplasma was associated with HLB symptoms, indistinguishable from those caused by liberibacters. This phytoplasma is called HLB phytoplasma and was found widespread in citrus orchards, although at low incidence. The same phytoplasma was found in Sunn Hemp (*Crotalaria juncea*) in 2008 and witches'-broom was commonly found associated with 16Sr group IX detection. The aim of this work was to assess the phytoplasma diversity in Sunn Hemp with emphasis at the detection of group 16Sr IX phytoplasma and to establish an association between the occurrence of HLB phytoplasma and symptoms. Sunn Hemp samples were harvested close to the blooming period. Plants were selected in the field when showing symptoms common to phytoplasma infection. We employed universal primers do amplify phytoplasmas in general and group specific primers for 16Sr group IX. PCR products were sequenced to allow grouping of phytoplasmas. We identified five phytoplasmas groups in 48 out of 99 Sunn Hemp plants, belonging to phytoplasma groups 16Sr I, III, VII, IX and XV. The most abundant phytoplasma was the group 16Sr IX, present in 70% of the samples, found in central and north São Paulo State. The occurrence of HLB phytoplasma in Sunn Hemp samples, showing 100% of similarity to the citrus phytoplasma, was highly related to virescence and the second most conspicuous symptom for this infection was witches'-broom.

*First author's dissertation in the Fundecitrus Professional Master on Control of Citrus Diseases and Pests.

9.13 P

First Report of 'Candidatus Liberibacter asiaticus' associated with huanglongbing in the weeds *Cleome rutidosperma, Pisonia aculeata* and *Trichostigma octandrum* in Jamaica

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Additional key words: citrus greening, alternative host plants

Citrus huanglongbing (HLB) also known as citrus greening is the most destructive disease of citrus worldwide. Three species of the causal organism have been identified. These are 'Candidatus Liberibacter asiaticus', 'Ca. L. africanus' and 'Ca. L. americanus' (Bové, 2006). In 2010 a survey of non-citrus plants was conducted on two major citrus producing farms in Clarendon and St Catherine in Jamaica. This was to determine the possibility of the existence of non-citrus hosts of HLB. A total of 120 plants belonging to 10 different species and nine families were collected over a period of two months. The plants collected included weeds as well as non-citrus trees. None of the plants collected exhibited any symptoms of HLB and at the time of sample collection, no citrus psyllids was observed on the plants. DNA was extracted from plant samples using the method of Dellaporta et al. (1983) and analysed by PCR using the primer pair OI1 (5` GCGCGTATGCAATACGAGCGGCA3`) and OI2c (5`GCCTCGCGACTTCGCAACCCAT 3`). DNA obtained from a confirmed HLB infected citrus plant from Florida served as the positive control whereas DNA from a citrus plant uninfected by HLB was used as the negative control.

Amplification of a 16S rDNA product of the expected size (1200 bp) confirmed the presence of HLB infection in three of the weed species that were tested. These included *Cleome rutidosperma* (Family Capparaceae) (1/9), *Pisonia aculeata* (Nyctaginaceae) (3/3) and *Trichostigma octandrum* (Phytolaccaceae) (2/8). No amplification was obtained for the following species: *Bidens pilosa* (0/17), *Parthenium hysterophorus* (0/18) (both Asteraceae), *Sida jamaicensis* (Malvaceae) (0/11, *Waltheria indica* (Sterculiaceae) (0/27), *Priva lappulacea* (Verbenaceae) (0/7), *Psidium guajava* (Myrtaceae) (0/12), *Hyptis capitata* (Lamiaceae) (0/8). Positive PCR products from the above mentioned weeds were digested with XbaI, and restriction fragments of approximately 530 bp and 650 bp were obtained which corresponded to the fragments expected for 'Ca. L. asiaticus' as well as the positive control that was used. PCR products from each of the weed species were cloned and sequenced (in both directions) and the

sequences were deposited in GenBank (Accession Nos. JN245977, JN245976, and JN245973). Blast analysis determined the consensus sequence to be most similar (98%) to 'Ca. L. asiaticus' found in Florida (EU265646), Belize (GQ502291) and Brazil (AY919311). Weeds have been known to act as reservoir hosts of many pathogens. However, to our knowledge this is the first report of 'Ca. L. asiaticus' in the weeds, *C. rutidosperma*, *P. aculeata* and *T. octandrum*. The presence of 'Ca. L. asiaticus' in weeds has very serious implications for the control of HLB in Jamaica. The current practices by farmers include the removal of infected citrus trees and control of the vector through the spraying of insecticides. The presence of the HLB pathogen in weeds would now imply that weed removal must be part of the HLB management program in Jamaica as they can serve as reservoirs for the disease as well as source of breeding for the vector.

Acknowledgements

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References

Bové, JM, 2006. Huanglongbing: A destructive newly emerging, century old disease of citrus. Journal of Plant Pathology 88, 7-37.

Dellaporta, SL, Wood J, Hicks JB, 1983. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1, 19-21.

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9.14 P

'*Candidatus* Liberibacter' in four indigenous Rutaceous species from South Africa

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'*Candidatus* Liberibacter africanus' (Laf), a phloem restricted, gram negative bacteria of the α-proteobacteria is the agent associated with Citrus greening disease in South Africa. A related bacterium *'Candidatus* Liberibacter africanus spp. capensis' (LafC) was previously described from an indigenous Rutaceae tree, *Calodendrum capense.* This led to the hypothesis that other indigenous Rutaceous trees may also be infected with Liberibacters related to either Laf or LafC. Samples from 289 *Vepris lanceolata*, 231 *Zanthoxylum capense*, and 234 *Clausena anisata* were collected from within the natural distribution of these trees in South Africa. Total DNA was extracted and tested for the presence of a Liberibacter using a generic Liberibacter real-time PCR. '*Candidatus* Liberibacters' present in positive samples were characterised by amplifying and sequencing the β-operon, 16S and *omp* gene regions. The percentage of Liberibacter positive samples differed per tree species with 6% *V. lanceolata*, 4% *Z. capense* and 11% *C. anisata* respectively, being infected. Phylogenetic analysis of the β-operon and *omp* gene regions, revealed unique phylogenetic clusters for Liberibacters associated with each tree species. Phylogenetic analysis from the 16S gene region however indicated that sequences obtained from *V. lanceolata* and *C. anisata* were similar to 16S sequences for LafC, whereas that obtained from *Z. capense* grouped on its own. Laf has not been identified from HLB-infected orchards from other Citrus producing countries other than Africa and the Mascarene Islands. The presence of related Liberibacters from indigenous Rutaceae species in South Africa may therefore suggest that Laf originated on the African continent.

9.15 P

Increases in '*Candidatus* Liberibacter asiaticus' viability and investigations of biofilm-like structures in citrus juice medium

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Huanglongbing disease of citrus, associated with infection by the bacterium '*Candidatus* Liberibacter asiaticus' (LAS), has spread rapidly in the US since 2005. Attempts to culture LAS *in vitro* have not yielded a consistently reproducible culture method; therefore, obtaining knowledge about the infection process is difficult. To determine conditions which sustain LAS viability, LAS inoculum obtained from seeds of fruit from infected pomelo trees (*Citrus grandis* 'Mato Buntan') was added to different media, and cell viability was monitored for several weeks using quantitative polymerase chain reaction (qPCR) in conjunction with ethidium monoazide (EMA). Among media tested, King's B (K) did not support viability of LAS cells, while grapefruit juice (G) allowed LAS cells to survive *in vitro* for ~20 days. In media that sustained LAS viability, a reproducible biofilm-like substance was formed over time at the air-liquid interface of culture flasks and glass slides inserted in cultures. Fluorescence *in situ* hybridization (FISH) showed the biofilm contains aggregates of LAS cells, which was confirmed by qPCR. 16S rDNA libraries of the biofilm samples have been constructed and will be sequenced via Illumina next-generation sequencing to determine their bacterial composition. To elucidate why juice-based media prolongs LAS viability, the elemental nutrient compositions of the media and the biofilm were analyzed via inductively coupled plasma optical emission spectrometry (ICP-OES). Compositions were compared, and specific elements, such as potassium and calcium, were more abundant in media that sustain LAS cell viability. Results will contribute to future development of a culture medium for LAS.

9.16 P

"Whole Genome PCR Sequencing Strategy for '*Candidatus*' Liberibacter asiaticus: Analyzing Sequence Diversity Among U.S. Isolates.

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The inability to culture the '*Candidatus'* Liberibacter asiaticus (CLas) bacterium has greatly hindered research on the etiology of the citrus disease Huanglongbing which is associated with this bacterium. This is especially true with respect to possible links between strain/isolate diversity and disease symptom variations and development. Past genetic marker research indicates that there is considerable CLas isolate diversity even within Florida (a location that has only recognized the presence of the disease since 2005); however, no effort has been made to correlate this diversity with symptom differences. To advance our understanding of CLas geographic spread and strain/isolate diversity, we have developed a whole-genome PCR amplification strategy that can be used in conjunction with next generation genome sequencing to rapidly obtain near whole genome sequence for specific isolates. This method was used to generate genome sequence data (~93% of the total genome with an average ~300x coverage) from numerous isolates within Florida that are known to be different with respect to previously characterized genetic markers, and also to compare genomic sequence with isolates that may induce different symptoms within citrus. Results will be presented that show the diversity among Florida CLas isolates and will also be contextualized within the diversity observed sequence variation among global isolates.

Session 10:

Host Tolerance and Resistance

10.1

Huanglongbing Resistance and Tolerance in Citrus

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Huanglongbing (HLB) is severely impacting Florida citrus. Productivity declines in many HLB-affected genotypes, often with greatly thinned canopies. Fruit size and quality are often adversely affected as the disease advances. HLB was assessed in diverse cultivars in commercial groves with high HLB-incidence. 'Temple' had the lowest HLB symptoms and Liberibacter (Las) titer, while 'Murcott' and 'Minneola' had the highest. The USDA Ft. Pierce, FL farm is managed to reveal genotype responses to HLB. Some current cultivars and hybrid seedlings demonstrate resistance/tolerance, at least to strain(s) of Las present. C. trifoliata is the best documented citrus resistance source with Las titers suppressed even when C. trifoliata is grafted onto severely-infected rootstocks. Some cultivars and hybrids have abundant foliage symptoms, but full canopies and seemingly normal fruit set and size. In 3-years of data from a replicated trial of 'Triumph'(T), 'Jackson'(J), 'Flame'(F), and 'Marsh'(M), HLB symptoms were severe in all trees and Liberibacter titers were similar. However, F&M were almost completely defoliated in some years while T&J had full canopies. Cumulative fruit/tree was greater for T&J (255&220) than for F&M (29&66). T&J fruit met commercial standards and had normal size but F&M fruit were unacceptable with many small and misshapen. Evidence mounts that useful resistance/tolerance to HLB is present in cultivated citrus and this is a focus of the USDA citrus breeding program.

10.2

Preliminary Evidence for Rootstock Effects on HLB Infection Frequency and Disease Severity in Sweet Orange and 'SugarBelle' Trees

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Evidence is accumulating that root system collapse is involved with HLB-induced tree decline, especially with trees on Swingle and Carrizo. Phytophthora resistance appears to be breaking down in HLB-infected trees on Swingle. Other stresses caused by blight, nematodes, cold, etc. also appear to be interacting with HLB to increase HLB disease frequency and severity. Improved rootstocks could help to mitigate these problems, allowing for sustainable production under appropriate nutrition. We are testing complex hybrid rootstock candidates (diploid and tetraploid) to determine their affect on HLB disease establishment and severity in trees grafted sweet orange scion; field and greenhouse experiments are underway. Rootstocks differentially translocate nutrients, phytohormones (plant growth regulators), micro-RNAs, small proteins (pathogenesis related?), and other metabolites to the scion. This could have both direct and indirect, quantitative and quantitative affects on scion gene expression, and possibly Liberibacter pathogenesis in citrus – especially with unique complex allotetraploid rootstocks. Data from two young field trials (both with the 'bad neighbor' effect) established to evaluate new rootstock candidates, previously not screened for HLB tolerance, will be presented. These include a trial of 3.5 year old trees of 'SugarBelle' that is nearly 100% infected with HLB, and a trial of 4.5 year old trees of sweet orange on >50 rootstocks that is approximately 15% infected. Rootstock differences regarding HLB disease frequency and severity are emerging. Complex 'tetrazyg' rootstock Orange #19 (Nova+HBPummelo x Cleopatra+Argentine trifoliate orange) is showing more HLB tolerance at both locations. Data on %'s of symptomatic fruit and fruit drop per rootstock will be presented.

We have also adjusted our rootstock breeding/greenhouse screening program to focus on HLB. Following a preliminary calcareous soil/Phytophthora screen, selected individual hybrid rootstock candidates are grafted with HLB-infected budsticks of Valencia sweet orange. The remaining rootstock top is then removed, forcing flush from the HLB- infected budstick. Trees are monitored for HLB symptoms, and healthy appearing trees are entered into a 'hot psyllid' house for 4 weeks, followed by field planting at Picos Farm (under DPI permit). Rootstocks capable of growing off healthy sweet orange trees are identified for further study (10 identified so far). Our goal is to develop rootstocks that will facilitate sustainable and profitable citriculture in an HLB-endemic Florida.

10.3

Screening of Transgenic Citrus for HLB Resistance

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Transgenic citrus scion (mostly grapefruit and sweet orange) and rootstock cultivars (Carrizo and experimental complex tetraploids) were transformed with gene(s) encoding antimicrobial peptides or systemic acquired resistance (SAR) proteins. Each transgene was under control of an enhanced CaMV 35S promoter. Several genes were also under control of a phloem specific *Arabidopsis* SUC2 (*AtSUC2*) promoter. A number of clones of each transgenic line (at least 3 replicate plants per clone) were evaluated for resistance to Huanglongbing (HLB, caused by *Candidatus* Liberibacter asiaticus). 650 clones, from over 180 individual transgenic lines planted in spring 2009 in a heavily HLB infected Martin County grove were tested using qPCR for infection to HLB after 30 months in field. 396 trees tested negative for the HLB bacterium. Approximately 200 clones were observed to be healthy and flushing after 40 months in the field and were again evaluated using qPCR during June 2012. We did not detect the *Ca.* Liberibacter asiaticus bacterium in a majority of these trees. In a separate trial in St. Lucie County, 300 clones, from over 80 individual transgenic lines planted during 2010 were evaluated in October 2012. Similar trends were observed to that seen in our Martin County site. 345 transgenic clones and controls containing the same transgene(s) were also placed in a greenhouse containing free flying HLB-infected Asian citrus psyllids (ACP) during April 2011. All trees were evaluated for infection after 12 months by qPCR, and 80% of the transgenic trees tested negative for the bacterium. These results suggest that some of the antimicrobial peptides and SAR-inducing proteins can provide long-term resistance against HLB.

10.4

HLB Progress on Tahiti acid lime grafted onto eight rootstocks

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The State of São Paulo is the main Tahiti (Persian) lime producer in Brazil with 65% of 43,000 ha grown in Brazil. In 2003, an experiment was planted in the Citrus Experimental Station (EECB), Bebedouro, Northern São Paulo State, with the objective of to characterize the performance of Tahiti acid lime grafted onto eight rootstocks: Davis A and Flying Dragon trifoliate oranges, Swingle citrumelo, HRS 849 ["citradia 1708" (Argentina trifoliate orange x Smooth Flat Sevile)], Morton citrange, Rangur lime and Volkamer lemon, at 8 x 5 m spacing. In 2004, citrus huanglongbing (HLB), was first reported in the São Paulo State and the trees of the experiment started to show HLB symptoms in 2009. From July 2010 to May 2012, disease severity was evaluated in four times and the bacteria titer quantified once. The numbers of qPCR positive replications were in a range of five to eight. Severity data was used to calculate the area under disease severity progress curve (*AUDSPC*). The data were analyzed by Fisher LSD test (5%). Flying Dragon and Davis A trifoliate oranges, Swingle citrumelo, had lower values of *AUDSPC*, differing from Morton citrange, Orlando tangelo, Rangpur lime and Volkamer lemon. The citradia HRS 849 [citradia 1708 (Argentina trifoliate orange x Smooth Flat Sevile) had intermediate behavior. The canopies were removed but the rootstocks are still alive, so, new studies using the rootstocks new shoots and the roots will be done aiming to quantify *Candidatus* Liberibacter asiaticus titer in them to confirm the rootstock tolerance degrees identified in the canopies.

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10.5

Resistance of *Poncirus* and *Citrus* x *Poncirus* Germplasm to the Asian Citrus Psyllid

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The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, has spread to citrus growing regions nearly worldwide and transmits phloem-limited bacteria (*Candidatus* Liberibacter spp.) that are putatively responsible for citrus greening disease. Host plant resistance may provide the most effective control, but ACP has a broad host range and resistance in *Citrus* and relatives to ACP has not been widely documented. Very low abundances of ACP were found on two accessions of *Poncirus trifoliata* L. in a field survey (Westbrook et al., 2011). Therefore, we tested whether 81 accessions of *P. trifoliata* and x*Citroncirus* sp. (hybrids of *P. trifoliata* and *Citrus* spp.) from the USDA-ARS National Clonal Germplasm Repository for Citrus and Dates were resistant to ACP by determining whether these accessions influence oviposition and lifespan of adults in no-choice tests. There was a higher abundance of eggs on the control (*Citrus macrophylla* Wester) than nearly all accessions of *P. trifoliata*, and zero eggs were laid on 36% of the accessions. Additionally, more eggs were laid on the control than 10 of 34 accessions of x*Citroncirus*. Lifespan of adults was ~2.5-5 times longer on 11 of the 17 trifoliates and trifoliate hybrids we tested. *P. trifoliata* appears to have antixenosis and antibiosis resistance to ACP, but we must next identify the traits that promote resistance. To identify chemical mechanisms that may promote resistance, we collected volatiles from several pairs of closely related susceptible/resistant accessions of trifoliates and trifoliate hybrids and found differences in the volatile profiles.

Citation

Westbrook, C. J., D. G. Hall, E. W. Stover, Y. P. Duan and R. F. Lee. 2011. Colonization of *Citrus* and *Citrus*–related germplasm by *Diaphorina citri* (Hemiptera: Psyllidae). HortScience. 46:997-1005

10.6

Screening of citrus and its close relatives for tolerance to huanglongbing

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Huanglongbing (HLB), a devastating disease of citrus, has become a serious problem for the citrus industries in Brazil and Florida, and both the disease and its psyllid vector, *Diaphorina citri* continue to spread to other citrus growing regions. Host resistance or tolerance to the pathogen would be extremely valuable to the citrus industry. A field trial was established in Fort Pierce, Florida where HLB has become endemic to assess the HLB tolerance level of different cultivars of citrus and citrus relatives. Over 800 seedlings representing over 100 accessions (8 replications of each) belonging to 18 genera of the subfamily Aurantioideae and family Rutaceae were evaluated over a period of four years. Leaf samples were collected at 6 month intervals during the spring and fall seasons and tested for the presence of HLB associated bacterium, *Candidatus* Liberibacter asiaticus (LAS) by real time PCR. While most accessions were found to be susceptible to HLB, the bacterium (LAS) was not detectable in about 20 accessions up to four years of analysis. These include many trifoliates and trifoliate hybrids, some species of *Bergera, Casimiroa, Clausena, Eremocitrus, Glycosmis, Microcitrus, Murraya, Naringi,* and *Zanthoxylum.* Information on varietal tolerance of citrus and its relatives to HLB is very important for management of the disease. While most accessions in Citrus were susceptible, partial resistance was observed in some clonal populations of *Citrus latipes*. The probable basis of resistance is being investigated.

10.7

Candidatus Liberibacter americanus induces significant reprogramming of the transcriptome of the susceptible citrus genotype

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In Brazil, *huanglongbing* (HLB) is caused by *Candidatus* Liberibacter americanus (CaLam) and *Ca.* L. asiaticus (CaLas). Both species are vectored by the Asian citrus psyllid and are restricted to the phloem of infected citrus, where they promote severe imbalance in the translocation of nutrients and other important substances along the plant. Several studies of the transcriptional response of citrus to HLB have been reported, but only for infection caused by CaLas. This study evaluated the transcriptional reprogramming of a susceptible genotype (Pera sweet orange) challenged with CaLam, using a customized 385K microarray chip. The analyses showed large number of genes and biological processes significantly altered upon CaLam infection. Among the changes we highlight induction of zinc transporters, modulation of enzymes related to sugar metabolism, depletion of photosynthesis, induction of several defense-related genes and modulation of enzymes regulating ROS production. Several biological processes reported as differentially modulated upon infection with CaLas responded similarly to CaLam. The large number of receptor-like proteins, PR genes, NBS-LRR and transcription factors (such as WRKY and MYB) found showed that even a susceptible citrus genotype is able to actively respond to infection by CaLam, as reported to CaLas. Twenty candidate genes were selected for validation in symptomatic and asymptomatic PCR-positive leaves of Hamlin sweet orange infected with CaLas or CaLam. Finally, using *in silico* approaches, we compared our results with all published studies using CaLas to hypothesize a global feature of the defense/susceptibility mechanisms of citrus in response to the bacteria. These results have been explored in selection of target genes for genetic engineering to control HLB. Also, further transcriptome (RNAseq) experiments using tolerant and susceptible citrus genotypes infected with CaLam or CaLas using different time points are in progress to investigate the dynamic of expression of these genes during early stages of infection.
(Support: Citrus Research & Development Foundation, and NISC Citrus)

10.8

Identification of differentially expressed genes in *Citrus sinensis* leaves and branches in response to *Candidatus* Liberibacter asiaticus and *Ca.* L. americanus.

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Several studies have addressed transcriptional changes in *Citrus sinensis* samples in response to *Candidatus* Liberibacter asiaticus (CaLas) with the objective to reveal the mechanisms underlying the development of *Huanglongbing* (HLB) and identify possible strategies to manage the disease. The aim of this work was to provide data using NGS technology (RNAseq) for a comprehensive analysis of differential expression changes in *C. sinensis* leaves and branches induced by HLB, caused either by CaLas or CaLas. Four treatments were evaluated; each of them consisted of RNA bulks extracted from five *C. sinensis* HLB symptomatic leaves or branches inoculated with CaLam or CaLas. The samples were subjected to RNAseq sequencing and the differential expression analyses were performed with Cuffdiff. In parallel, we performed a simple parametric test based on the mean and standard deviation to select statistically significant differentially expressed genes (DEG), named RSDA (Relative standard deviation analysis). For this approach, we considered standard deviation values of <0.7, and p-value = 0.01. Several genes associated with disease response, transcription factors involved in the activation of pathways such as the jasmonic acid, salicylic acid and ethylene, as well as genes involved in oxidative stress proved to be differentially expressed in our analyses. In leaves, we identified genes belonging to the WRKY transcription factors, ankyrin repeat family, NB-ARC domain-containing disease resistance, ethylene-forming enzymes and chaperones. In branches, we found many cytochromes, as well as gene involved in callose deposition, AP2/B3 transcriptional factor family and LEA proteins as being differentially expressed. Validation by RT-qPCR was performed for ten DEG.

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10.9

A quick evaluation method of AtNPR1 transgenic plants for resistance to HLB

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We have produced a number of 'Carrizo' citrange (*Citrus sinensis x Poncirus trifoliata)* transformed with the *Arabidopsis thaliana* NPR1, a transcriptional co-activator that is key in the regulation of systemic acquired resistance (SAR) and the expression of pathogenesis related (PR) genes. Over-expression of this gene has been shown to induce broad spectrum disease resistance in several species. One of the limitations in obtaining genetically resistant citrus plants to HLB is how lengthy it is to propagate and evaluate the transgenic plants. Using grafting with infected budwood takes several months, is labor intensive and normally requires specialized greenhouse space which can be limited. We have developed a system to quickly screen AtNPR1 transgenic lines and determine if they exhibit an enhanced defense response to *Candidatus* Liberibacter asiaticus PAMPs. First, we used a synthetic peptide of L-flg22 (22 amino acid flagellin epitope derived from CLas) capable of triggering immunity in citrus. Second, using real time PCR, we determined changes in the expression levels of a battery of genes associated with defense in citrus in a time course of up to 72 hours after infiltration with L-flg22 and compared it with the expression in wild type plants. Certain lines consistently showed an enhanced defense response when exposed to L-flg22 thus identifying the ones with the most potential. The advantage of this method as a first step in the screening process is that is quick, controlled and does not require specialized greenhouse space. The selected lines are being further evaluated through graft inoculation for their tolerance to HLB.

10.10

Lflg22, a Pathogen-Associated Molecular Pattern (PAMP) of *Candidatus* Liberibacter asiaticus, initiated differential PAMP-Triggered Immunity (PTI) in Grapefruit and Sun Chu Sha

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'Duncan' Grapefruit (*Citrus paradisi* Macf.) and 'Sun Chu Sha' mandarin (*C. reticulalta* Blanco) represent two citrus genotypes that have different levels of tolerance to citrus greening or huanglongbing (HLB), a bacterial disease caused by *Candidatus* Liberibacter sp. In this study, the response of the two genotypes to the conserved 22 amino acid domain of the Liberibacter flagellin (Lflg22), a Pathogen-Associated Molecular Pattern (PAMP), were compared. The expression levels of citrus defense-associated genes including AZI1, EDS1, NDR1, SGT1, RAR1, PAL1, ICS1, PAL1, NPR1, NPR2, NPR3, PR1 and RdRp in response to Lflg22 were analyzed. The HLB moderately tolerant Sun Chu Sha showed a stronger response to Lflg22 than the HLB-sensitive grapefruit. These results suggest that differences in the levels of PAMP-triggered Immunity (PTI) between the two genotypes are associated with the observed levels of HLB tolerance. Interestingly, although the *Ca*. L. asiaticus flagellin gene has been shown to be functional, no flagellum has been observed in this bacterium.

10.11

Genetic transformation of sweet orange to overexpress a *CsPR-8* gene aiming *Candidatus* Liberibacter asiaticus resistance

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A strategy to produce HLB-resistant citrus using genetic engineering is the overexpression of genes identified in citrus genome. Plants respond to pathogen attacks by producing several pathogenesis-related (PR) proteins. Therefore, individual PR overexpression in transgenic plants can lead to a increased resistance. In this study, we have chosen to use one *PR-8* isoform cloned from *Citrus sinensis* (*CsPR-8*). The PR-8 is an endochitinase that also has lysozyme activity, to be potentially used against bacteria attacks. We constructed an expression transformation vector (pCAMBIA2201) containing *CsPR-8* gene and the selection gene *npt*II that confers kanamycin resistance in plants, both driven by the CaMV35S constitutive promoter. Epicotyl segments collected from *in vitro* seedlings of 'Hamlin' sweet orange (*Citrus sinensis* L. Osbeck) were used for transformation via *Agrobacterium tumefaciens* strain EHA105. The developed shoots were excised from the explants and *in vitro* grafted onto Carrizo citrange [*C. sinensis* x *Poncirus trifoliata* (L.) Raf] seedlings. The grafted plants were analyzed by PCR, using specific primers for detection of the *npt*II gene. Acclimation of transgenic plants is on the way in order to be transferred to the greenhouse. These plants will be analyzed by Southern blot to confirm the integration of the transgene and by RT-qPCR to evaluate the transgene expression, prior to their evaluation for *Candidatus* Liberibacter asiaticus resistance.

10.12

Analysis and evaluation of China-native citrus and citrus related germplasm on their susceptibility to the infestation by *Diaphorina citri* Kuwayama (Homotera: Psyllidae)

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Abstract: Huanglongbing (HLB) is the most devastating disease of citrus worldwide and vectored by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). The pathogen associated with HLB maintains uncultural in vitro, and there are few effective options against HLB-affected plants. Identification and deployment of ACP- resistance traits of citrus and citrus related germplasm to suppress ACP populations may be a potential management strategy for the management of HLB. In the present study, the susceptibilities of 71 Chinese citrus genotypes to ACP infestation were evaluated and analyzed in a free-choice situation under field conditions by using the method of systematic clustering and dynamic clustering. The results showed that there was significant difference in susceptibility to ACP infestation among the genotypes of citrus. These genotypes can be graded according to the number of psyllids on the trees. Grade I: highly susceptible with a total of 8 genotypes. Grade II: moderate susceptible with a total of 18 genotypes. Grade III: lower susceptible with a total of 45 genotypes. The 71 genotypes of citrus are used to be classified into 8 groups according to Chinese classification system "Citrus Varieties in China" (Chinese Citrus Association). There existed significant differences among the 8 groups. Lemons and Pummelos were highly susceptible hosts to ACP, no significant difference with *Murraya panciculata* L., which was the most suitable host to ACP. Mandarins, Hybrid citrus, Sweet oranges and Tangerines were moderate susceptible hosts. Kumquat and Sour oranges were lower susceptible hosts. Some genotypes of Kumquat, like *Citrus medurenisi* var. Variegated Calamondin, *Fortunella hindsii* var. Chintou, and Sour orange, *Citrus aurantium* var. Variegated sour orange, were seemly the lowest susceptible to ACP infection. Further experiment would be taken to confirm the low ACP- susceptibility of genotypes from Kumquat and Sour orange.

Keywords: *Diaphorina citri* Kuwayama; Huanglongbing, Susceptibility, Citrus and Citrus related germplasm

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10.13 P

Results on attempts in management of HLB under small scale in Vietnam and initiation in screening for HLB tolerant from varieties/clones belonging to Rutaceae

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In the Mekong Delta, Vietnam, Citrus Huanglongbing (HLB) was officially announced in 1994 and its causal organism was described by Bove and Garnier in 1995 to be *Candidatus* Liberibacter asiaticus. Throughout the years, intensive works have been carried out for HLB control under small scale orchards and the achievements are discussed, the model for effective control of HLB under small scale which could elongate the life cycle of citrus tree for better and longer harvesting. In addition, there were 130 rutaceae related accessions had been collected and screened for HLB tolerant; the results revealed that the serverity of HLB infection was less on Hanh/Tac/Quat (*Citrus microcarpa*) and Long Co co pummelo (*Citrus maxima*) than that on orange and mandarin. The wild Rutaceae species/clones such as Quyt Dang, Quyt rung, Cam rung, Buoi Rung, Buoi Dang, Buoi Bung, Mac Run, Mac Mat, Can Thang, Quach, Nguyet Que, Kim quit, Truc, Com Ruou, Dau dau ba la, Ca ri, Da tu bien and Gioi Lom gave symptomless under transmission conditions and negative reaction by PCR tests. In molecular study, 38 primers has been designed and used for screening of HLB tolerant capacity of 49 varieties/clones belonging to Rutaceae. The preliminary results shown that the tolerant varieties was grouped into Group D, which somehow matched with the tolerants screened under screenhouse conditions.

10.14 P

Mandarin and mandarin hybrid genetic transformation for resistance to *Candidatus* Liberibacter asiaticus

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Brazil is one of the largest producer and exporter of citrus. Currently, Huanglongbing disease (HLB) associated to *Candidatus* Liberibacter asiaticus (CLas) is the main threat to citrus industry. The aim of this work is to study the genetic transformation of mandarin/mandarin hybrid 'Thomas' (*Citrus reticulata* Blanco) and 'Fremont' (*C. clementina* hort. ex Tanaka x *C. reticulata* Blanco) with the gene that encodes attacin antibacterial peptide (*att*A) driven by phloem-specific promoters. The genetic transformation experiments were performed with epicotyl segments, via *Agrobacterium tumefaciens* (EHA 105), with the gene constructs pCAtSUC2/attA and pCAtPP2/attA, containing the *att*A gene controlled by AtSUC2 and AtPP2 promoters. Transgenic plants were identified by PCR analysis and acclimatized to greenhouse conditions. The plants will be propagated and evaluated for resistance to CLas.

Support: FAPESP, CNPq

10.15 P

Cell Penetrating Peptides as an Alternative Transformation Method in Citrus

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Huanlongbing (HLB) has caused the loss of thousands of trees in Florida's multi-billion dollar citrus industry. An effective, long-term strategy to controlling this disease will be by the incorporation of genetic resistance into commercial genotypes. Because conventional breeding is limited by the lack of natural resistance in citrus to HLB, genetic engineering is now considered a significant alternative to incorporating such characteristics. In fact, despite general concerns from the public against genetically modified organisms (GMOs), one National Academy report[1] stated that genetic engineering will be the way to fully exterminate HLB, while growers' support of a transgenic approach for disease resistant traits also continues to rise. The primary transformation method of citrus typically uses *Agrobacterium*, in which explants are suspended with the bacterium and subsequently placed on selection media. After treatment, the explants produce shoots that can ultimately lead to stable transgenic plants. Due to the slow growth and lengthy maturation, this process takes several years to produce reproductive trees and must be optimized for each cultivar. Consequently, transformation efficiency is substantially less than other model systems. The commercialization of transgenic disease resistant cultivars is even slower due to regulations limiting GMOs worldwide. In order to decrease the dependence upon bacterial vectors and increase transformation efficiency, we have researched an alternative method for introducing nucleic acids into plants that does not involve *Agrobacterium* and instead uses cell penetrating peptides (CPPs). CPPs are short, positively charged amino acid sequences that bind to negatively charged molecules and subsequently translocate across cellular membranes. Most surprisingly plant cell walls can also be bypassed, as CPPs are currently used in plants in transient expression and gene silencing assays. Until now, CPPs have not been examined in citrus or other woody crops for stable transformation protocols. We have developed a method for the transient expression of reporter genes (GUS and GFP) using plasmid DNA and CPPs. Our data indicate that up to 50% of treated explants express GUS when CPPs are used alone. Several optimization steps have been tested and the expression efficiency can be increased up to 100% when CPPs are used in conjunction with a lipid transfection reagent. We have also produced hypocotyl segments which survived kanamycin selection. Some produced shoots that rooted and were planted in soil and have been maintained in a growth chamber. PCR and reporter gene analysis will confirm if stable integration has occurred. Our novel protocol could have far reaching effects for the successful integration of disease resistant GMOs in global markets by limiting the perceived negative effect of bacterial vectors.

10.16 P

Breaking citrus juvenility by modulating endogenous miR156 and miR172 levels

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The ability to either transform mature citrus directly or to transform juvenile citrus and also induce it to flower and set fruit within a few years is critical for evaluation of fruit quality, quantity and of horticultural performance of any transgenic trees. In plants, the transition from juvenile to adult stage is regulated by the sequential and complementary action of microRNAs miR156 and miR172. miR156 suppresses the expression of specific transcriptional factors that would otherwise promote the juvenile to adult phase transition, including factors that activate miR172, which directly promotes the transition. Here, we created a target mimic for miR156 to attempt to sequester miR156 and reduce its levels in juvenile citrus. We cloned the nonprotein coding gene IPS1 from Arabidopsis and replaced its native microRNA target with the predicted citrus target of citrus miR156, resulting in a citrus miR156 mimic gene, cMIM156. Five sweet orange (Hamlin) seedling transgenic plants expressing cMIM156 were produced and the endogenous miR172 levels were monitored over time. All five transgenic plants showed enhanced expression levels of miR172 (two exhibited 10X higher levels) compared to non-transgenic control plants regenerated from explants at the same time as transformants. By 1 year after transformation, miR172 levels were still 5X less than that observed using mature sweet orange, and no flowering has yet been observed. The levels of miR172 expression have increased over time, and we are attempting to establish a timeline for flowering of 3 years after transformation.

Funding: USDA-APHIS CPHST

10.17 P

One-for-all: a monoclonal antibody specific to different recombinant proteins in transgenic citrus plants

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The easy and rapid identification of a recombinant protein in transgenic plants is becoming increasingly relevant as more transgenic plants are used for research and commercial applications. Tagging recombinant proteins with a small peptide (epitope) can perform such a task using a variety of immunological methods. Epitope tags are short, hydrophilic peptide sequences recognized by specific antibodies. Compared with larger protein fusions, the small size of epitope tags makes them less likely to interfere with protein folding and function.

We describe herein the detection of the c-myc epitope using different immunological methods in citrus transgenic plants. A c-myc tag sequence (N-EQKLISEEDL-C, corresponding to the C-terminal amino acids (410-419) of human c-myc protein) was added to the DNA sequence by PCR and the resulting proteins are being tested at the CREC. Our experiments with a genetically altered endogenous citrus gene modified to produce a protein with the c-myc tag demonstrate the utility of this technique for detection of trans-proteins in Citrus. Since this tag can be incorporated in the C terminal end of any protein, this technology simplifies different assays that require recognition by protein specific antibodies. We could detect different trans-proteins using the same antibody against the Myc epitope by ELISA or Western blotting. Moreover, expression of recombinant proteins bearing epitope tags can also eliminate the need of isolating proteins and producing antibodies for each new recombinant protein to be studied, which requires more cost and time, and can be problematic as a result of low antigenicity or high background cross-reaction with other proteins.

Our protocol would also accelerate the characterization of the transgenic plants in a timely fashion. This would help to isolate transgenic lines that produce optimum levels of the foreign trans-protein thereby improving and enhancing methods to evaluate and screen new priority transgenic commercial citrus scions and rootstock cultivars for resistance and/or tolerance to Huanglongbing, citrus canker and the Asian citrus psyllid.

10.18 P

Evidence that 'flying dragon' trifoliate orange delays HLB symptom expression for four sweet orange cultivars, Tahiti lime and Okitsu mandarin

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Huanglongbing (HLB), caused by *Candidatus* Liberibacter asiaticus and vectored by *Diaphorina citri*, was first reported in 2004 in Brazil and is currently widespread in São Paulo State. Brazil is the world's largest sweet orange producer and has 49,000 ha cultivated with 'Tahiti' lime acid lime. Mandarin cultivation represents 5.5% of total citrus production in the country. In 2001, three experiments were planted in the Citrus Experimental Station (EECB), Bebedouro, Northern São Paulo State, where the first HLB symptomatic tree was detected on 2006. The initial objective was to evaluate the performance of 'Folha Murcha' sweet orange, 'Tahiti' acid lime and 'Okitsu' mandarin grafted on twelve rootstocks including Rangpur lime, Swingle citrumelo, Rubidoux and Flying Dragon (FD) trifoliate oranges. Cumulative HLB incidence (CI) was calculated in 2009. Folha Murcha and Tahiti lime trees on FD had lower CI values (6.7 and 10%) than trees on Rangpur lime (33.3 and 80%), Swingle citrumelo (46.7 and 66.7%) and Rubidoux (46.7 and 60%). CI was similar for Okitsu on FD, Carrizo citrange and HRS 827 (11.1%). In another field trial at EECB, Valencia, Hamlin and Natal were planted on FD in November 1994. Drip irrigation was installed in 2001. The first symptomatic plants in the area were detected in November 2008. In 2010, the 16 yr-old trees on FD have a lower CI (5.6%) than 20 yr-old sweet orange trees on Swingle citrumelo in an adjacent trial (CI = 35.4%). Our results indicated that FD rootstock could prolong the longevity of citrus orchards but more well-controlled studies of scion-rootstocks combinations with and without vector management are required.

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10.19 P

A Tomato Detached Leaf Assay for Chemical Genomics of an HLB Model System

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To better understand plant-pathogen interactions in Huanglongbing disease and develop control strategies we investigated a novel approach known as chemical genomics with Tomato "Psyllid Yellows", caused by *Candidatus* Liberibacter psyllaurous (CLps), as model of HLB. Chemical genomics involves three key stages starting with designing and performing high-throughput chemical screening, identifying chemicals inducing desired effects and dissecting the genetic targets of the candidate chemical. Our study has been focused on developing a high throughput chemical screen assay using model plants such as tomato and Arabidopsis that can be infected by Candidatus *Liberibacter psyllaurous* (Clps). The key objective is to identify chemicals that induce plant defense against CLps infection or its transmission via psyllids. We evaluated *Arabidopsis thaliana* and tomato in different media such as MS sterile media and hydroponic culture, however qPCR results indicated very low and inconsistent numbers of CLps positive plants. We designed a modified detached leaf assay for tomato based on the citrus detached leaf assay (Eldesouky Ammar, USDA-ARS) that has resulted in consistently high (80-85%) number of CLps-positive leaf petioles. We are currently evaluating application of the detached leaf assay in screening chemicals using a tomato CaBP22-GUS transgenic line. This line was developed by Dr. Isgouhi Kaloshian and Dr. Thomas Eulgem (University of California, Riverside) based on a transgenic Arabidopsis reporter line with the promoter $CaBP22^{-333}$ promoter fused to *GUS* (Knoth et.al. 2009). The *Arabidopsis* transgenic line has been used successfully in many high-throughput chemical screens to identify chemicals inducing defense responses. In our study, we will test chemical uptake and its effect on the transgenic tomato line using GUS expression by RT-PCR. To develop methods to test responses to candidate chemicals in citrus, we are testing gene expression of sweet orange seedlings following exposure to four chemicals known to induce defense responses in other plants.

Citations:

Knoth C, Salus MS, Girke T, Eulgem T. 2009. The synthetic elicitor 3,5- Dichloroanthranillic acid (DCA) induces *NPR1*-dependent and *NPR1*-independent mechanisms of disease resistance in *Arabidopsis thaliana.* Plant Physiology 150:333-347